Are sulfur-containing metabolites involved in the circadian-regulated pathogen defense? - Influence of the sulfur metabolism on the pathogen defense in *Brassica napus*

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Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 5.

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Publikation (Kapitel 2)

Weese, A., Pallmann, P., Papenbrock, J., Riemenschneider, A. (2015) *Brassica napus* L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack. *Front Plant Sci. Frontiers*; 2015;6. doi:10.3389/fpls.2015.00009

Die Idee für die Experimente wurde von J. Papenbrock, A. Riemenschneider und A. Rumlow geb. Weese entwickelt. Die Experimente mit verschiedenen *Brassica napus* Sorten wurden von A. Riemenschneider und A. Rumlow geb. Weese durchgeführt. Ein Teil der morphologischen Untersuchungen mit selbigen Pflanzenmaterial wurden von A. Riemenschneider durchgeführt. Der andere Teil der morphologischen Untersuchungen sowie die Analyse der physiologischen Aspekte, die Analyse ausgewählter schwefelhaltiger Metabolite und die Expressionsanalyse verschiedener Gene wurden von A. Rumlow geb. Weese durchgeführt. Auch das Auswerten der Daten sowie das Erstellen von Graphen und Tabellen erfolgte von A. Rumlow geb. Weese. Die statistische Auswertung der Daten erfolgte von P. Pallmann. Große Teile des Manuskripts, besonders der Material und Methodenteil sowie Teile der Einleitung und Diskussion, wurden von A. Rumlow geb. Weese verfasst.

Publikation (Kapitel 3)

Rumlow, A., Keunen, E., Klein, J., Pallmann, P., Riemenschneider, A., Cuypers, A., Papenbrock, J. (2016) Quantitative expression analysis in *Brassica napus* by Northern blot analysis, semi-quantitative and reverse transcription-quantitative PCR in a complex experimental setting. *PLoS ONE* 11(9): e0163679. doi:10.1371/journal.pone.0163679

Die Idee für die Experimente wurde von J. Papenbrock, A. Riemenschneider und A. Rumlow geb. Weese entwickelt. Die Pflanzenanzucht von *Brassica napus* wurde von A. Riemenschneider und A. Rumlow geb. Weese durchgeführt. Ein kleiner Teil der Expressionsanalysen sowie die Aufstellung der Kosten für die einzelnen Methoden wurden von J. Klein durchgeführt. Der große Anteil der Expressionsanalysen wurde von A. Rumlow geb. Weese durchgeführt, sowie das Erstellen von Graphen und Tabellen. Die Auswertung der Daten der Expressionsanalyse mittels des GrayNorm Algorithmus wurden von E. Keunen durchgeführt. Die statistische Auswertung der Daten erfolgte durch P. Pallmann. Große Anteile des Manuskripts wurden von A. Rumlow geb. Weese geschrieben und von E. Keunen, A. Cuypers und J. Papenbrock teilweise überarbeitet.

Manuskript, in Vorbereitung (Kapitel 4)

Rumlow, A., Hornbacher, J., Pallmann, P., Riemenschneider, A., Papenbrock, J. The circadian clock influences the levels of sulfur-containing metabolites in *Brassica napus* and its defense status against the fungal pathogen *Verticillium longisporum*

Die Idee für die Experimente wurde von J. Papenbrock, A. Riemenschneider und A. Rumlow geb. Weese entwickelt. Die Pflanzenanzucht von *Brassica napus* für die Untersuchungen im Tagesverlauf wurde von A. Riemenschneider und A. Rumlow geb. Weese durchgeführt. Die Infektionsversuche wurden allein von A. Rumlow geb. Weese durchgeführt. Die Analyse der schwefelhaltigen Metabolite Glutathion, Cystein und die Glucosinolate sowie die Analyse des Gesamtschwefels wurden von A. Rumlow geb. Weese durchgeführt. Die Messung des Sulfats wurde von E. Bloem durchgeführt. Die Identifizierung der GSL in B. napus sowie die Optimierung der Methode zur Quantifizierung wurde von J. Hornbacher durchgeführt. Die Expressionsanalyse wurde ebenfalls von A. Rumlow geb. Weese durchgeführt. Die statistische Auswertung zur 24 h Rhythmik erfolgte durch P. Pallmann. Die statistische Analyse der restlichen Daten erfolgte durch A. Rumlow geb. Weese. Große Anteile des Manuskripts wurden von A. Rumlow geb. Weese geschrieben und von J. Papenbrock überarbeitet.

Summary

A possible enhancement of defense mechanisms of crop plants to pathogens by the application of increased amounts of sulfur-containing fertilizers, is based on the fact that sulfur-containing compounds of the primary and secondary metabolism accumulate in the plant and may act as defense compounds. Therefore, diseases derived from plant pathogens can be restricted in an effective and environmentally friendly way. A possible regulation of the sulfur metabolism by the circadian clock can give information about the right time of the application of sulfur-containing fertilizers that will enhance the defense even further as the susceptibility of plants varies in the course of a day. Therefore, the aim of this thesis was to investigate the involvement of the sulfur metabolism in the circadian regulated immunity in the agriculturally important crop species *Brassica napus*. In the first place different canola cultivars exhibiting different features and different levels of resistance to the fungus *Verticillium longisporum* were analyzed in their reaction to sulfur deficiency and infection with the fungus. Long term sulfur-deficient conditions led to a drastic reduction in biomass and in the content of the sulfur-containing metabolites involved in the pathogen defense. Under these conditions the plants were more susceptible to the infection as the biomass production as well as the efficiency of the photosynthesis were further decreased. Under sulfur-sufficient as well as under sulfur-deficient conditions the total sulfur content was increased in the leaves of infected plants in comparison to non-infected plants. The cultivar Genie showed in this context the most obvious reactions to the sulfur deficiency and infection. Therefore, further experiments were performed with this cultivar. For investigating the circadian regulation of the sulfur metabolism plants grown with sufficient sulfur and deficient sulfur supply were harvested in the course of a day under diurnal and circadian conditions. For the expression analysis Northern blot analysis was chosen after careful comparisons with qPCR analysis. For Northern blot analysis the RNA could directly be used without the need of transcribing the RNA into cDNA. Furthermore, the transcripts of the genes of interest could be directly visualized on the membrane and the resulting band intensity represented the expression level. For evaluating the data obtained by Northern blot analysis in a quantitative way the method had to be optimized first. This was achieved by using reference genes for normalization. The selected reference genes were affected in different ways by the experimental conditions. With the help of an algorithm a suitable set of reference genes could be validated. Transcript levels of the selected genes involved in the sulfur assimilation as well as the content of glutathione showed diurnal oscillations with a period ranging from 20 to 23 h independent from the sulfur status. Under continuous light the period remained the same, whereas the amplitude of this oscillations were in most cases lowered and a shift in the phase occurred. An exception was on one hand the transcript amount of *Sultr4;2* as the expression was unaffected by light and on the other hand the content of the glucosinolates which showed ultradian oscillations. The oscillations of the latter one were altered by the sulfur status and led to a loss in the rhythmic oscillations of the aliphatic glucosinolates under circadian conditions. In a last experiment it should have been determined, whether the susceptibility of *B. napus* to the fungus *V. longisporum* is dependent on the time point of infection. As the infection itself might have not been successful in this experiment no final conclusions in this context could have been drawn yet.

Keywords: *Brassica napus,* circadian rhythm, defense, diurnal, metabolites, sulfur, *Verticillium longisporum*

Zusammenfassung

Eine mögliche Verbesserung der Abwehrmechanismen in Pflanzen gegenüber Pathogenen durch die vermehrte Zugabe von schwefelhaltigen Düngern beruht auf der Tatsache, dass die Metabolite des primären und sekundären Schwefelmetabolismus in der Pflanze angereichert werden und womöglich als Abwehrstoffe fungieren. Somit bietet sich hier eine effektive und umweltfreundliche Möglichkeit Pflanzenkrankheiten einzuschränken. Eine mögliche Regulation des Schwefelmetabolismus durch die circadiane Uhr kann Aufschluss darüber geben, ob die Zugabe von schwefelhaltigem Dünger zu einer bestimmten Zeit eine gesteigerte Verbesserung der Abwehr bewirken kann, da die Anfälligkeit von Pflanzen im Tagesverlauf variiert. Daher war das Ziel der Arbeit die Rolle des Schwefelmetabolismus in der circadian regulierten Pathogenabwehr in der landwirtschaftlich wichtigen Nutzpflanze *Brassica napus* zu verstehen. Zunächst wurden verschiedene Rapssorten mit unterschiedlichen Eigenschaften und unterschiedlicher Anfälligkeit gegen den Pilz *Verticillium longisporum* im Hinblick auf die Reaktion gegenüber Schwefelmangel und Infektion untersucht. Durch die mangelnde Versorgung mit Schwefel kam es zu einer drastischen Reduktion der Biomasse sowie der schwefelhaltigen Metabolite, von denen einige an der Pathogenabwehr beteiligt sind. Es konnte zudem gezeigt werden, dass unter diesen Umständen die Pflanzen anfälliger für die Infektion waren, da die Biomasseproduktion in den infizierten Pflanzen weiter zurückging und die Photosyntheseleistung weiter abnahm. Sowohl mit ausreichender Schwefelversorgung als auch unter Schwefelmangel war der Gesamtschwefel erhöht in infizierten im Vergleich zu nicht infizierten Pflanzen. Die Sorte Genie zeigte in dieser Hinsicht die offensichtlichsten Reaktionen im Hinblick auf den Schwefelmangel und auf die Infektion. Daher wurden weitere Untersuchungen mit dieser Sorte durchgeführt. Für die Untersuchung einer möglichen circadianen Regulation des Schwefelmetabolismus wurden die Pflanzen mit ausreichend Schwefel sowie unter Schwefelmangel im Tagesverlauf unter diurnalen, sowie unter circadianen Bedingungen geerntet. Für die Expressionsanalyse ausgewählter Gene, die an der Schwefelassimilation beteiligt sind, wurde die Northern Blot-Analyse gewählt, nachdem diese sorgfältig mit der qPCR verglichen worden war. Für die Northern Blot-Analyse konnte die RNA direkt verwendet werden, ohne diese vorher in cDNA umschreiben zu müssen. Weiterhin konnten die Transkripte der Gene direkt auf der Membran visualisiert werden und die resultierenden Bandenintensitäten repräsentierten die Expressionsstärke. Um diese Daten quantitativ auswerten zu können, war es notwendig, die Methode zu optimieren. Dies konnte durch den Einsatz von Referenzgenen ermöglicht werden. Unter den experimentellen Bedingungen wurden die ausgewählten Referenzgene in ihrer Expression unterschiedlich beeinflusst. Mit der Hilfe eines Algorithmus konnte ein geeignetes Set von Referenzgenen ausgewählt werden. Die Transkriptlevel der ausgewählten Gene, die an der Schwefelassimilation beteiligt sind, sowie der Gehalt von Glutathion zeigten diurnale Schwingungen mit einer Periode zwischen 20 und 23 h unabhängig von dem Schwefelstatus. Bei konstantem Licht blieb zwar die Periode gleich, aber die Amplitude dieser Schwingungen war kleiner und es zeigte sich eine Verschiebung der Phase. Ausnahmen machten hierbei zum einen die Expression des Sulfattransporters *Sultr4;2,* da diese nicht vom Licht beeinflusst wurde, und zum anderen der Gehalt der Glucosinolate, die ultradiane Schwankungen im Tagesverlauf zeigten. Die Schwankungen der Glucosinolate wurden durch die Schwefelversorgung beeinflusst und führten sogar zu einem Verlust der rhythmischen Schwankungen der aliphatischen Glucosinolate unter circadianen Bedingungen. In einem letzten Versuch sollte überprüft werden, ob die Anfälligkeit von *B. napus* gegenüber dem Pilz *V. longisporum* von dem Zeitpunkt der Infektion abhängig ist. Da die Infektion in diesem Versuch nicht erfolgreich war, konnten in dieser Frage noch keine Aussagen getroffen werden.

Schlüsselwörter: Abwehr, *Brassica napus,* circadianer Rhythmus, diurnal, Metabolite, Schwefel, *Verticillium longisporum*

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CHAPTER 1

General introduction

The importance of controlling pathogen-derived diseases in crop plants

The world population is growing extremely fast and led to an increased world food crop demand and production over the past years. This was achieved by improved cultivation techniques including the use of varieties providing higher yields. These, however, showed often a higher susceptibility to diseases resulting in increased infection rates (Oerke, 2005). Yield losses caused by plant pathogens comprise up to 20% of the world's harvest (Flood, 2010). In the near future the percentage might be even increased as pathogens are further spreading by human activity. In this context plant diseases had become a major issue of the global food security (Flood, 2010). There are a number of common methods controlling pathogen-derived diseases such as soil tillage measures, crop rotation, mixed cropping systems, and the breeding of internal resistances in plant cultivars specific to the pathogen. The latter one was extensively used in the recent years and nowadays a number of cultivars are available with internal resistances to certain pathogens. Due to climatic changes and modification in the natural environment by human activity new species of pathogens might evolve which might be able to overcome these internal resistances (Fisher et al., 2012). The use of pesticides is also a strategy for disease control, however, especially in the case of organic farming this is no option. A promising possibility for long term control and without the use of pesticides would be fertilizing strategies based on the nutrient enhanced defense in plants. For applying such strategies in agriculture effectively, better understanding of the regulation of the essential elements associated metabolism is required as well as the plant-pathogen interaction.

Verticillium longisporum - a serious threat for the cultivation of *Brassica napus*

The biggest proportion of biotic threats on crop plants comprises the fungi and oomycetes (Fisher et al. 2012). The crucial effect of fungal infections is long known as in the 19th and 20th century single fungi led to starvation and baring forest landscapes. Modifying the natural environments by human activity led to an increased appearance of fungal diseases (Institute of Medicine, 2011; Pennisi 2010). In fact, the fungi and the oomycetes were the most widespread crop pest and pathogen (CPP) in the recent years compared to other CPPs. Despite their more restricted host range they were also the most rapidly spreading CPP (Bebber et al., 2014). In general, control of these pathogens can be achieved by using fungicides. However, diseases deprived from soil-borne pathogens are challenging in agriculture, as these cannot be controlled effectively due to their persistence in soil by forming survival structures such as oospores, chlamydospores, and sclerotia. These root-infecting pathogens are able to cause significant reduction in yield and quality of many crop plants (Okubara and Paulitz, 2005; Okubara et al., 2014). One of those soil-borne pathogens is the ascomycete *Verticillium longisporum* which is

mainly restricted to crucifers causing Verticillium wilt. The fungus is an allopolyploid that might be evolved from *Verticillium dahliae* and *Verticillium alboatrum* (Clewes et al., 2008; Inderbitzin et al., 2011). First infections of the agriculturally important crop plant *Brassica napus* with the fungus occurred in Sweden in 1969 (Kroeker, 1970). Upon spreading in the recent years *V. longisporum* is found in Europe (Gladders et al., 2011; Karapapa et al., 1997; Steventon et al., 2002; Zeise and Tiedemann, 2002), Russia (Pantou et al., 2005) and, recently, in Canada (CFIA, 2015). As Europe and Canada represent important areas for the cultivation of oilseed rape, *V. longisporum* became a serious threat in the production of this oilseed plant. The suggested yield loss due to infection with *V. longisporum* ranges between 10% and 50% (Dunker et al. 2008).

The disease cycle of *V. longisporum* can be divided in three stages: dormant, parasitic, and a limited saprophytic stage (Figure 1). In the first stage root exudates of the plant induce the germination of the melanized microsclerotia that can remain in the soil until the infection of the host. The growth of hyphae out of the microsclerotium, is directed to the roots enabling the fungus to colonize the surface of the root hairs. Thus penetration of rhizodermal cells take place enabling the fungus to enter the roots, whereas this is only possible if the endodermis is physically damaged or as in the root tips not yet developed. Inside the plant the hyphae grow in the root cortex towards the central cylinder. In the parasitic stage conidia are formed and carried upwards through the vascular elements. New conidias are formed and the plant becomes increasingly colonized. The limited sacrophytic stage occurs at senescence of leaves, where the stem parenchyma is invaded and formation of microsclerotia is carried out leading to dark unilateral striping on the stem during the ripening of the crop. Typical symptoms upon infection are wilting, stunting, chlorosis, vascular discoloration, and early senescence (Fradin and Thomma, 2006). As wilting symptoms of the infection of oilseed rape are absent due to prematurely ripening, disease symptoms cannot be distinguished from natural senescence. Furthermore, as the stem striping occurs only at later stages in the growing season (Heale and Karapapa, 1999) early detection of the infection is difficult. As no effective fungicide for *V. longisporum* is available yet other ways have to be developed guaranteeing a long-term control.

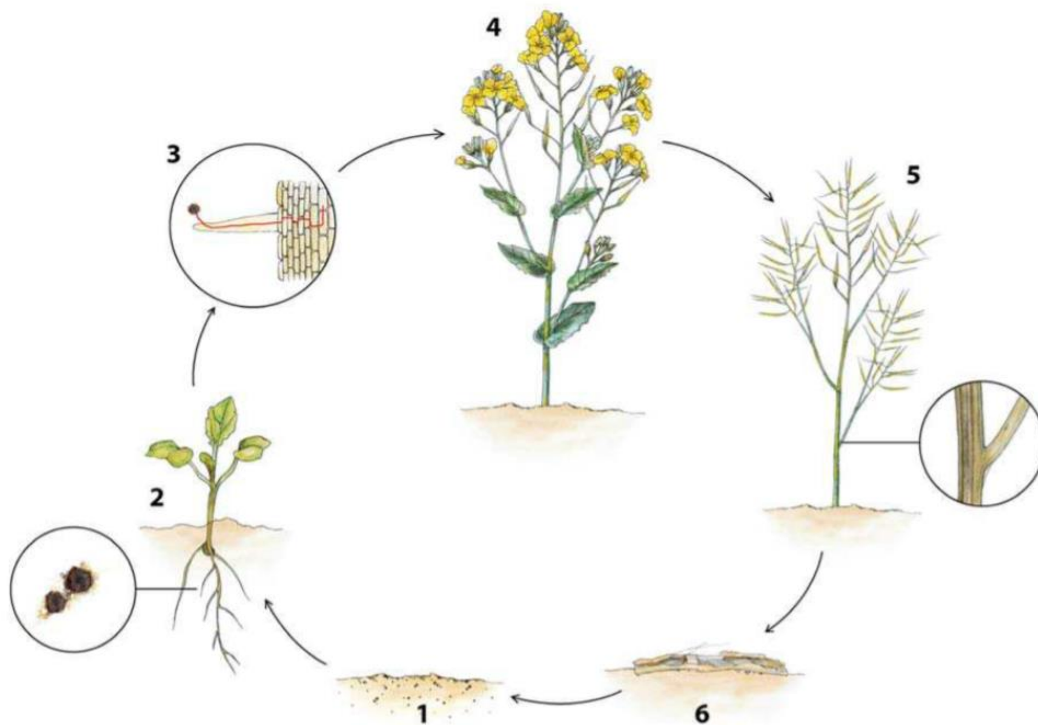

Figure 1: Disease cycle of *Verticillium longisporum* on oilseed rape (taken from Depotter et al., 2015). Microsclerotia as persistent resting structures remain in the soil until the infection (1). Germination of the microsclerotia is induced by root exudates (2). Penetration of epidermal cells of lateral roots or root hairs and growth of the hyphae towards the central cylinder (3). Absence of disease symptoms during the growing season (4). Developement of dark unilateral striping on the stems during the ripening and formation of microsclerotia (5). Release of the microsclerotia into the soil (6).

Brassica napus as an agriculturally important crop plant

Enhancing the resistance in *B. napus* against the fungus *V. longisporum* by applying internal resistances is one of the aims in breeding new cultivars, as oilseed rape has become an important crop plant in agriculture over the past years. Besides its cultivation as leaf vegetable or leafy fodder crop, the role as an oilseed is nowadays the most important one. In the world oilseed production oilseed rape takes after soybean the second place with a percentage of 12,8 % (Figure 2). With a share in the world production of oilseed rape of 32 % the EU-28 takes the leading role. About 57% of the cultivable land for oilseeds is comprised by oilseed rape cultivation and is therefore, the most important crop plant in the EU-28 (Goldhofer and Schmid, 2016). Approximately 20 % of the cultivable area in Germany is used for oilseed rape cultivation where winter oilseed rape cultivation dominates, as it comprises 99,7% of the cultivable area (Statistisches Bundesamt, 2016). With a market share of 40% is canola oil the most sold oil in Germany (UFOP, 2016). Looking at the history of *B. napus* in agriculture the road to its role as valuable oilseed took some time and effort.

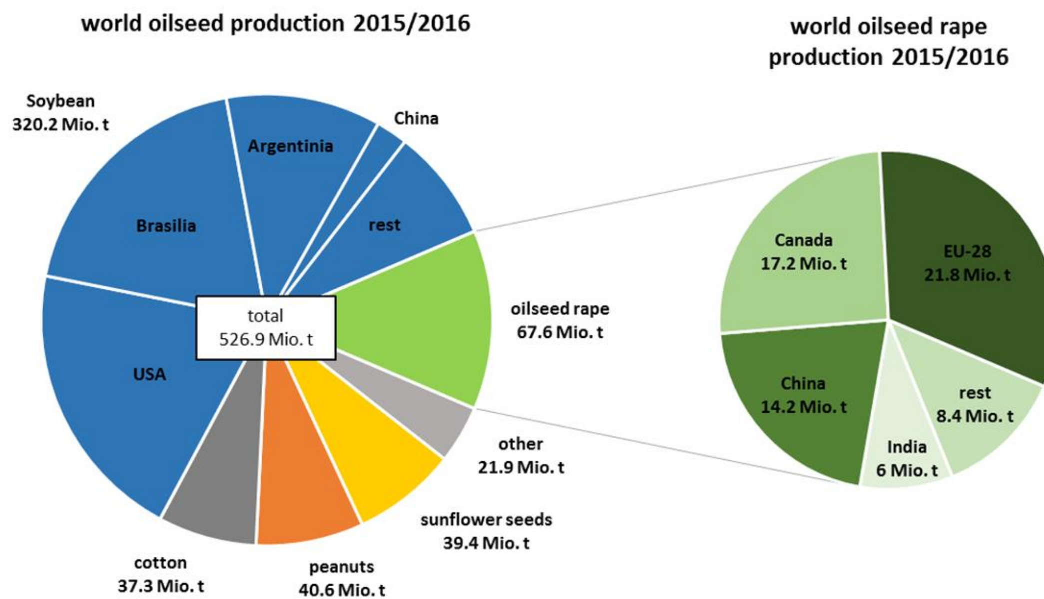

Figure 2: **Oilseed and oilseed rape production in the world 2015/2016 (data taken from Goldhofer and Schmid, 2016).**

Vegetables and oilseed of *Brassica* plants are known to be the earliest cultivated plants by humanity. Oilseed rape as a member of the Brassicaceae is a very recent species based on the fact that there are no wild forms. With an age of only a few hundred years *Brassica napus* is the youngest species among our crop plants, whereas researchers agree that this plant is a product created by humankind ~7500 years ago. Among closely-related *Brassica* species spontaneous chromosome doubling in crossing occurred in the recent years. When the species *Brassica oleracea* and *Brassica rapa* were cultivated in geographical proximity the allypolyploid hybrid *B. napus* evolved containing 18 chromosomes from *B. oleracea* and 20 chromosomes from *B. rapa.* Until the 20th century cultivation of *B. napus* for leafy fodder was not attractive, as the high content of erucic acid and glucosinolates (GSL) led to disruption in the metabolism in animals. Furthermore, these components led to a bitter tasting oil and causing cardiac health problems, when consumed in high doses, making it unsuitable for human consumption. Nevertheless, the oil could be used as biofuel and as a source of lamp oil production. In the 1970s and 1980s oilseed rape underwent targeted selection reducing the content of these compounds resulting in the single and double low rapeseed varieties (Stefansson 1983; Downey and Röbbelen, 1989; Downey, 1990). These cultivars are called canola when the content of erucic acid is less than 2 % and the meal must contain less than 30 µmol g^{-1} seed DM of aliphatic GSLs. Therefore, the canola oil became a valuable source of

nutritional oils and fats and the oil no longer used solely as biofuel. With these achievements in the breeding, the consumption of the rapeseed oil in the world increased from 11.7 Mio t in 1995 up to 26.7 Mio t in 2015/16 (Goldhofer and Schmid, 2016). Meeting the increasing demand, the seed yield potential was improved by developing functional male sterility system also known as the Male Sterility Lembke (MSL) system for the production of fully restored rapeseed hybrids without any yield or quality penalty. With the invention of those hybrids, line-bred cultivars became less important as the potential yield as well as the yield assurance was much higher in hybrids than in line-bred cultivars. In fact in Germany more than 84% of the oilseed rape area is used for the cultivation of hybrids. In France hybrids comprises 77% and in North America about 99% (DSV, 2016). Nowadays a number of cultivars for summer and winter oilseed rape are available with different properties based on its role and utility in human agricultural systems. As the seed quality traits for breeding the double low cultivars were continuous selected since the 1970s the genetic diversity in those cultivars was relatively low and lacks a broad spectrum of disease resistances (Allender and King 2010). In 2003 the extensive screening of the germplasm of *B. napus* revealed that there are no sources for the resistance to *V. longisporum* (Happstadius et al., 2003). By interspecific hybridization of the parental species *B. oleracea* and *B. rapa* the transfer of *V. longisporum* resistance into resynthesized *B. napus* lines was successful (Rygulla et al., 2007). Newer cultivars show now internal resistances against *V. longisporum.* However, the internal resistance might not solely prevent an infection or guarantee a long-term disease control. Therefore, a strategy needs to be developed enhancing the natural resistance and thus improving the plant-pathogen tolerance. This might be achieved by developing a nutrient-based fertilizer strategy as the natural resistance in plants is dependent on the nutrient status in the plant.

Sulfur and its role in the disease control

The involvement of the nutritional status in the susceptibility of plants against pathogens was already introduced in 1873 by Justus von Liebig. Thus a sufficient supply of essential nutrients by fertilizing the right amount can enhance the natural resistance in plants (Huber and Haneklaus, 2007; Walters and Bingham, 2007). The nutritional requirements for plants comprises 18 important nutrients. All essential nutrients are involved in the plant pathogen response, but among them the importance of the macronutrient sulfur increased in the recent years. Already in 1802 the fungicidal effect of elemental sulfur foliar-applied to the plants was determined by Williams Forsyth. This became even more prominent, as the atmospheric sulfur

due to enhanced emission controls in the 1980s decreased resulting in sulfur-deficient soils. The reduction of crop-available sulfur in the soil was even more increased, as fertilizers were used containing little or no sulfur (Schnug and Haneklaus, 1994). Deficient sulfur supply can affect the use of other nutrients such as carbon and nitrogen negatively resulting in deficiencies, as well as decreases in protein biosynthesis, chlorophyll content and yield (Lunde et al., 2008; Mazid et al., 2011; Iqbal et al., 2013). Furthermore, the susceptibility in plants under sulfur-deficient conditions was increased and infection rates, especially in crop plans with a high sulfur demand increased as well (Schnug, 1997). In sulfur fertilization experiments in the greenhouse the disease index for various host/pathogen relationships were reduced by 5 to 50%. In field experiments the reduction ranged from 17 to 35 % respectively. In this context in 1995 the term sulfur-induced resistance (SIR) was introduced by Schnug et al., describing the enhanced stimulation of metabolic processes involving sulfur by applying fertilizer to the soil. In recent studies the term sulfur-enhanced defense (SED) occurred as to prevent misinterpretation of the term resistance (Rausch und Wachter, 2005; Kruse et al., 2007).

The sulfur assimilation as initial point for the sulfur-enhanced defense

The SED mechanism is mediated by a variety of sulfur-containing defense compounds (SDCs). The precursor of these compounds is the amino acid cysteine which is the product of the sulfate assimilation und thus the prerequisite for the biosynthesis of the SDCs (Figure 3). Plants have the ability to take up the sulfur in its most frequent form as sulfate from the soil. The uptake into the roots across the plasma membrane takes place under energy consumption through a proton/sulfate co-transport. The biggest portion of the sulfate is transported via xylem vessels to the shoot mediated by the transpiration stream. The uptake, transport, as well as the distribution of sulfate is mediated by a number of tissue specific transporters which are divided in 5 groups according to their translocation and specific role in the sulfate transport (Buchner et al., 2004; Hawkesford, 2003). In *B. napus* 14 members homolog to the transporters in *Arabidopsis thaliana* were identified (Parmar et. al., 2007). The primary uptake of sulfate from the soil is enabled through members of the group 1 that are mainly located in the roots, exhibiting a high sulfate affinity. Members of group 2 with a low sulfate affinity are found in the vascular tissue mediating the transport within the stele via xylem and phloem. Excessive sulfate can be stored in the vacuole and be restored by members of group 4 sulfate transporters localized in the tonoplast under sulfur-deficient conditions. Transportation into the chloroplasts of the leaves is mediated by the group 3 transporters where the sulfur assimilation takes place. In the first step adenosine 5'-phosphosulfate (APS) is generated, catalyzed by ATP sulfurylase

under energy consumption. The second reduction is mediated by the enzyme APS-reductase (APR) forming sulfite with glutathione (GSH) as electron donor. Further activation of APS by APS kinase leads to the formation of 3'-phosphoadenylylsulfate (PAPS) which is among other sulfation reactions required for the biosynthesis of GSLs which are mostly found in the family Brassicaceae (Brown and Morra, 1997). For the synthesis of cysteine the sulfite is further reduced to sulfide by the sulfite reductase. The last step is mediated by the O-acetylserine(thiol)lyase (OAS) fixating the sulfide in cysteine, the first stable organic sulfur compound of the sulfur metabolism. The uptake as well as the sulfur assimilation are mainly regulated by the sulfate availability in the soil. Therefore, by using increased amounts of sulfur-containing fertilizers the metabolism of the SDCs might be increased, enhancing the resistance in plants against certain pathogens.

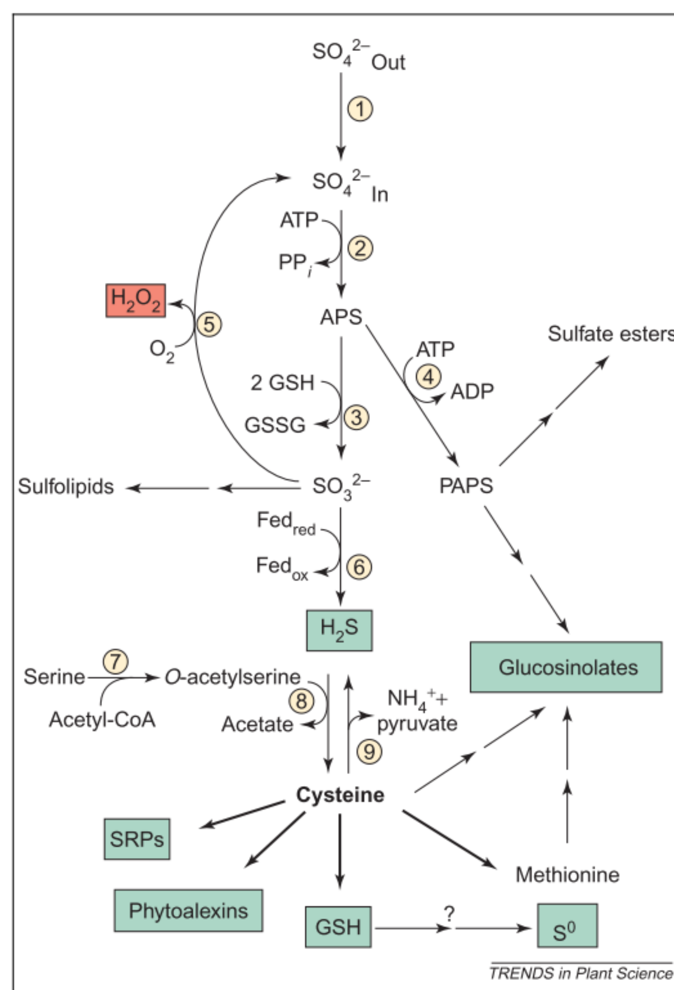

Figure 3: Sulfur assimilation and biosynthesis of sulfur-containing defense compounds (SDCs) (taken from Rausch and Wachter, 2005). Sulfate is taken up from the soil by high-affinity transporters (1). Inside the plant sulfate is mainly transported to the shoot and activated under energy consumption via the ATP sulfurylase generating 5'-adenylylsulfate (APS) (2). APS is reduced by the APS-reductase to sulfite (3). Alternatively, further activation of APS by the APS kinase lead to the formation of 3'-phosphoadenylylsulfate (PAPS) which is required for the glucosinolate synthesis (4). Sulfite is reduced to H_2S via the sulfite reductase (6). H_2S is incorporated into *O*-acetylserine generating cysteine (8). Cysteine is incorporated in sulfur-containing compounds. H_2S can be released from cysteine (9). Excess sulfite is converted to sulfate, releasing H_2O_2 (5).

Sulfur availability influences the formation of sulfur-containing defense compounds

Applying sulfate as sulfur-source to the soil for the SED might be preferred due to the rapid availability of sulfates for the plant resulting in a fast significant effect on the resistance (Schnug et al., 1995). With increasing sulfur supply GSH und secondary plant metabolites accumulate, which is strongly dependent on the cysteine pool. Therefore, the OAS is upgraded, which in turn induces the expression of sulfate transporters and a number of genes involved in the sulfur assimilation (Rausch and Wachter, 2005). Upon infection the biosynthesis of the SDCs is likely to be increased, which is the case for a wide range of pathogens, whereas this is most prominent for fungal-derived infections (Kruse et al., 2007). The increase is accompanied by a higher demand of sulfur. Therefore, a constantly high plant available sulfur reserve in the soil is required to satisfy the enhanced sulfur demand for plant defense during infection (Haneklaus, 2009). An optimal sulfur supply provides a better protection against pathogens and plants are additionally able to activate resistance and mechanisms faster and intensely compared to plants grown under sulfur-deficient conditions (Kruse et al. 2012). Furthermore, symptom developement and fungal spread can be reduced under high sulfur nutrition (Klikocha et al., 2005; Bollig et al., 2012). In contrast, under sulfur-deficient conditions the susceptibility of the plants is likely to be promoted as the SED is restricted under these conditions (Dubuis et al., 2005). Special attention in this context should be given to oilseed rape as the demand for sulfur is elevated due to high contents of proteins and sulfur-containing GSLs (Schnug and Haneklaus, 2005). Especially double low cultivars of *B. napus* have a high requirement of sulfur due to the modified GLS metabolism (Haneklaus et al., 2007). An oilseed crop removes between 20 and 30 kg sulfur ha^{-1} whereas about 10 to 15 kg sulfur ha^{-1} is removed by cereals (Walker and Booth, 1992). According to the high demand oilseed rape plants are very sensitive to sulfur deficiency. Already a sulfur content of 3.5 mg sulfur g^{-1} DM in the double low varieties is considered to be a critical value leading to symptoms of sulfur deficiency (Scherer, 2001). Therefore, a sulfur supply for *B. napus* is required which covers the metabolic sulfur needs as well as the enhanced demand for the plant-pathogen interaction. In summary, optimal sulfur nutrition can enhance the capability of a plant to cope with stress and the extra demand under stress can be met. Regarding the effect of sulfur on the resistance in plants, the use of sulfur-containing fertilizers is required to prevent sulfur-deficient conditions.

Sulfur-containing defense compounds are involved in the defense response in plants

As the SED seems to be an effective protection against the plant diseases the involvement of the SDCs in the plants defense should be clarified. In the sulfur metabolism of plants a broad range of SDCs is given (Figure 4). Some of them exhibit direct fungicidal effects, such as the sulfur-rich proteins, phytoalexins, elemental sulfur, H_2S, and the GSLs (Kuc, 1994; Cooper et al., 1996; Wallsgrove et al., 1999; Hughes et al., 2000). Whereas, for the latter one only the breakdown products are known to be toxic for the fungus. (Mithen, 1992; Wallsgrove et al., 1999). Other SDCs such as the thiols are directly involved in the defense immunity. To understand their role in the plant defense it is necessary to understand the plant immune system. As an adaption to pathogens plants evolved a robust multi-layered innate immune system. The first layer of the defense, also known as the basal defense response is triggered by the pathogen invading the plant. If the pathogen is non-specific to the host the infection can be avoided by the pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones and Dangl, 2006). Transmembrane pattern recognition receptors (PRR) are able to detect the PAMPs derived from the pathogen leading to molecular and physiological responses such as callus deposition in the cell wall or accumulation of reactive oxygen species (ROS). As some pathogens evolved effectors suppressing the PTI, secure nutrients and water from the host, plants developed the effector triggered immunity (ETI) (Dangl and Jones, 2001; Macho and Zipfel, 2015). These effectors can be detected directly or indirectly by plant resistance (R) proteins. This mechanism is based on the gene for gene resistance (Flor 1971), where the virulent gene as aviurlent gene from the pathogen is complementary to a resistance gene from the plant. The R-mediated resistance is important for the defense against biotrophic pathogens (Glazebrook, 2005). This form of resistance is accompanied by an oxidative burst, which is required for the hypersensitive response (HR) a type of programmed cell death. The HR induces the systematic acquired resistance, which is dependent on salicylic acid (SA). Expression of pathogenesis-related proteins (PR) is induced by SA and accelerate the oxidative outburst as an early signal for defense activation. The oxidative outburst is accompanied by the formation of ROS. With the function of GSH as a redox buffer the connection of the sulfur metabolism to the defense response in plants were given by the *Foyer-Halliwell-Asada* pathway as here in the detoxification of ROS the involvement of GSH was verified (Foyer and Halliwell, 1976). Cysteine, as the precursor of GSH, is also involved in the R-mediated defense as it is essential for the HR (Alvarez et al., 2012). The resistance against necrotrophic pathogens can either be solely jasmonic acid (JA) dependent or dependent on JA and ethylene (ET). The JA is known to induce the biosynthesis of indolic GSLs and a set of genes involved in the sulfur metabolism

such as the serineacetyltransferase (SAT) and APR (Jost et al., 2005). It is also involved in the regulation of the GSH formation leading to an increase in the content (Cai et al., 2011). This demonstrates that the sulfur metabolism and the corresponding metabolites play an important role in the defense response against biotrophic as well as against necrotrophic pathogens. As mentioned before there is a linear relationship between the sulfur supply and the SDCs, however, the relationship between the sulfur supply and the fungal infection is not always predictable as there are a number of factors which needs to be taken into consideration as well (Bloem et al., 2004; Salac et al., 2005). One important factor is the timing and the extent of the plants defense response. It might be more beneficial applying sulfur at a certain time of the day, as the susceptibility of the plant might be dependent on the daytime. This would require a regulation by an endogenous rhythm. Plants have evolved such a mechanism also known as the circadian rhythm.

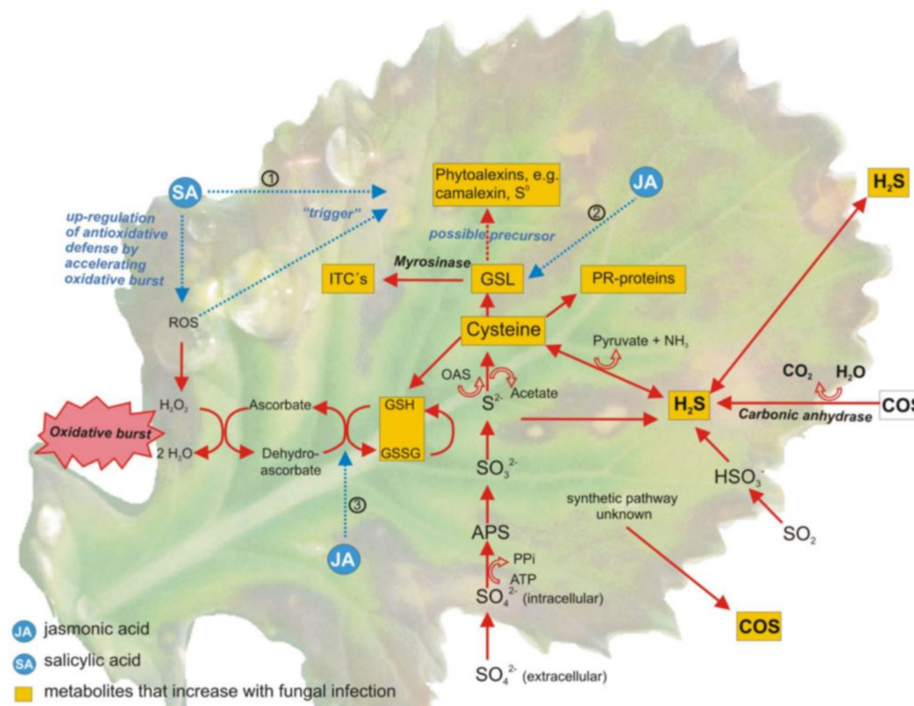

Figure 4: **The involvement of the sulfur metabolism in the plant defense (taken from Bloem et al., 2014).** Salicylic acid (SA) induces the oxidative burst and triggers the camalexin biosynthesis (1). Jasmonic acid (JA) induces the biosynthesis of indolic GSL and sulfur-related genes (2). Increase of the ascorbate and glutathione (GSH) content under stress by JA (3).

The role and function of the plant circadian clock

In organisms a number of processes are regulated by an endogenous mechanism enabling the anticipation of daily events in a more predictable way. Fluctuations in the behavior and

biological processes in response to the daily changes in light and dark conditions due to the rotation of the earth are known as diurnal. If these fluctuations persists in the absence of an external cue they are called circadian. The term circadian, from the Latin words “circa” (about) and “dies” (day) in this context is referred to the period, which is defined as the time to complete one cycle of ~24 h (Halberg 1959). Circadian rhythms persist under constant environmental conditions such as constant light or temperature as they are endogenously generated and self-sustaining. Under those called free-running conditions the period can diverge from the 24 h period. Another defining characteristic of the circadian rhythm is that the periodicity is maintained relatively constant over a broad range of physiological temperatures, which is also known as temperature compensation. The entrainment of the clock to the environment is an important aspect in the circadian system. Entrainment is achieved by environmental time cues such as light or temperature also known as ‘zeitgebers’, resetting the clock to synchronize the inner clock with the local time. In the case of light the sunrise and sunset synchronize the clock every day. Evolving such endogenous mechanism in plants with the same period as the Earth’s rotation had a beneficial effect in the adaption to the environment as plants are able to predict daily as well as seasonal changes.

The first report of the circadian rhythm in plants goes back to 1729 as the movement of the leaves from plants kept in dark followed a 24 h rhythm (de Mairan, 1729). Henceforward researches in this field increased, illuminating the complexity of the circadian system in plants (McClung, 2006). In general, it can be divided in three parts: the oscillator that generates rhythmicity, the input pathways setting the oscillator to the environment, and regulated by the oscillator the output rhythms. The model of the circadian clock had been best described so far in *A. thaliana* (Figure 5). The circadian clock of *A. thaliana* consists of three interlocked feedback loops. The central loop represents the core oscillator of the circadian clock with two single Myb domain transcription factors, CIRCADIAN AND CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) together with a member of the *PSEUDO-RESPONSE REGULATOR* (PRR) family TIMING OF CAB1 (TOC1). Upon upregulation of *CCA1* and *LHY* in the morning activation of the daytime-expressed genes *PSEUDO-RESPONSE REGULATOR (PRR)* occur which in turn repress transcription of *CCA1* and *LHY*. Thus the proteins CCA1 and LHY decrease in the day and cannot repress the expression of *TOC1* anymore. In the evening the produced TOC1 activates the evening genes and represses the genes of the morning loop. Upon degradation of the TOC1 protein by the ZEITLUPE (ZTL) protein in the night the expression of *CCA1* and *LHY* is not repressed anymore. Thus the proteins can be produced again in the morning. The genes of the circadian

clock are not only regulating themselves, they are also involved in the regulation of a number of genes. In this way physiological processes such as the photosynthesis or hormone signaling pathways are coordinated to the daily changes for optimizing their growth (Farré, 2012). The stress signaling in plants is also under circadian control enabling plants to cope with abiotic and biotic stress in a more predictable way and to gate appropriate responses in a timely manner (Seo and Mas, 2015). The circadian clock indeed plays an important role in the plant-pathogen interaction by balancing the immune responses with the cellular metabolism due to the closely association of the pathogens life cycle with the diurnally regulated host metabolism.

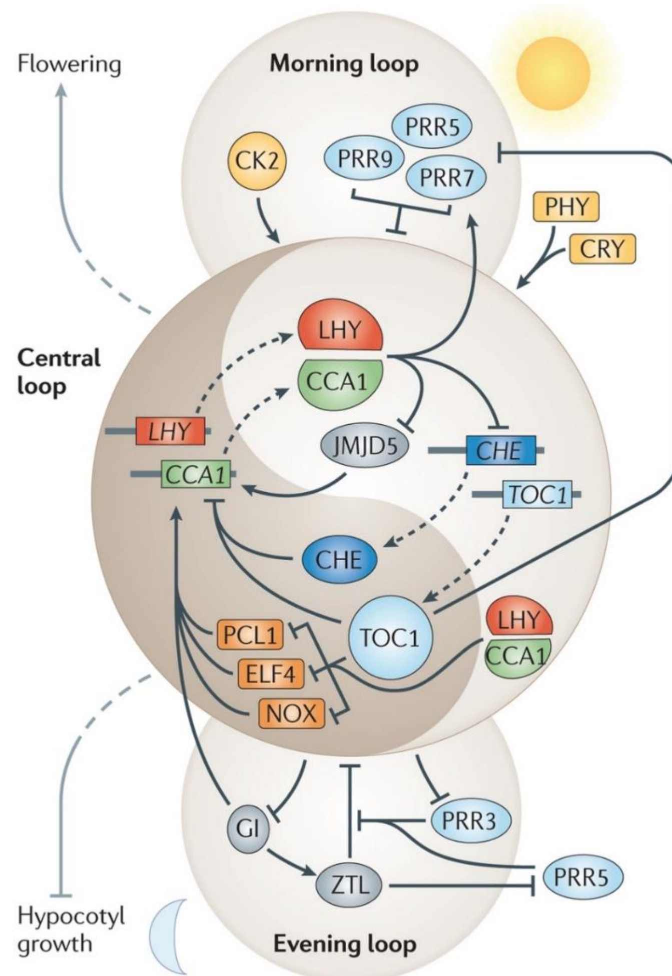

Figure 5: The regulatory network of the circadian clock in *Arabidopsis thaliana* (taken from Chen, 2013). The circadian clock consists of a central loop and two side loops. The CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and their regulator TIMING OF CAB EXPRESSION 1 (TOC1) represent the central loop. CCA1 and LHY are activating the gene expression of the genes in the morning loop. In the course of the day CCA1 and LHY decrease and TOC1 can be produced as the expression is inhibited by CCA1 and LHY. The protein TOC1 accumulates in the evening inhibiting the expression *CCA1* and *LHY*. Expression of the genes in the evening loop are induced TOC1. In the night TOC1 decreases and *CCA1* and *LHY* can be expressed again at dawn.

The circadian-regulated pathogen defense in plants

The regulation of central components of the defense pathway by the circadian clock enables the plant to anticipate the pathogen attack in a more predictable way. The role of the circadian clock in the immunity of the plants got more prominent as it was shown that a functional clock is beneficial for an enhanced fitness (Dodd et al., 2005). Transcriptome analysis in *A. thaliana* led to the identification of circadian clock regulated genes involved in the PTI and the R-mediated defense (Sauerbrunn and Schlaich, 2004; Wang et al., 2011). The defense hormone signaling in plants is also under circadian control as the accumulation of SA and JA is clock regulated (Goodspeed et al., 2012). In this context variations in the susceptibility dependent on the time of the day and pathogen are likely to occur (Bhardwaj et al., 2011; Ingle et al., 2015). The circadian control of the immunity might have been evolved as a response to the timed stages of the pathogens to be strongest at the time when they are most susceptible to an infection. The fungal sporulation as an example is likely to occur at night, whereas the spore dissemination mainly occurs at dawn (Wang et al., 2011; Slusarenko and Schlaich, 2003). Therefore, it would be beneficial for the plant maximizing the level of defense compounds at the time of the day when the encounter with the pathogen is more likely to occur. Applying sulfur at a certain time of the day might improve the SED in plants as the immunity in plants is dependent on the time of the day. The dynamic nutrient fluxes are tightly linked to rhythmic physiology and nutrient uptake is likely to be under circadian control (Haydon et al., 2015). As the nutrient uptake mainly occurs at dawn applying sulfur at that time would be more beneficial. In this context a circadian regulation of the sulfur metabolism might result in a maximized defense response. Based on that fertilizer strategies can be developed maximizing the resistance response in plants when applying sulfur at the right time of the day. This leads ultimately to the question: Is it possible to maximize the SED by applying sulfur at the right time resulting in further enhancement of the plant defense?

Aims of the thesis

- Comparison of different *B. napus* cultivars in response to induced sulfur deficiency and pathogen attack
- Development of a growth system in a suitable and reproducible way for analyzing the circadian rhythm in *B. napus*
- Optimization of the method for the expression analysis in a complex experimental setting in *B. napus*
- Determining a circadian regulated sulfur assimilation regarding the sulfur status in the plants by analyzing key enzymes of the sulfur metabolism as well as sulfur-containing compounds
- Analyzing the circadian regulated immunity in *B. napus* by infecting the plants with *V. longisporum* at different times of the day

References

Allender, C.J. and King, G.J., (2010) Origins of the amphiploid species *Brassica napus L.* investigated by chloroplast and nuclear molecular markers. *BMC Plant Biol.*, 10(1), p.54. doi:10.1186/1471-2229-10-54 .

Álvarez, C., Bermúdez, M.Á., Romero, L.C., Gotor, C. and García, I., (2012) Cysteine homeostasis plays an essential role in plant immunity. *New Phytol.*, 193(1), pp.165–77. doi:10.1111/j.1469-8137.2011.03889.x .

Bebber, D.P., Holmes, T., Smith, D. and Gurr, S.J., (2014) Economic and physical determinants of the global distributions of crop pests and pathogens. *New Phytol.*, 202(3), pp.901–10. doi:10.1111/nph.12722 .

Bhardwaj, V., Meier, S., Petersen, L.N., Ingle, R.A. and Roden, L.C., (2011) Defense responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. *PLoS ONE* 6(10): e26968. doi:10.1371/journal.pone.0026968 .

Bloem, E., Riemenschneider, A., Volker, J., Papenbrock, J., Schmidt, A., Salac, I., Haneklaus, S. and Schnug, E., (2004) Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus L. J. Exp. Bot.*, 55(406), pp.2305–12. doi:10.1093/jxb/erh236 .

Bloem, E., Haneklaus, S. and Schnug, E., (2014) Milestones in plant sulfur research on sulfur-induced-resistance (SIR) in Europe. Front. Plant Sci., 5, p.779. doi:10.3389/fpls.2014.00779 .

Bollig, K., Specht, A., Myint, S.S., Zahn, M. and Horst, W.J., (2012) Sulphur supply impairs spread of *Verticillium dahliae* in tomato. *Eur. J. Plant Pathol.*, 135(1), pp.81–96. doi:10.1007/s10658-012-0067-5 .

Buchner, P., Stuiver, C.E.E., Westerman, S., Wirtz, M., Hell, R., Hawkesford, M.J. and De Kok, L.J., (2004) Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulfate nutrition. *Plant Physiol.*, 136(2), pp.3396–408. doi:10.1104/pp.104.046441 .

Cai, Y., Cao, S., Yang, Z. and Zheng, Y., (2011) MeJA regulates enzymes involved in ascorbic acid and glutathione metabolism and improves chilling tolerance in loquat fruit. *Postharvest Biol. Technol.*, 59(3), pp.324–326. doi:10.1016/j.postharvbio.2010.08.020 .

CFIA (2015) Verticillium wilt –*Verticillium longisporum*. Canadian Food Inspection Agency. Available at: http://www.inspection.gc.ca/plants/plant-pests-invasive-species/diseases/verticillim-wilt/eng/1420746212959/1420746213803. Accessed: 20.10.2016

Chen, Z.J., (2013) Genomic and epigenetic insights into the molecular bases of heterosis. *Nat. Rev. Genet.*, 14(7), pp.471–82. doi:10.1038/nrg3503 .

Clewes, E., Edwards, S.G. and Barbara, D.J., (2008) Direct molecular evidence supports long-spored microsclerotial isolates of *Verticillium* from crucifers being interspecific hybrids. *Plant Pathol.*, 57(6), pp.1047–1057. doi:10.1111/j.1365-3059.2008.01897.x .

Dangl, J. L., and Jones, J. D. (2001) Plant pathogens and integrated defense responses to infection. *Nature*, 411(6839), 826-833.

De Mairan, J. (1729). Observation botanique. *Hist. Acad. Roy. Sci*, 35, 36.

Depotter, J.R.L., Deketelaere, S., Inderbitzin, P., von Tiedemann, A., Höfte, M., Subbarao, K. V., Wood, T.A. and Thomma, B.P.H.J., (2015) *Verticillium longisporum* , the invisible threat to oilseed rape and other *Brassicaceous* plant hosts. *Mol. Plant Pathol.*, 17: 1004–1016. doi:10.1111/mpp.12350 .

Dodd, A.N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd, J.M., Millar, A.J. and Webb, A. a R., (2005) Plant circadian clocks increase photosynthesis , growth , survival , and competitive advantage. *Science*, 309, pp.630–634.

Downey, R. K., Janick, J., & Simon, J. E. (1990). Canola: a quality brassica oilseed. In advances in new crops. *Proceedings of the first national symposium New crops: research, development, economics'*, Indianapolis, Indiana, USA, 23-26 October 1988. (pp. 211-215). Timber Press.

DSV: Deutsche Saatveredelung AG. Benefits of hybrid breeding. Available: https://www.dsv-uk.co.uk/company/Benefitshybrids.html Accessed 9 November 2016

Dubuis, P.-H., Marazzi, C., Stadler, E. and Mauch, F., (2005) Sulphur deficiency causes a reduction in antimicrobial potential and leads to increased disease susceptibility of oilseed rape. *J. Phytopathol.*, 153(1), pp.27–36. doi:10.1111/j.1439-0434.2004.00923.x .

Dunker, S., Keunecke, H., Steinbach, P. and von Tiedemann, A., (2008) Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *J. Phytopathol.*, 156(11–12), pp.698–707. doi:10.1111/j.1439-0434.2008.01429.x .

Farré, E.M., (2012) The regulation of plant growth by the circadian clock. *Plant Biol.*, 14(3), pp.401–410. doi:10.1111/j.1438-8677.2011.00548.x .

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. and Gurr, S.J., (2012) Emerging fungal threats to animal, plant and ecosystem health. *Nature*, 484(7393), pp.186–94. doi:10.1038/nature10947 .

Flood, J., (2010) The importance of plant health to food security. *Food Secur.*, 2(3), pp.215–231. doi:10.1007/s12571-010-0072-5 .

Flor, H. H. (1971) Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.*, 9(1), 275-296.

Forsyth, W. (1802) *A Treatise on the Culture and Management of Fruit Trees. London*: Nichols and Son.

Foyer, C.H. and Halliwell, B., (1976) The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta*, 133(1), pp.21–5. doi:10.1007/BF00386001 .

Fradin, E.F. and Thomma, B.P.H.J., (2006) Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. alboatrum*. *Mol. Plant Pathol.*, 7(2), pp.71–86. doi:10.1111/j.1364-3703.2006.00323.x .

Gladders, P., Smith, J.E., Kirkpatrick, L., Clewes, E., Grant, C., Barbara, D.J., Barnes, A. V. and Lane, C.R., (2011) First record of verticillium wilt (*Verticillium longisporum*) in winter oilseed rape in the UK. *New Dis. Reports*. 23(8). ISSN 2044-0588

Glazebrook, J., (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.*, 43, pp.205–27. doi:10.1146/annurev.phyto.43.040204.135923 .

Goldhofer, H. and Schmid, W. (2016) Ölsaaten und Eiweißpflanzen. *Agrarmärkte 2016.* Bayerische Landesanstalt für Landwirtschaft.

Goodspeed, D., Chehab, E.W., Min-Venditti, A., Braam, J. and Covington, M.F., (2012) *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. *Proc. Natl. Acad. Sci. U. S. A.*, 109(12), pp.4674–7. doi:10.1073/pnas.1116368109 .

Halberg, F., Halberg, E., Barnum, C. P., & Bittner, J. J. (1959). Physiologic 24-hour periodicity in human beings and mice, the lighting regimen and daily routine. *Photoperiodism and related phenomena in plants and animals*, 55, 803-878.

Happstadius, I., Ljungberg, A., Kristiansson, B. and Dixelius, C., (2003) Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. *Plant Breed.*, 122(1), pp.30–34. doi:10.1046/j.1439-0523.2003.00774.x .

Haneklaus, S., Bloem, E., and Schnug, E. (2007) Sulfur and plant disease. *Mineral elements and plant disease. APS Press: St Paul, Minneapolis, USA*.

Haneklaus, S., Bloem, E., & Schnug, E. (2009) Plant disease control by nutrient management: sulphur. *Disease Control in Crops: Biological and Environmentally Friendly Approaches*, 221-236.

Hawkesford, M.J., (2003) Transporter gene families in plants: the sulphate transporter gene family - redundancy or specialization? *Physiol. Plant.*, 117(2), pp.155–163. doi:10.1034/j.1399-3054.2003.00034.x .

Haydon, M.J., Román, Á. and Arshad, W., (2015) Nutrient homeostasis within the plant circadian network. *Front. Plant Sci.*, 6(April), p.299. doi:10.3389/fpls.2015.00299 .

Heale, J.B. and Karapapa, V.K., (1999) The verticillium threat to canada's major oilseed crop: canola. *Can. J. Plant Pathol.*, 21(1), pp.1–7. doi:10.1080/07060661.1999.10600114 .

Huber, D. M., & Haneklaus, S., (2007) Managing nutrition to control plant disease. *Landbauforschung Volkenrode*, 57(4), 313.

Hughes, P., Dennis, E., Whitecross, M., Llewellyn, D. and Gage, P., (2000) The Cytotoxic Plant Protein, β-Purothionin, Forms Ion Channels in Lipid Membranes. *J. Biol. Chem.*, 275(2), pp.823–827. doi:10.1074/jbc.275.2.823 .

Inderbitzin, P., Davis, R.M., Bostock, R.M. and Subbarao, K. V, (2011) The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS One*, 6(3), p.e18260. doi:10.1371/journal.pone.0018260 .

Ingle, R.A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L.C. and Denby, K.J., (2015) Jasmonate signalling drives time-of-day differences in susceptibility of *Arabidopsis* to the fungal pathogen *Botrytis cinerea*. *Plant J.* doi:10.1111/tpj.13050 .

Institute of Medicine (2011) *Fungal Diseases*, Washington, D.C.: National Academies Press. doi:10.17226/13147 .

Iqbal, N., Masood, A., Khan, M.I.R., Asgher, M., Fatma, M. and Khan, N.A., (2013) Cross-talk between sulfur assimilation and ethylene signaling in plants. *Plant Signal. Behav.*, 8(1), p.e22478. doi:10.4161/psb.22478 .

Jones, J.D.G. and Dangl, J.L., (2006) The plant immune system. *Nature*, 444(7117), pp.323–9. doi:10.1038/nature05286 .

Jost, R., Altschmied, L., Bloem, E., Bogs, J., Gershenzon, J., Hähnel, U., Hänsch, R., Hartmann, T., Kopriva, S., Kruse, C., Mendel, R.R., Papenbrock, J., Reichelt, M., Rennenberg, H., Schnug, E., Schmidt, A., Textor, S., Tokuhisa, J., Wachter, A., Wirtz, M., Rausch, T. and Hell, R., (2005) Expression profiling of metabolic genes in response to methyl jasmonate reveals regulation of genes of primary and secondary sulfur-related pathways in *Arabidopsis thaliana. Photosynth. Res.*, 86(3), pp.491–508. doi:10.1007/s11120-005-7386-8 .

Karapapa, V. K., Bainbridge, B. W., and Heale, J. B., (1997) Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycol. Res.*, 101(11), 1281-1294.

Klikocka, H., Haneklaus, S., Bloem, E. and Schnug, E., (2005) Influence of sulfur fertilization on infection of potato tubers with *Rhizoctonia solani* and *Streptomyces scabies*. *J. Plant Nutr.*, 28(5), pp.819–833. doi:10.1081/PLN-200055547 .

Kroeker, G. (1970) Wilt of rape and turnip rape in sweden caused by *Verticillium*. *Svensk Frotidning*, 39(1), 10-13.

Kruse, C., Jost, R., Lipschis, M., Kopp, B., Hartmann, M. and Hell, R., (2007) Sulfur-enhanced defense: effects of sulfur metabolism, nitrogen supply, and pathogen lifestyle. *Plant Biol.* 9(5), pp.608–19. doi:10.1055/s-2007-965432 .

Kruse, C., Haas, F.H., Jost, R., Reiser, B., Reichelt, M., Wirtz, M., Gershenzon, J., Schnug, E. and Hell, R., (2012) Improved sulfur nutrition provides the basis for enhanced production of sulfur-containing defense compounds in *Arabidopsis thaliana* upon

inoculation with *Alternaria brassicicola*. *J. Plant Physiol.*, 169(7), pp.740–3. doi:10.1016/j.jplph.2011.12.017 .

Kuc, J., (1994) Relevance of phytoalexins – a critical review. *Acta. Horticulturae* 381, 526–539.

Lunde, C., Zygadlo, A., Simonsen, H.T., Nielsen, P.L., Blennow, A., and Haldrup, A., (2008) Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. *Physiol. Plant.*, 134(3), pp.508–21. doi:10.1111/j.1399-3054.2008.01159.x .

Macho, A. P., and Zipfel, C., (2015) Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Curr. Opin. Microbiol.*, 23, 14-22.

Mazid, M., Khan, T. A., and Mohammad, F., (2011) Role of secondary metabolites in defense mechanisms of plants. *Biology and medicine*, 3(2), 232-249.

McClung, C.R., (2006) Plant circadian rhytms. *Plant Cell*, 18, pp.792–803. doi:10.1017/CBO9781107415324.004 .

Mithen, R., (1992) Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. *Euphytica*, 63(1-2), 71-83.

Oerke, E.-C., (2005) Crop losses to pests. *J. Agric. Sci.*, 144(1), p.31. doi:10.1017/S0021859605005708 .

Okubara, P., and Paulitz, T. (2005). Root defense responses to fungal pathogens: a molecular perspective. In Root Physiology: from Gene to Function, vol. 4, *Plant Ecophysiology*, H. Lambers, and T. Colmer, eds. (Springer), p.215–226.

Okubara, P.A., Dickman, M.B. and Blechl, A.E., (2014) Molecular and genetic aspects of controlling the soilborne necrotrophic pathogens *Rhizoctonia* and *Pythium*. *Plant Sci.*, 228, pp.61–70. doi:10.1016/j.plantsci.2014.02.001 .

Pantou, M.P., Strunnikova, O.K., Shakhnazarova, V.Y., Vishnevskaya, N.A., Papalouka, V.G. and Typas, M.A., (2005) Molecular and immunochemical phylogeny of *Verticillium* species. *Mycol. Res.*, 109(8), pp.889–902. doi:10.1017/S0953756205003345 .

Pennisi, E., (2010) Armed and dangerous. *Science*, 327(5967), pp.804–5. doi:10.1126/science.327.5967.804 .

Rausch, T. and Wachter, A., (2005) Sulfur metabolism: a versatile platform for launching defense operations. *Trends Plant Sci.*, 10(10), pp.503–9. doi:10.1016/j.tplants.2005.08.006 .

Röbbelen, S., Downey, R. K., & Ashri, A. (1989). Oilcrops of the world: their breeding and utilization. McGraw-Hill Publishing company.

Rygulla, W., Snowdon, R.J., Eynck, C., Koopmann, B., von Tiedemann, A., Lühs, W. and Friedt, W., (2007) Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97(11), pp.1391–6. doi:10.1094/PHYTO-97-11-1391 .

Salac, I., Haneklaus, S. H., Bloem, E., Booth, E. J., Sutherland, K. G., Walker, K. C. and Schnug, E., (2005) Sulfur nutrition and its significance for crop resistance: a case study from Scotland. *Landbauforsch. Volk. Spec.* 283, 111-119.

Sauerbrunn, N. and Schlaich, N.L., (2004) PCC1: a merging point for pathogen defense and circadian signalling in *Arabidopsis*. *Planta*, 218(4), pp.552–61. doi:10.1007/s00425-003-1143-z .

Scherer, H.W., (2001) Sulphur in crop production — invited paper. *Eur. J. Agron.*, 14(2), pp.81–111. doi:10.1016/S1161-0301(00)00082-4 .

Schnug, E., and Haneklaus, S., (1994) Sulphur deficiency in *Brassica napus*-biochemistry-symptomatology-morphogenesis. *Landbauforschung Voelkenrode. Sonderheft (Germany).* no. 144.

Schnug, E., Booth, E., Haneklaus, S., and Walker, K. C., (1995) Sulphur supply and stress resistance in oilseed rape. *In Proceedings of the 9th international rapeseed congress*, Cambridge Vol. 1995, pp. 229-231.

Schnug, E., (1997) Significance of sulphur for the quality of domesticated plants. *Sulphur metabolism in higher plants: molecular, ecophysiological and nutritional aspects.*

Schnug, E., and Haneklaus, S., (2005) Sulphur deficiency symptoms in oilseed rape (*Brassica napus L.*)-the aesthetics of starvation. *Phyton* (Austria), 45.3 (2005): 79.

Seo, P.J. and Mas, P., (2015) Sressing the role of the plant circadian clock. *Trends Plant Sci.*, 20(4), pp.230–7. doi:10.1016/j.tplants.2015.01.001 .

Slusarenko, A.J. and Schlaich, N.L., (2003) Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). *Mol. Plant Pathol.*, 4(3), pp.159–70. doi:10.1046/j.1364-3703.2003.00166.x .

Statistisches Bundesamt, (2016) Anbaufläche ausgewählter Anbaukulturen im Zeitvergleich. Available at: https://www.destatis.de/DE/ZahlenFakten/Wirtschaftsbereiche/LandForstwirtschaftFischerei/FeldfruechteGruenland/Tabellen/FeldfruechteZeitreihe.html Accessed: 20.10.2016

Stefansson, B. R., (1983) The development of improved rapeseed cultivars. *High and Low Erucic Acid Rapeseed Oils,* 143-159.

Steventon, L.A., Fahleson, J., Hu, Q. and Dixelius, C., (2002) Identification of the causal agent of Verticillium wilt of winter oilseed rape in Sweden, *V. longisporum*. *Mycol. Res.*, 106(5), pp.570–578. doi:10.1017/S0953756202005865 .

UFOP E. V., (2016) Rapsöl bleibt weiter vorn. Available at: http://www.presseportal.de/pm/15598/3272537 Accessed: 20.10.2016

Walker, K. C., and Booth, E. J., (1992) Sulphur research on oilseed rape in Scotland. *Sulphur Agric*., 16, 15-19.

Wallsgrove, R., Benett, R., Kiddle, G., Bartlet, E., and Ludwig-Mueller, J., (1999) Glucosinolate Biosynthesis and Pest Disease Interactions. *Canbarra,CBR: Proceedings of the 10th Intenational Rapeseed Congress.*

Walters, D.R. and Bingham, I.J., (2007) Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. *Ann. Appl. Biol.*, 151(3), pp.307–324. doi:10.1111/j.1744-7348.2007.00176.x .

Wang, W., Barnaby, J.Y., Tada, Y., Li, H., Tör, M., Caldelari, D., Lee, D., Fu, X.-D. and Dong, X., (2011) Timing of plant immune responses by a central circadian regulator. *Nature*, 470(7332), pp.110–4. doi:10.1038/nature09766 .

Zeise, K. and von Tiedemann, A., (2002) Host Specialization among Vegetative Compatibility Groups of *Verticillium dahliae* in Relation to *Verticillium longisporum*. *J. Phytopathol.*, 150(3), pp.112–119. doi:10.1046/j.1439-0434.2002.00730.x .

CHAPTER 2

Brassica napus L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack.

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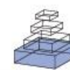

Brassica napus L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack

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Under adequate sulfur supply, plants accumulate sulfate in the vacuoles and use sulfur-containing metabolites as storage compounds. Under sulfur-limiting conditions, these pools of stored sulfur-compounds are depleted in order to balance the nitrogen to sulfur ratio for protein synthesis. Stress conditions like sulfur limitation and/or pathogen attack induce changes in the sulfate pool and the levels of sulfur-containing metabolites, which often depend on the ecotypes or cultivars. We are interested in investigating the influence of the genetic background of canola (*Brassica napus*) cultivars in sulfur-limiting conditions on the resistance against *Verticillium longisporum*. Therefore, four commercially available *B. napus* cultivars were analyzed. These high-performing cultivars differ in some characteristics described in their cultivar pass, such as several agronomic traits, differences in the size of the root system, and resistance to certain pathogens, such as *Phoma* and *Verticillium*. The objectives of the study were to examine and explore the patterns of morphological, physiological and metabolic diversity in these *B. napus* cultivars at different sulfur concentrations and in the context of plant defense. Results indicate that the root systems are influenced differently by sulfur deficiency in the cultivars. Total root dry mass and length of root hairs differ not only among the cultivars but also vary in their reaction to sulfur limitation and pathogen attack. As a sensitive indicator of stress, several parameters of photosynthetic activity determined by PAM imaging showed a broad variability among the treatments. These results were supported by thermographic analysis. Levels of sulfur-containing metabolites also showed large variations. The data were interrelated to predict the specific behavior during sulfur limitation and/or pathogen attack. Advice for farming are discussed.

Keywords: canola, diurnal rhythm, elemental sulfur, metabolites, *Verticillium longisporum*

INTRODUCTION

Oilseed rape or canola (*Brassica napus* L.) belongs to the Brassicaceae family. Oilseed rape is used for the production of green fuel, human consumption, as animal feed, in the chemical and pharmaceutical industry (Friedt and Snowdon, 2009), and has an enormous economical importance for many farmers in Europe (European Commission Eurostat, 2014).[1] Compared with crops such as wheat, soybean and rice, which have a long history of evolution and domestication, rapeseed is a recently domesticated species. It possibly arose as a result of interspecific hybridizations and genome doubling between diploid genotypes of turnip rape (*Brassica rapa*, $2n = 2 \times 10 = 20$, genome AA) and cabbage (*Brassica oleracea*, $2n = 2 \times 9 = 18$, genome CC) that occurred spontaneously during medieval times or earlier (Iñiguez-Luy and Federico, 2011). Vollmann and Rajcan (2009) summarized that in general, the breeding of oil crops is a more complex undertaking than breeding of cereals or legumes because most of the oil crops are dual- or multi-purpose crops. Often simultaneous manipulations are required to create different characteristics of quality.

Oilseed rape has higher requirements for nitrogen, phosphorus and sulfur than cereals and other crops. *Brassica napus* plants need approximately 40–50 kg of nitrogen (30% more than wheat), 8 kg phosphorus and 10 kg sulfur per metric ton of grain produced. In fact, wheat needs 15–25 kg sulfur ha^{-1}, whereas *B. napus* needs 30–50 kg sulfur ha^{-1} (Bloem and Haneklaus, 2002).

The high demand of sulfur supplementation in rapeseed occurred as a cause of sulfur-deficient soils and dramatic reduction in atmospheric deposition of sulfur in recent years due to enhanced emission controls (Dämmgen et al., 1998; Lewandowska and Sirko, 2008). This reduction has had significant impact on agriculture; most notably as oilseed rape has been exhibiting sulfur deficiency symptoms. (Schnug et al., 1995).

[1]European Commission Eurostat (2014). Available online at: epp.eurostat.ec.europa.eu/tgm/table.do?tab=table&init=1&language=de&pcode=tag00099&plugin=1. accessed 15.09.2014

Under sulfur limitation [for definitions of the sulfur status see Scherer (2001)], crops begin to develop sulfur deficiency symptoms such as reduced plant growth and chlorosis of the younger leaves (Grant and Kovar, 2012). Symptoms become visible in crop plants in the following order: first in oilseed rape, potato, sugar beet, beans, peas, cereals and finally in maize. The total sulfur concentration in tissues corresponding to the first appearance of deficiency symptoms is highest in oilseed rape (3.5 mg sulfur g^{-1} dry weight, DW) and lowest in the Gramineae (1.2 mg sulfur g^{-1} DW) (Haneklaus et al., 2007). Long term sulfur deficiency can lead to reduced yield and crop quality (Ahmad and Abdin, 2000; Scherer, 2001). An increase of diseases due to enhanced emission controls led to the hypothesis that there might be a relationship between the sulfur supply, the high sulfur demand of rape (Holmes, 1980), and defense mechanisms against fungal diseases. The hypothesis of sulfur-induced resistance (SIR) (Schnug et al., 1995) and a sulfur-enhanced defense (SED) (Kruse et al., 2007) was proposed.

In recent years, lab-scale experiments have produced substantial amounts of data supporting the conjecture that sulfur-containing compounds play a role in pathogen defense. *Arabidopsis thaliana* wild-type and knockout plants were used to investigate the role of cysteine in response to pathogen attack by using *Pseudomonas syringae* pv. *tomato, Botrytis cinerea* (Álvarez et al., 2011), and *Alternaria brassicicola* (Kruse et al., 2012). Cysteine is a precursor for essential vitamins, cofactors, and many defense compounds such as glucosinolates (GSL), thionins, or phytoalexins (Smith and Kirkegaard, 2002; Van Wees et al., 2003; Rausch and Wachter, 2005). In infected plants, the cysteine content decreased by 24–28% but a 14–15% increase in glutathione content was observed (Álvarez et al., 2011).

Williams et al. (2002) demonstrated that elemental sulfur was formed and accumulated in tomato plants in response to infection with *Verticillium dahliae.* In older leaves, the sulfate content increased after 14 days post-infection (dpi), indicating that sulfate levels in infected plants were dependent on the leaf age. Significant changes in cysteine levels of plants infected with *V. dahliae* were measured in the stem vascular tissue. The same was observed for the glutathione content in leaves. In the past, reduction of sulfur containing metabolites especially in young leaves was shown in *B. napus* under sulfur limitation (Blake-Kalff et al., 1998).

Field experiments with *B. napus* and *Pyrenopeziza brassicae* have shown an increase of the thiol concentration. Crops were able to react to a fungal infection and had a greater potential to release H_2S, which is reflected by a positive correlation between L-cysteine desulfhydrase enzyme activity and fungal infection levels (Bloem et al., 2004). Consecutive greenhouse experiments have shown that already after one dpi, the H_2S emission of plants grown under full sulfur supply increased strongly (Bloem et al., 2012).

Haneklaus et al. (2007) concluded that under sulfur deficiency, *B. napus* develops the most distinctive and most specific expression of phenotypic symptoms. No difference in the symptomatology of sulfur deficiency was observed in high and low containing GSL cultivars. Differences in their susceptibility against fungal infections were not unambiguously demonstrated so far.

Several winter rape cultivars with low erucic acid and low total GSL contents, $<30\,\mu mol\ g^{-1}$ defatted seed meal, were investigated to determine their contents of GSL in leaves and roots. Also in these organs, the GSL levels are rather low. In leaves, the concentrations of total GSL were about $3\,\mu mol\ g^{-1}$ DM and in roots, about $18\,\mu mol\ g^{-1}$ DM (Eberlein et al., 1998). In very recent high-performance cultivars, the GSL contents are even lower (see **Table 1**) and probably do not contribute much to the total sulfur amount in modern *B. napus* cultivars (Eberlein et al., 1998).

As mentioned before, yield reductions of *B. napus* is caused by not only a sulfur deficiency, but also a Verticillium infection (Dunker et al., 2008). The soil-borne vascular fungal pathogen *Verticillium longisporum* is one of the most important yield-minimizing pathogens of oilseed rape, and there has been no approved fungicide available until now (Heale and Karapapa, 1999; Friedt and Snowdon, 2009). In addition, the fungus survives in the soil for long periods through the production of microsclerotia. Therefore, selection of suitable resistant cultivars and optimized cultivation methods need to be developed. As was reported previously, many pathogens attack plants during dawn by dispersing their spores (Wang et al., 2011). However, there is no knowledge of exactly when *V. longisporum* attacks. Interestingly, expression studies of adenosine 5′-phosphosulfate reductase (APR) indicate that sulfur assimilation is controlled in a diurnal way (Kopriva et al., 1999).

Results from Burandt et al. (2001) indicate that the capability to use available soil sulfur is genetically controlled. Therefore, different high-performance cultivars currently cultivated in Europe were analyzed. The high-performance cultivar plants were compared at early stages of development under controlled conditions, irrespective of the final yield. (I) We were interested in the metabolic reaction to sulfur limitation and the reaction of *B. napus* cultivars to pathogen attack of *V. longisporum.* (II) To better understand the mechanisms of SED of the high-sulfur-demanding *B. napus* plants, the influence of time points during the day was analyzed. (III) The plants were comprehensively analyzed by measuring biometrical and physiological parameters, levels of several sulfur- and non-sulfur containing metabolites, and gene expression levels. After analyzing *B. napus* plants in an early stage of development, a recommendation for a promising cultivar to avoid yield loss is given.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Plant material

Winter oilseed rape seeds from the cultivars Compass, Exocet, Genie and King10 were obtained from the D[2] eutsche Saatveredelung AG (DSV) (Lippstadt, Germany) (http://www.dsv-saaten.de/raps/winterraps/sorten). The most important traits are summarized in **Table 1**. Compass is an MSL-hybrid (MSL, Male Sterility Lembke), has an excellent resistance to lodging of insects and has been on the market in Germany since 2009. Exocet is an OGURA-hybrid, which has been introduced to the market in 2005 (OGURA, expressed as cytoplasmatic male

[2]Deutsche Saatveredelung, A. G. (2014). Available online at: http://www.dsv-saaten.de/raps/winterraps/sorten [Accessed 01. 07. 2014]

Table 1 | Summary of available data about the different cultivars.

Cultivar	Type	Vigor	Resistance to *Verticillium* wilt	Root system	Oil content	GSL content [μmol g^{-1} seed DM]
Compass	H	+++	++++	++++	++++++	<18
Exocet	H	++++++	+++	++++++	++++	<25
Genie	H	++++++	++++	+++	+++++	<25
King10	L	++++++	++++++	–	++++++	<25

H, hybrid; L, line-bred; +, intensity; –, no information. The intensity is rated based on parameter values found on the websites given in Materials and Methods (Range from + very low to ++++++ very high). DM, dry mass; GSL, glucosinolates.

sterility, originated from *Raphanus sativus*) (Ogura, 1968). It has a high grain yield potential and excellent resistance against blackleg. Genie is an MSL-hybrid and has been introduced in 2010. It is very vital and has an extremely low temperature resistance. In addition to the three hybrid varieties, we have chosen line King10 that is a line-bred cultivar and was market-authorized in 2009. The yield of line King10 is comparable with new high-performance hybrids. The three varieties and the line will be summed up as cultivars. More information about the cultivars can be found on several websites (www.rapool.de[3], www.roth-agrar.de[4] and www.dsv-saaten.de/raps/winterraps/sorten).

Plant growth

For infection experiments, 30 seeds per plate of all four cultivars were sterilized and placed on plates containing solidified Blake-Kalff medium (Blake-Kalff et al., 1998) with 1 mM $MgSO_4$. After 7 day of germination in a climatic chamber [22°C, 70% humidity, 12 h light/12 h dark, 480 μmol m^{-2} s^{-1}(lamp type CMT 360LS/W/BH-E40, Eye Lighting Europe Ltd, Uxbridge, UK)], 45 seedlings were mock-inoculated with water or root dip-inoculated for 30 min (the production of spores is described below) and transferred in pots (8 cm diameter) filled with sand (0–2 mm grain size, Hornbach, Hannover, Germany). After pre-experiments reclined to experiments of Blake-Kalff et al. (1998), three different sulfur regimes with respect to sulfur concentration and volume of nutrient solution per week were chosen. As a control, one third of the pots were irrigated with Blake-Kalff medium containing 1 mM $MgSO_4$ (full sulfur supply, optimal growth conditions), the other pots were treated with Blake-Kalff medium containing 0.025 mM (moderate sulfur limitation) or 0.010 mM $MgSO_4$ (severe sulfur limitation) and additional $MgCl_2$. Each pot was watered with 150 ml of a nutrient solution weekly. After 14 dpi, the leaves and the stems of three plants of each treatment were harvested every 4 h, beginning 1 h before light was switched on (labeled 0, 4, 8, 12, and 16 h in the graphs). Plant material was pooled and directly frozen in liquid nitrogen for further analyses.

Pathogen cultivation

For the production of *Verticillium longisporum* spores, 500 μl of a frozen spore culture (isolate VL43, Enyck et al., 2009) was cultivated in 500 ml potato dextrose liquid medium (Difco PDB, Becton, Dickinson and Company, New Jersey, USA) in 1 L flasks. The flasks were incubated at 23°C in a rotary incubator at 150 rpm in darkness for 2 weeks until a dense spore suspension was produced. The concentration of the filtered spores per ml suspension was determined using a Thoma chamber and diluted with sterile water (pH 7.0) to $1*10^6$ spores per ml.

THERMOGRAPHIC ANALYSIS

The evaporative cooling as water is lost through stomata is an important component of the local leaf energy balance. Thus, leaf temperature can provide a sensitive indicator of leaf conductance to water vapor (Jones, 2007). In several studies, leaf temperature was used as an indicator of water stress, salt stress and nutrition deficiency induced stress (Chaerle et al., 2007; James and Sirault, 2012; Guretzki and Papenbrock, 2013). Furthermore, an influence due to pests and disease on the leaf temperature was observed (Allègre et al., 2007). Therefore, in this study, thermographic analysis was carried out to analyze early symptoms of stress caused by sulfur limitation or pathogen attack. The thermal imaging investigation was carried out with the camera T360 (FLIR Systems, Wilsonville, USA) according to Grant et al. (2006), in order to measure the surface temperature on plant leaves. For an optimal signal-to-noise ratio, the camera was turned on at least 30 min before the first thermographic picture was taken. For analyzing the pictures of three plants per cultivar and per treatment, the program ThermoCam Researcher 2.10 FLIR QuickReport 1.2 SP2 (FLIR Systems, Wilsonville, USA) was used. The parameters were set for each image to emissivity 0.95, reflected apparent temperature 22°C, atmospheric temperature 22°C, relative humidity 70% and distance 0.8 m.

CHLOROPHYLL FLUORESCENCE MEASUREMENTS

Chlorophyll fluorescence was determined by a PAM M series device and ImagingWin v2.32 software (Heinz Walz, Effeltrich, Germany). The measurements were performed with up to six areas of interest (AOI, points on the leaves where the measurement data points were taken) on different expanded leaves. Light curves using different photosynthetically active radiations (PAR) were examined as presented in the manufacturer's handbook. Because of the use of the filter plate IMAG-MAX/F, the effective PAR values were about 15% lower. Before taking the measurement, the plants were dark adapted for 20 min. The parameters F_v/F_m (maximal PS II quantum yield) and Y(II) (effective PS II quantum yield) were analyzed (for background information:

[3]Rapool (2014). Available online at: www.rapool.de [Accessed 01. 07. 2014]
[4]Roth-Agrar (2014). Available online at: roth-agrar.de/produkte/saatgut/saatraps/sortenliste/sortenliste_saatraps_herbst_2013 [Accessed 01. 07. 2014]

Baker, 2008; Sperdouli and Moustakas, 2012). F_v/F_m values were obtained from the false-color images, created by ImagingWin software. Measurements ($n = 5$–8) were performed 1 h after light was switched on.

BIOMASS MEASUREMENTS

For the analysis of the biomass, all plants of the cultivars were harvested, and material was divided into shoot, stem, and root categories, before being weighed. When weighing the roots, all soil particles were removed by washing the whole root system of one plant and drying it carefully with tissue. The plant material was dried in paper bags at 80°C for 4 day, and the dry mass (DM) was determined.

MORPHOLOGY

Ten sterilized seeds per plate were germinated for 5 day on three plates with Blake-Kalff medium, containing 1 mM $MgSO_4$/0.010 mM sulfate. Additionally, on half of the plates 0.5 μl of a $1*10^6$ spore suspension was applied. The roots of the seedlings were sectioned in an investigation zone that lay 1–2 cm below the root crown. Pictures of the primary roots were taken with a camera installed on a binocular (Olympus SZ2-ILST, Tokyo, Japan). The root hair lengths of five plants were determined at the computer using a ruler.

METABOLIC ANALYSIS

Elemental analysis of plant material

For the analytical measurements, pooled samples were measured at least three times, up to six times. Dry plant material was ground to fine powder (MM 400, Retsch GmbH, Haan, Germany). About 38 mg of the ground powder was incinerated for a minimum of 8 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA) for each cultivar and treatment. After cooling the samples to room temperature (RT) (between 21 and 23°C), 1.5 ml of 66% nitric acid was added. After 10 min, 13.5 ml of ultrapure water was pipetted to the samples. The solutions were filtered (0.45 μm pore size, Carl Roth, Karlsruhe, Germany) and stored in vials at −20°C before final analysis. The samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation). Fluctuations of the results were around 5% for sulfur and phosphorus. Iron measurement results showed fluctuations of about 20%.

Sulfate determination and analysis of soluble thiol compounds

Sulfate was determined by capillary electrophoresis (CE) in the following way: 30 mg of deep-frozen, fine-ground plant material was solved in 700 μl HPLC grade H_2O, mixed for 1 min, incubated at RT for 10 min, mixed again for 1 min and centrifuged for 10 min at 13,200 × g at 4°C. The supernatant was transferred to a new reaction tube, frozen overnight, and after thawing, centrifuged another 10 min. The supernatant was transferred to a 500 μl reaction tube and used for CE analysis (P/ACE™ MDQ Capillary Electrophoresis System with MDQ-PDA detector, Beckman Coulter, Krefeld, Germany). Separations were performed in a Beckman Coulter eCAP™ CE-MS capillary (fused silica, 75 μm i.d., 57 cm total length, 50 cm effective length). Before starting the analyses, the capillary was rinsed with HPLC grade H_2O for 10 min and equilibrated with the background electrolyte Basic Anion Buffer for HPCE (Agilent Technologies, Waldbronn, Germany) at 14.5 psi for 10 min. Injection was done by applying 0.7 psi for 6 s. Separation of the samples was performed by applying 14 kV, 22°C, reverse polarity for 10 min. Samples were detected at 350 nm with a bandwidth of 20 nm. Calibration graphs for sulfate were generated with 78–10,000 μM Na_2SO_4. The detection limit for this method is about 10^{-13}–10^{-16} mol. Evaluation of the electropherograms was done with Karat 32 7.0 software. The determination of thiols was done according to Riemenschneider et al. (2005).

Determination of phenols and flavonoids

To 50 mg of ground leaf material 800 μl of 80% methanol was added, mixed for 10 min and centrifuged for 5 min. The pellet was re-extracted three times with 400 μl methanol and the supernatants were combined. The whole sample was centrifuged and the supernatant stored at −70°C.

Based on the method of Dudonné et al. (2009), 100 μL of water was pipetted into a 96-well microtiter plate. Triplicates of 10 μL sample, blank (80% methanol) or gallic acid standard (5–250 μg mL^{-1}) and finally 10 μL Folin Ciocalteu reagent were added. After incubation for 8 min and addition of 100 μL 7% sodium carbonate, the plate was incubated for 100 min and measured at 765 nm. Total phenols were calculated from a standard curve.

Flavonoids were analyzed based on Dewanto et al. (2002). To each well of a clear 96-well microtiter plate 150 μl water, 25 μl sample or catechin hydrate standard (10–400 μg ml^{-1}) or 80% methanol as blank (in triplicate) and 10 μl 3.75% $NaNO_3$ were added. After 6 min incubation, 15 μL 10% $AlCl_3$ were added. After 5 min incubation, 50 μl 1 M NaOH was added and the total flavonoids were calculated from a standard curve based on the absorption at 510 nm.

Measurements of anthocyanins

For the determination of the anthocyanin content, 1 ml 1% HCl in methanol and 0.5 ml H_2O were added to 50 mg of ground plant material and incubated overnight at 4°C. Then the samples were centrifuged for 15 min at 21,000 × g at RT. The supernatants were measured at 530 and 675 nm for the anthocyanin concentration and degraded products of the chlorophyll determination, respectively (Rabino and Mancinelli, 1986). The formula for the calculation was: $c_{anthocyanin} = AU(A_{530}-(0.25^* A_{657}))/g$ FM.

SEQUENCE ANALYSIS

For the primer design, sequences homologous of *A. thaliana* DNA sequences for APR2 and APR3 sequences were searched in the *B. napus* database (Computational Biology and Functional Genomics Laboratory, 2014) using BLAST. The data bank uses parts of short homologous sequences (high-fidelity virtual transcripts; TC-sequences, tentative consensus sequences) to generate EST sequences (Quackenbush et al., 2000) that were used for the primer pair design (Primer Design version 2.2, Scientific & Educational Software, Cary, USA). For the design of the primer pairs for the amplification of cDNA fragments of sulfate transporter, the respective homologous sequences from

Brassica oleracea were used (Buchner et al., 2004a), because the *B. napus* sequences were still not available. The primers were used to amplify cDNA fragments between 339 and 973 bp (**Table 2**).

NORTHERN BLOTTING

Total RNA was extracted according to Sokolowsky et al. (1990) from ground plant material and quantified spectrophotometrically. Fifteen μg of the RNA were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium bromide. After RNA transfer onto nylon membranes, they were probed with digoxigenin-labeled cDNA probes obtained by PCR (PCR DIG probe synthesis kit, Roche, Mannheim, Germany). To amplify the respective probes, the sequence-specific primers listed in **Table 2** were used. The colorimetric detection method with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates for alkaline phosphatase was applied. Quantitative analysis of the Northern blot results was done by GelAnalyzer[5] (http://www.gelanalyzer.com). None of the common housekeeping genes was expressed under sulfur deficiency, pathogen attack, and diurnal rhythm in a constitutive way. Therefore, calculations were done in the following way: For each membrane, the band intensity of the first sample (0 h) was set to 100%. The intensities of the following bands were referred to the intensity of the first band.

STATISTICAL ANALYSIS

The biomass data were evaluated using a Three-Way ANOVA with DM as dependent variable (log-transformed to meet linear model assumptions such as normality and homogeneity of variances) and cultivar, infection, and S concentration as independent factors. Significance of factors and their interactions was assessed by means of *F*-tests; all interaction terms except cultivar: infection proved non-significant and were thus eliminated from the model. To pinpoint significant differences among factor levels, we applied Tukey tests (i.e., pairwise mean comparisons) controlling the rate of type I errors at 5%. In the presence of interactions, the Tukey comparisons were carried out separately for each level of the interacting factor.

A similar ANOVA model was fit to the (logarithmized) shoot-to-root ratios; here all interaction terms turned out to be non-significant, so we simplified the model to main effects only before performing Tukey comparisons.

Leaf temperatures and quantum yields were analyzed with ANOVA-type linear mixed-effects models including leaf-specific random effects to account for correlation among 10 replicated measurements from each of five leaves. These models could not be simplified due to all two- and three-way interactions of the factors cultivar, infection, and S concentration being significant and hence not omissible. In consequence, pairwise Tukey comparisons were carried out separately for each combination of factor levels.

All statistical computations were done in R 3.1.1 (R Core Team, 2014). The graphs were generated with SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

RESULTS

MORPHOLOGY

For the determination of the root morphology, sterilized oilseed rape seeds were grown for 5 day on Blake-Kalff medium with and without an adjusted spore suspension. The roots were separated in sections and the pictures were taken in the section one to two cm below the crown (**Figure 1A**). As visible on the pictures, Genie (D) has significantly shorter root hairs when comparing them with the cultivars Compass (B) and Exocet (C). The root hairs of Genie have an average length of 0.58 ± 0.08 mm. **Figure 1** shows that the length of the hairs is different between King10 (E) and either of Exocet and Genie. As demonstrated by the statistical analyses (Table S1), there are no significant differences between the cultivars Compass and Exocet, with an average root hair length of 1.16 and 1.14 mm. No differences in the root length could be observed at seedlings grown with less sulfate or when incubated with *V. longisporum* spores in comparison to control conditions.

BIOMASS PRODUCTION

Because of the remarkable differences in the root hair length, the biomass of the organs and especially the root system was analyzed in more detail. Oilseed rape plants were cultivated as described, weighed and dried. No visible symptoms due to infection were observed. The total DM and the shoot to root ratio was calculated (**Figure 2**). The total DM of all non-infected (C) and infected

[5]Gelanalyzer (2014). Available online at: GelAnalyzer.com [Accessed 16. 09. 2014]

Table 2 | Primers used in this study.

Primer pairs	*B. napus* DFCI TC No.	*A. thaliana* accession No.	Sequences from 5′ to 3′	bp
P226BoST4;2s	–	At3g12520	CGTTCCATAAGTCACTCAGTC	968
P227BoST4;2as			GTGTACGCTTCTGGATACTGC	
P743_BnAPR2_for	TC162152	At1g62180	CAAGAAGGAAGATGACACCACC	377
P744_BnAPR2_rev			GCGAATCGACATCTCTATGCTC	
P745_BnAPR3_for	TC186950	At4g21990	CATCAAGGAGAACAGCAACGCA	339
P746_BnAPR3_rev			TCGGGAACACTAGTATCGTCGG	

Known sequences from A. thaliana genes were used to find homologous gene sequences from B. napus or B. oleracea. Blasted sequences (compbio.dfci.harvard.edu) were used to create the primer pairs (Primer Design version 2.2). The annealing temperature was 55.3° C for all primer pairs. S, sense; as, antisense; for, forward; rev, reverse; DFCI, Dana-Faber Cancer Institute; TC, tentative consensus.

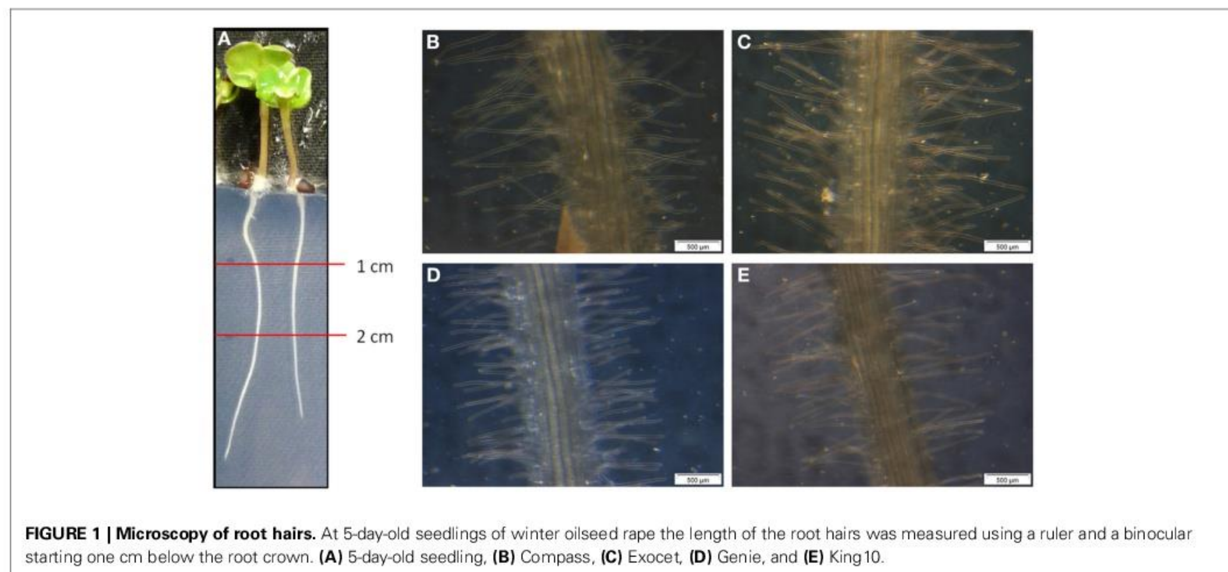

FIGURE 1 | Microscopy of root hairs. At 5-day-old seedlings of winter oilseed rape the length of the root hairs was measured using a ruler and a binocular starting one cm below the root crown. **(A)** 5-day-old seedling, **(B)** Compass, **(C)** Exocet, **(D)** Genie, and **(E)** King10.

(INF) cultivars is significantly decreased under sulfur deficiency, whereas no significant differences were observed between the deficiency conditions. Plants of the cultivar Exocet developed the highest leaf biomass with an average DM of up to 0.540 ± 0.147 g as mean values. Under sulfur limitation the biomass of leaves (−31.3%) and stems (−27.3%) decreased significantly in non-infected plants (data not shown).

Depending on the cultivar, the treatment with the fungus led to different DM. Highly significant reductions of DM in comparison to control were measured for Exocet and Genie. In infected plants, Exocet is the only cultivar showing significantly decreased biomass in all three organs (data not shown). Control plants of King10 developed significantly less DM compared to Exocet and Compass, Genie developed significantly less DM than Compass. Upon infection a significantly higher DM was determined for Compass in comparison to all other cultivars (Table S2).

As expected, the shoot to root ratio is significantly higher in plants grown under full sulfur supply compared to sulfur deficiency conditions, whereas the fungal infection has no effect. To distinguish Compass plants achieved the significantly lowest shoot to root ratio (**Figure 2B**, Table S3).

PHYSIOLOGICAL MEASUREMENTS

To detect small effects of sulfur limitation and especially of pathogen attack on the cultivars at an early stage, physiological measurements were conducted. The non-invasive method of analyzing the leaf temperature with a thermo camera was used (**Figure 3**).

In non-infected plants, the fertilization of the different cultivars generated no significant differences in the leaf temperature. Only for plants grown under sulfur deficiency did the infection led to increasing leaf temperature. Statistical analyses revealed significantly substantial increased leaf temperature as compared with 1 and 0.01 mM $MgSO_4$ in the cultivars Compass and King10 grown with 0.025 mM MgSO4 after 14 dpi. The temperature increased significantly in Compass and King10 (0.025 mM $MgSO_4$), whereas the temperature decreased under 0.01 mM $MgSO_4$ in Compass in comparison to the other cultivars. Thus, differences were measured with low sulfur supply (0.01 mM) and infection, in which King10 showed the highest and Compass the lowest temperature. Results obtained from infected plants with the thermo camera clearly indicate that significant differences between the different cultivars only occur under sulfur limitation (Table S4).

Chlorophyll fluorescence measurements were done with non-infected and infected plants grown with different sulfur concentrations (**Figure 4**). Independently of the sulfur supply, the F_v/F_m values range between 0.714 and 0.831 (data not shown), indicating photosynthetic activity in the same range when grown at higher or lower sulfur concentrations (Kitajima and Butler, 1975). Under full sulfur supply, Compass and King10 performed better (around 5%) than Exocet and Genie. A slight decrease of the quantum yield was observed in Compass and Exocet under sulfur limitation. The sulfur limitation decreased the quantum yield of photosystem II in King10 strongly. The quantum yield of Genie plants remains almost constant.

No significant differences were obtained for plants grown under full sulfur supply after infection. Infection with the fungus *V. longisporum* led to a significant decrease of the quantum yield of plants grown under sulfur limiting conditions from approximately 4.5–41%. The performance of King10 was not significantly influenced by infection (Table S5).

METABOLIC ANALYSIS

Elements

Plant material of three plants per time point were dried and used for the elemental analysis (**Figure 5**). The control plants fed with 1 mM sulfate showed amounts in a range between 4 and 7 mg sulfur per g DM (**Figure 5**). Under sulfur limitation in all cultivars,

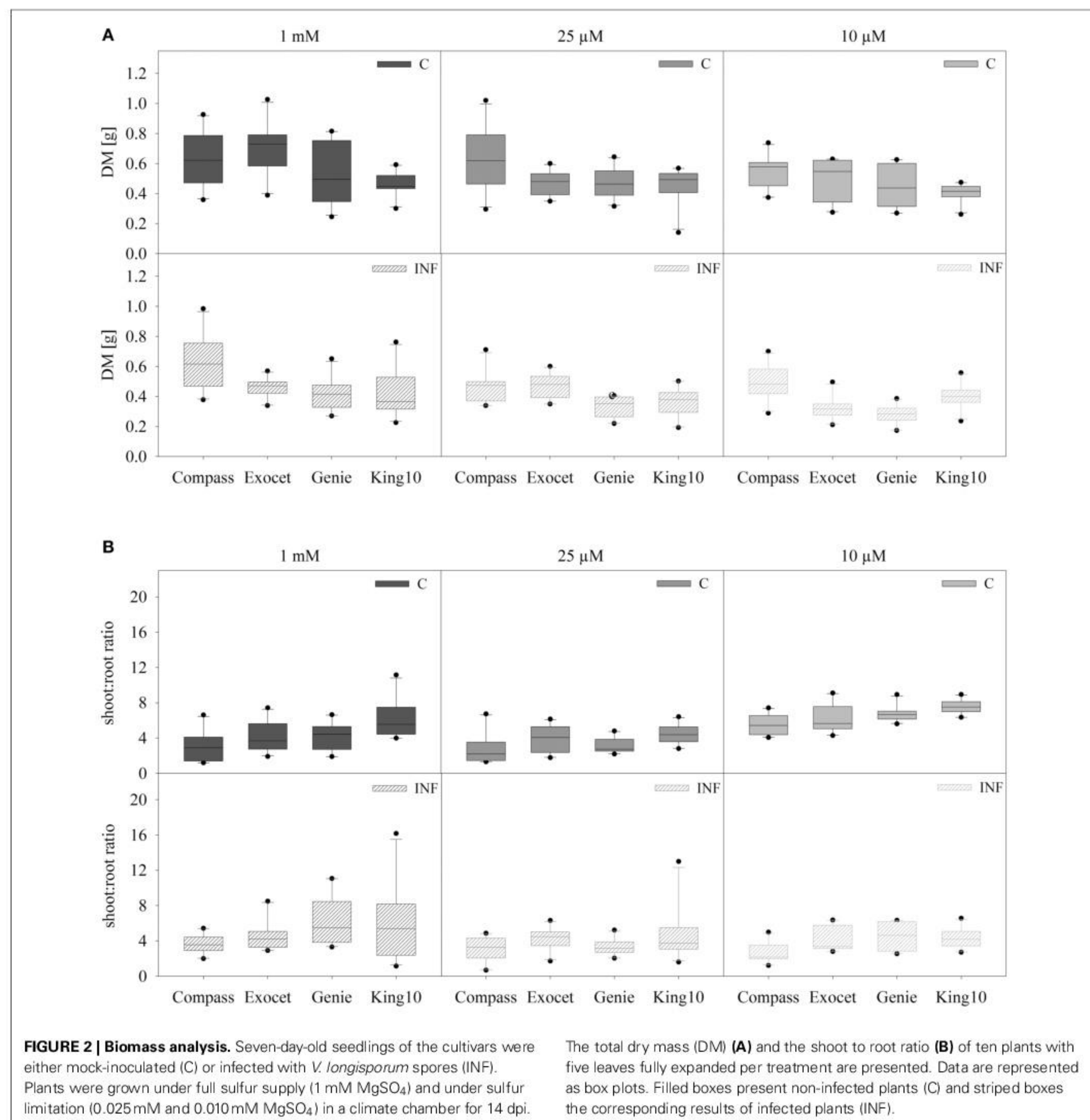

FIGURE 2 | Biomass analysis. Seven-day-old seedlings of the cultivars were either mock-inoculated (C) or infected with *V. longisporum* spores (INF). Plants were grown under full sulfur supply (1 mM $MgSO_4$) and under sulfur limitation (0.025 mM and 0.010 mM $MgSO_4$) in a climate chamber for 14 dpi. The total dry mass (DM) **(A)** and the shoot to root ratio **(B)** of ten plants with five leaves fully expanded per treatment are presented. Data are represented as box plots. Filled boxes present non-infected plants (C) and striped boxes the corresponding results of infected plants (INF).

the sulfur content decreased below 2.18 mg g^{-1} DM. Calculated over the time range of the day Genie incorporated the highest amounts of sulfur in control plants followed by Compass. On average, Exocet accumulated 20% less sulfur than Genie. King10 incorporated 3.78 times less sulfur in plants with 0.01 mM than control plants. In control plants of Compass sulfur, values show deviations over the day with a maximum at 4 h with 7.20 mg g^{-1} DM.

Infected plants of all cultivars fed with 1 mM $MgSO_4$ incorporated more sulfur in the shoots than the control plants. Interestingly, King10 incorporated over the whole time range higher sulfur amounts (7.26 mg sulfur g^{-1} DM) in the plants grown with full sulfur supply. Under sulfur limiting conditions plants infected with the fungus incorporated more sulfur than the control plants. In Genie, the sulfur content increased by 30, 66, and 80% compared with the control plants fed with 1, 0.025 and 0.01 mM $MgSO_4$.

Subsequently, iron and phosphorus were analyzed because both elements are, e.g., indispensable for energy transfer and structural components (Expert et al., 2012). Deficiencies of

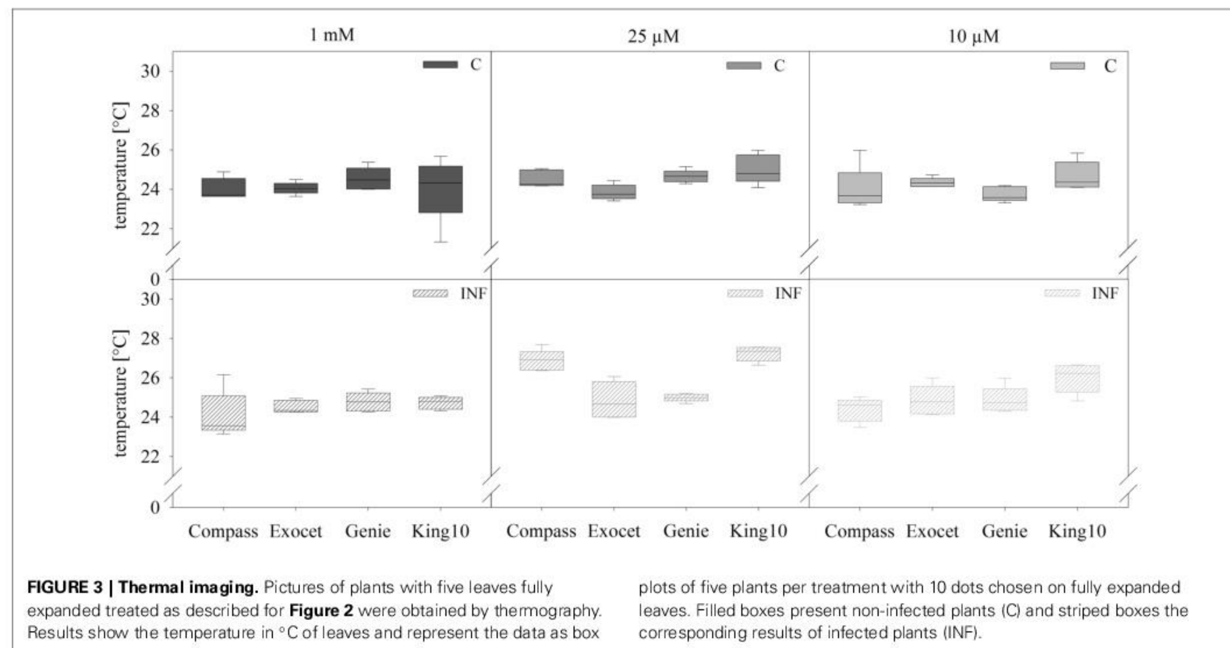

FIGURE 3 | Thermal imaging. Pictures of plants with five leaves fully expanded treated as described for **Figure 2** were obtained by thermography. Results show the temperature in °C of leaves and represent the data as box plots of five plants per treatment with 10 dots chosen on fully expanded leaves. Filled boxes present non-infected plants (C) and striped boxes the corresponding results of infected plants (INF).

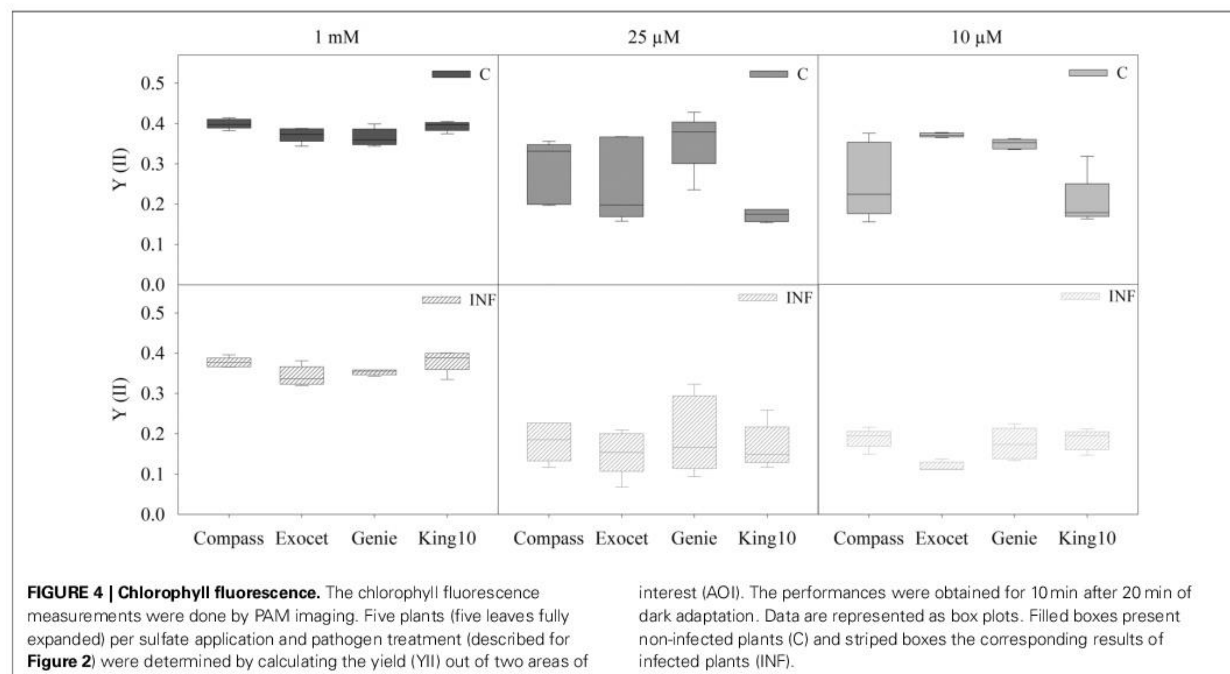

FIGURE 4 | Chlorophyll fluorescence. The chlorophyll fluorescence measurements were done by PAM imaging. Five plants (five leaves fully expanded) per sulfate application and pathogen treatment (described for **Figure 2**) were determined by calculating the yield (YII) out of two areas of interest (AOI). The performances were obtained for 10 min after 20 min of dark adaptation. Data are represented as box plots. Filled boxes present non-infected plants (C) and striped boxes the corresponding results of infected plants (INF).

phosphorus are common and frequently limit canola yields (Prabhu et al., 2007).

The plants of the cultivars incorporated under control conditions approximately 0.03–0.11 mg iron g^{-1} DM (**Figure S1**). Apart from a few sample values, the iron content decreased in plants fed with less sulfur except in the cultivar Compass. The highest amounts of iron were measured in the plants fed with 1 mM sulfate in King10 (0.06–0.11 mg iron g^{-1} DM). The iron content increased by 16.5–63%, due to infections in all cultivars.

In the cultivars, only slight differences were observed in the phosphorus content (**Figure S1**). Exocet showed the highest phosphorus values without big fluctuations. In Genie plants, the phosphorus in mg g^{-1} DM increased in the plants fed with

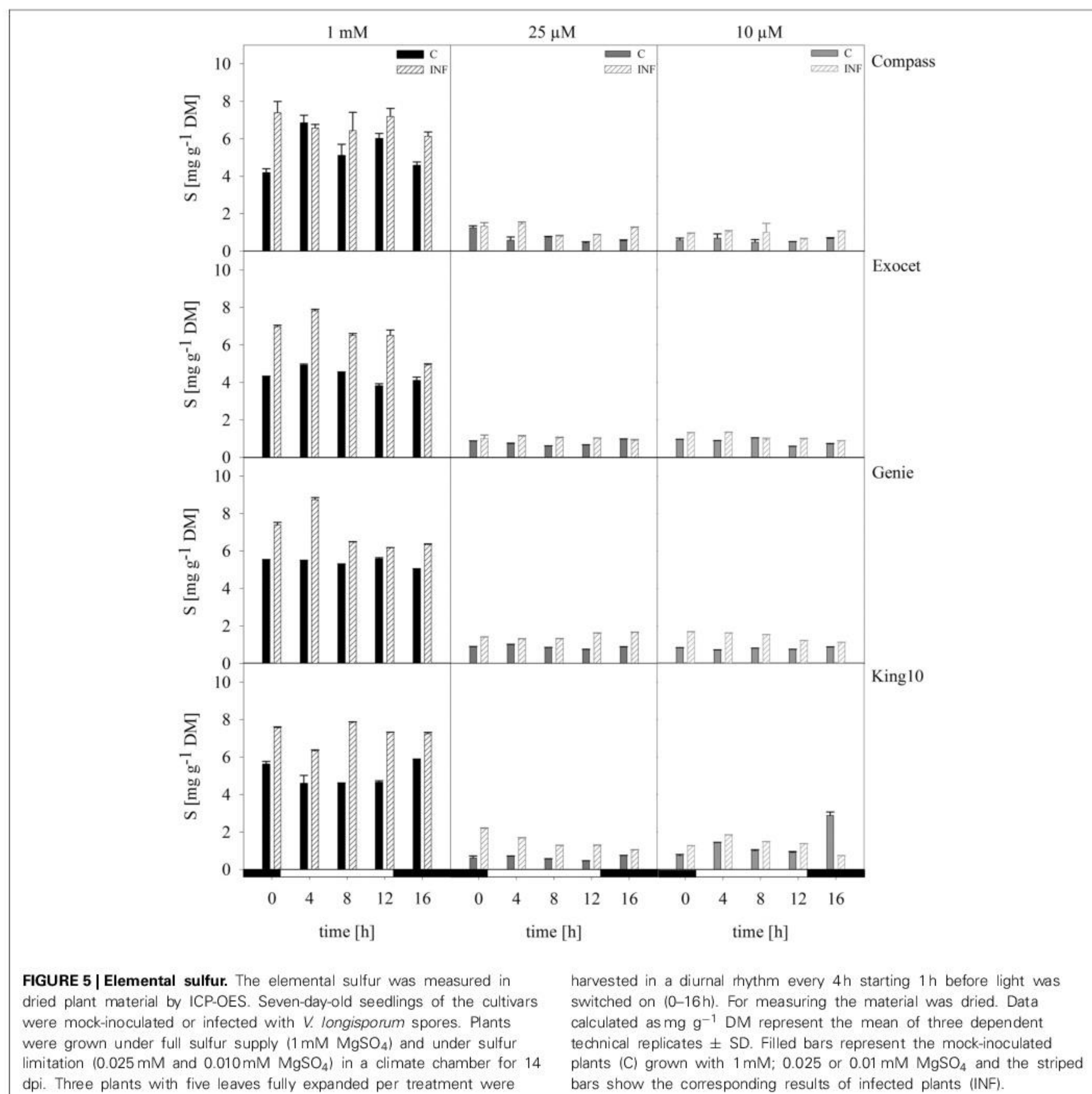

FIGURE 5 | Elemental sulfur. The elemental sulfur was measured in dried plant material by ICP-OES. Seven-day-old seedlings of the cultivars were mock-inoculated or infected with *V. longisporum* spores. Plants were grown under full sulfur supply (1 mM $MgSO_4$) and under sulfur limitation (0.025 mM and 0.010 mM $MgSO_4$) in a climate chamber for 14 dpi. Three plants with five leaves fully expanded per treatment were harvested in a diurnal rhythm every 4 h starting 1 h before light was switched on (0–16 h). For measuring the material was dried. Data calculated as mg g^{-1} DM represent the mean of three dependent technical replicates ± SD. Filled bars represent the mock-inoculated plants (C) grown with 1 mM; 0.025 or 0.01 mM $MgSO_4$ and the striped bars show the corresponding results of infected plants (INF).

0.025 mM SO_4^{2-}. The values in the line King10 remained without high fluctuation; the content of phosphorus increased under the condition of sulfur limitation in non-infected plants.

As a tendency, Compass showed less incorporated phosphorus compared to the other cultivars. The measurements furthermore showed hints that the phosphorus content in infected plants increased.

Sulfate

The anion sulfate which is taken up by the roots was measured in leaves from non-infected and infected plants using capillary electrophoresis. In the samples from plants grown with 0.025 and 0.01 mM $MgSO_4$, the sulfate contents were below the detection limit of the method used. Exemplary for dark and light conditions, the amount of sulfate, given in µmol g^{-1} FM, was analyzed at 0 (dark) and 4 h (light) in control and infected plants (**Figure 6**). For non-infected plants of the three varieties, an increase in the sulfate amount from the harvesting time point in the dark to the harvesting time point in the light was measured, whereas in line King10 the sulfate content slightly decreased. At 0 and 4 h, Genie showed the highest values with 10.93 and 13.14 µmol g^{-1} FM, respectively. An increase in the

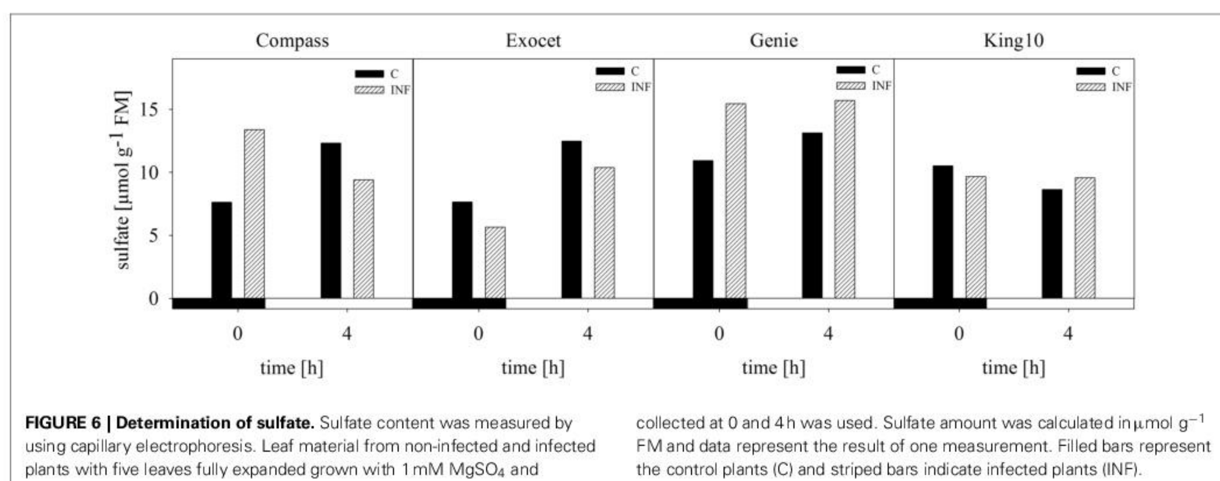

FIGURE 6 | Determination of sulfate. Sulfate content was measured by using capillary electrophoresis. Leaf material from non-infected and infected plants with five leaves fully expanded grown with 1 mM $MgSO_4$ and collected at 0 and 4 h was used. Sulfate amount was calculated in μmol g^{-1} FM and data represent the result of one measurement. Filled bars represent the control plants (C) and striped bars indicate infected plants (INF).

sulfate content in infected Compass and Genie plants at 0 h up to 43 and 30% was observed. In contrast, Exocet and King10 did not show any increase of sulfate in infected plant at 0 h. In Genie, the highest amounts of sulfate (about 16 μmol g^{-1} FM) were observed after infection, both in light and dark.

Thiols

The compounds cysteine and glutathione, which contain reduced sulfur, were analyzed in control and infected plants by HPLC. Under full sulfur, supply an increase in the cysteine content was measured ranging from 11.25 up to 27.52 nmol g^{-1} FM over the day. Highest amounts were observed after 8 h, and lowest amounts at 0 and 16 h (**Figure 7**). When comparing the amounts of cysteine among the cultivars, Exocet accumulated with full sulfur supply the highest amounts of cysteine. In plants grown under sulfur limitation, the cysteine content decreased by more than 30%. The lowest levels were obtained in the middle of the light phase. The glutathione content showed similar fluctuations over the day. However, the decrease under sulfur limitation by up to 90% is more drastic.

In infected plants grown under full sulfur supply, the amounts of cysteine and glutathione increased only slightly. Under sulfur deficiency, more cysteine and glutathione is accumulated in the leaves of infected plants. Among the cultivars, King10 showed the highest amounts of accumulated thiols in leaves of infected plants. After 8 h, the plants of King10 accumulated up to 40% more cysteine than the control plants under sulfur deficiency. Infected plants of King10 accumulated three times more glutathione than control plants.

Phenols and flavonoids

Total phenols. Phenolic compounds are bioactive components and are discussed to have high health-promoting activity. The content and composition of phenolic compounds can be used to distinguish among plant cultivars and varieties (Klepacka et al., 2011). **Figure 8** shows the phenolic contents of the cultivars. The results of the control plants showed that they accumulated the highest amounts of phenolic acids. Only slightly decreasing amounts were observed in plants grown under sulfur deficiency. The control plants of the cultivar Genie showed thereby the lowest levels (**Figure 8**). In infected plants, the phenolic levels were reduced independently of the sulfur supply.

Total flavonoids. Measurements of the total flavonoid content (**Figure S2**) showed that there were only slight differences. The values ranged between 0.6 and 1.4 μg catechin equivalent per mg FW. Interestingly, in the plants of Genie, the control plants showed more accumulated flavonoids than infected plants, independently from the amounts of sulfur applied. By contrast, King10 showed the lowest flavonoid levels in the control plants and under sulfur limitation (0.025 mM $MgSO_4$), the infected plants produced more flavonoids than the control plants.

Anthocyanins. Anthocyanins are a subgroup of the flavonoids. The values range between 0.56 and 3.45 absorption units (AU) of anthocyanins per g FM for the control plants (**Figure 9**). The lowest levels were determined in the control plants (0.56–1.37 AU g^{-1} FM). Plants grown under sulfur limitation showed increased levels. Notably, Exocet reached the highest values.

Infected plants especially from Exocet and Genie showed no differences to control plants at full sulfur supply. The anthocyanin levels increased only slightly under sulfur limitation. With less sulfur, the anthocyanin levels in King10 and Compass increased, but only in Compass the amounts exceed the levels of the non-infected plants at time points 12 and 16 h.

NORTHERN BLOT ANALYSIS

To analyze the key steps of sulfur assimilation in the oilseed rape cultivars, the expressions of *sulfate transporter 4;2* (*SULTR4;2*), and two *APR* genes were determined (**Figures 10 A–C**, **Figure S3**). As an indicator for sulfur-induced stress the expression of *SULTR4;2* was analyzed (Parmar et al., 2007). In addition, this is the only transporter expressed in leaves that rapidly responds to S deficiency (Buchner et al., 2004b). Only under sulfur limitation did the expression of the tonoplast-localized *SULTR4;2* increase strongly, indicating that S fertilization in our experiments is sufficient. The highest degree of up-regulation was detected in Compass. Light seemed to influence the expression

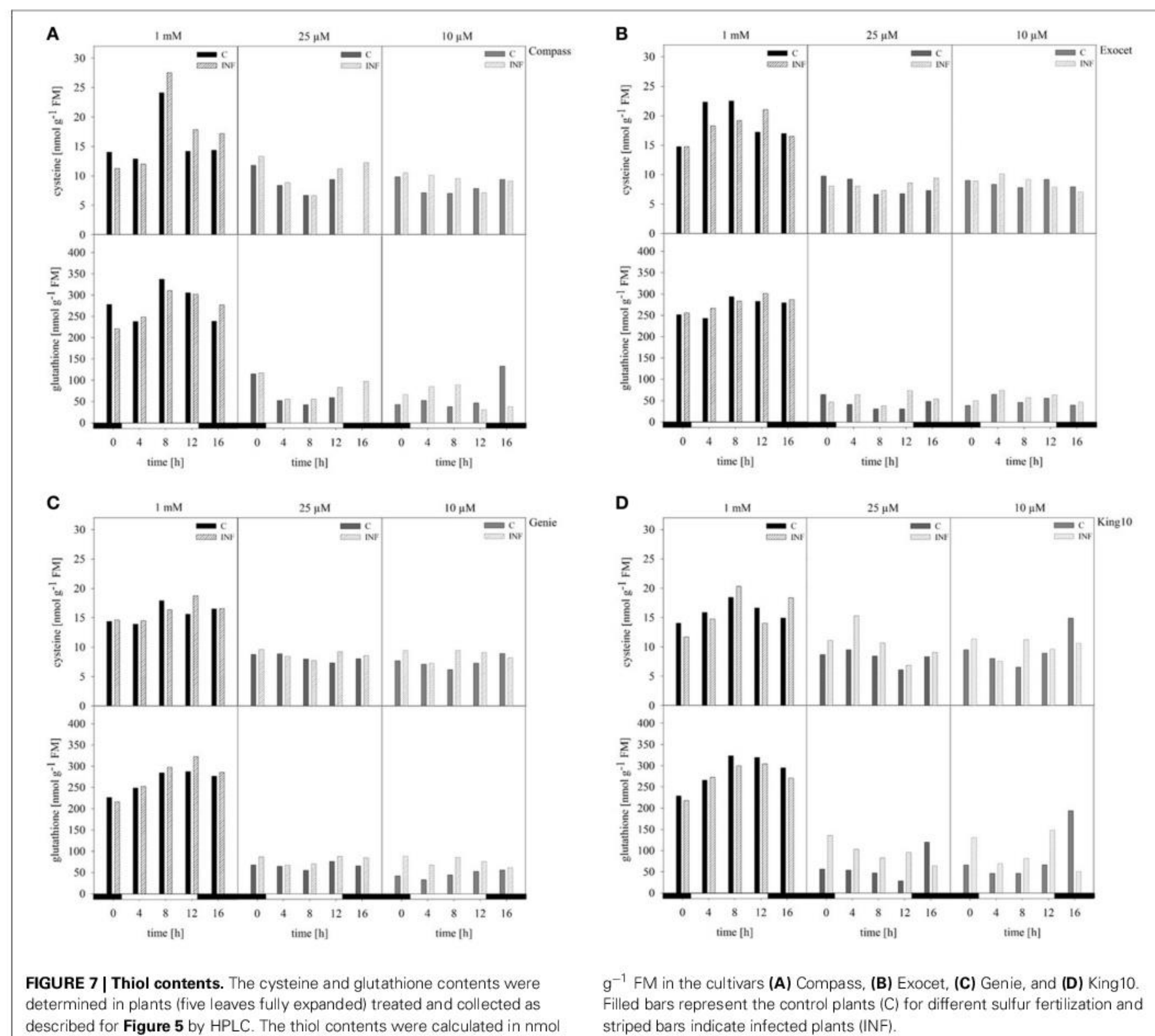

FIGURE 7 | Thiol contents. The cysteine and glutathione contents were determined in plants (five leaves fully expanded) treated and collected as described for **Figure 5** by HPLC. The thiol contents were calculated in nmol g^{-1} FM in the cultivars **(A)** Compass, **(B)** Exocet, **(C)** Genie, and **(D)** King10. Filled bars represent the control plants (C) for different sulfur fertilization and striped bars indicate infected plants (INF).

slightly in the time range 3–7 h after light was switched on. Described as one of the primary regulatory points in sulfate reduction, adenosine 5′-phosphosulfate (APS) is reduced to sulfite catalyzed by APS reductase. Therefore, the homologs of both *APR2* and *APR3* were analyzed because both isoforms reveal a different expression behavior in *A. thaliana* (Kopriva et al., 1999). In *B. napus*, the expression of both genes was influenced by the sulfur status of the plants. Under limitation, the *APR* expression increased in all cultivars. The lowest degree of up-regulation was detected for *APR2* in Compass, the most in Genie. Regarding the expression of *APR3*, the highest increase under sulfur limitation was determined in King10.

In the experiments, the expression of *BnSULTR4;2* is affected by the pathogen. Especially under sulfur limiting conditions, the expression decreased, most strongly in Compass. The pathogen controls the expression of *APR2* in the following intensity order: Compass, Exocet and Genie. The line King10 showed higher expression levels of *APR2* than Genie, but here the expression is clearly up-regulated in infected plants. In the case of the expression pattern of *APR3*, in Genie and King10 the highest expressions in infected plants were detected 3 h after light was switched on. The difference among the *APR3* expression in infected and non-infected plant was higher in King10 than in all other cultivars.

ANALYSIS OF THE AMPLIFIED DNA FRAGMENTS OF *B. napus* GENES

The analysis of the four different *B. napus* cultivars revealed a number of different reactions with respect to the sulfur supply. Therefore, we investigated whether the cultivars differ in the sequences of key genes in sulfur metabolism. The respective DNA fragments of about 500 bp were amplified by PCR from the transcribed cultivar-specific cDNAs, cloned and sequenced.

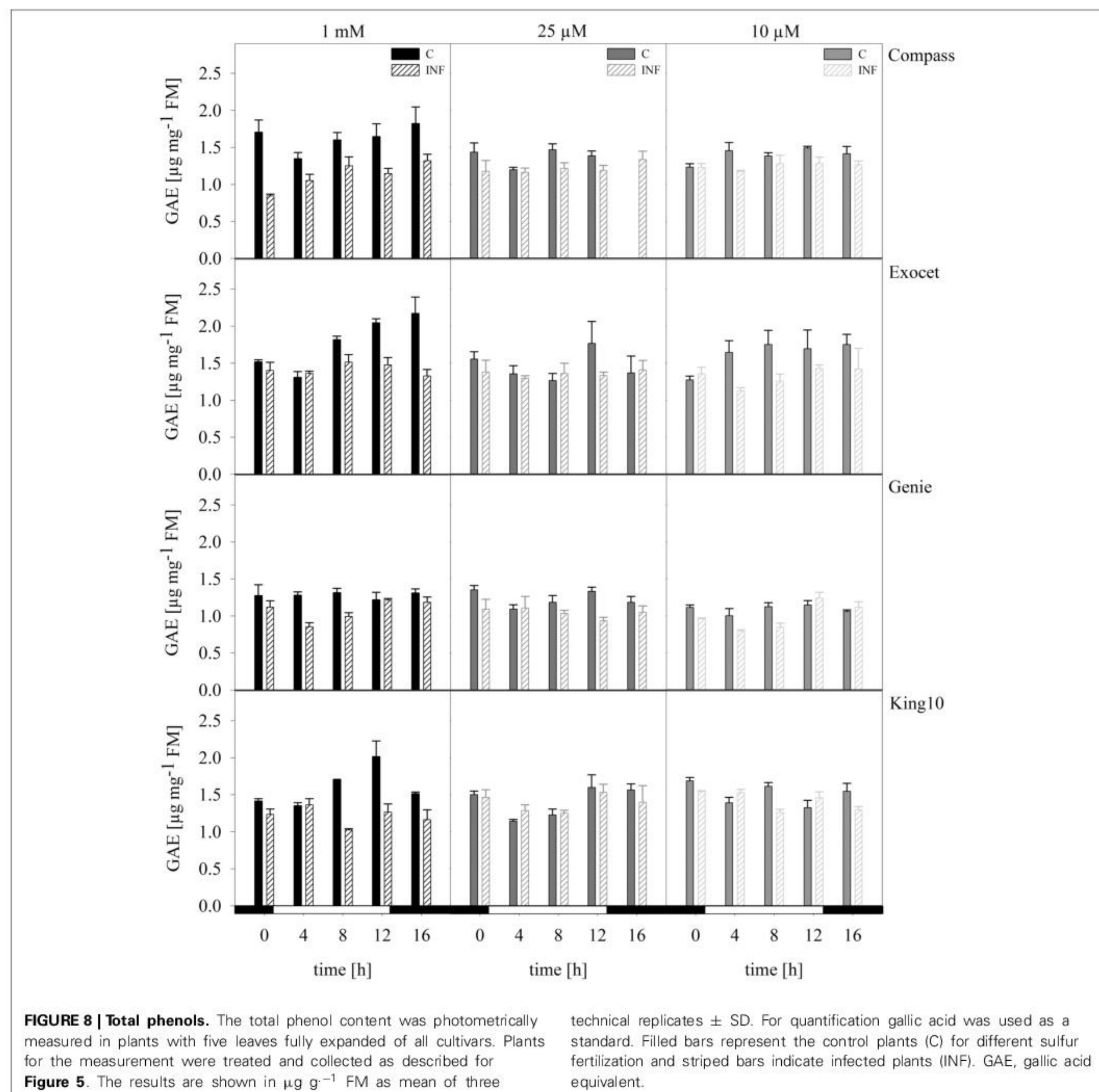

FIGURE 8 | Total phenols. The total phenol content was photometrically measured in plants with five leaves fully expanded of all cultivars. Plants for the measurement were treated and collected as described for **Figure 5**. The results are shown in µg g^{-1} FM as mean of three technical replicates ± SD. For quantification gallic acid was used as a standard. Filled bars represent the control plants (C) for different sulfur fertilization and striped bars indicate infected plants (INF). GAE, gallic acid equivalent.

A considerable difference among *APR2* and *APR3* sequences was observed (78%), indicating the correct choice of primer pairs for amplification of specific fragments. In comparison to the *B. rapa* consensus, sequences the identity was for APR2 93% and APR3 96%, respectively. In comparison to *B. oleracea*, the identity was 100% for both, APR2 and APR3. The alignment of the *APR2* and *APR3* fragments from the four cultivars revealed no different bases among them. A longer fragment was amplified from the *B. napus* sulfur transporter *SULTR4;2.* The identity of the 975 bp fragment chosen was 99% between the sequences from Genie and King10. The identity of these *SULTR4;2* fragments with the homologous partial sequence of the *B. oleracea* transporter was 97% for both sequences.

DISCUSSION

WHICH CULTIVARS SHOW THE HIGHEST RESISTANCE AGAINST PATHOGEN INFECTION?

Before we can decide which cultivar is the most promising according to our results, we need to discuss first the recorded data for biomass, chlorophyll fluorescence and leaf temperature. Therefore, the best cultivar in this study is characterized by high biomass of all organs at all conditions chosen,

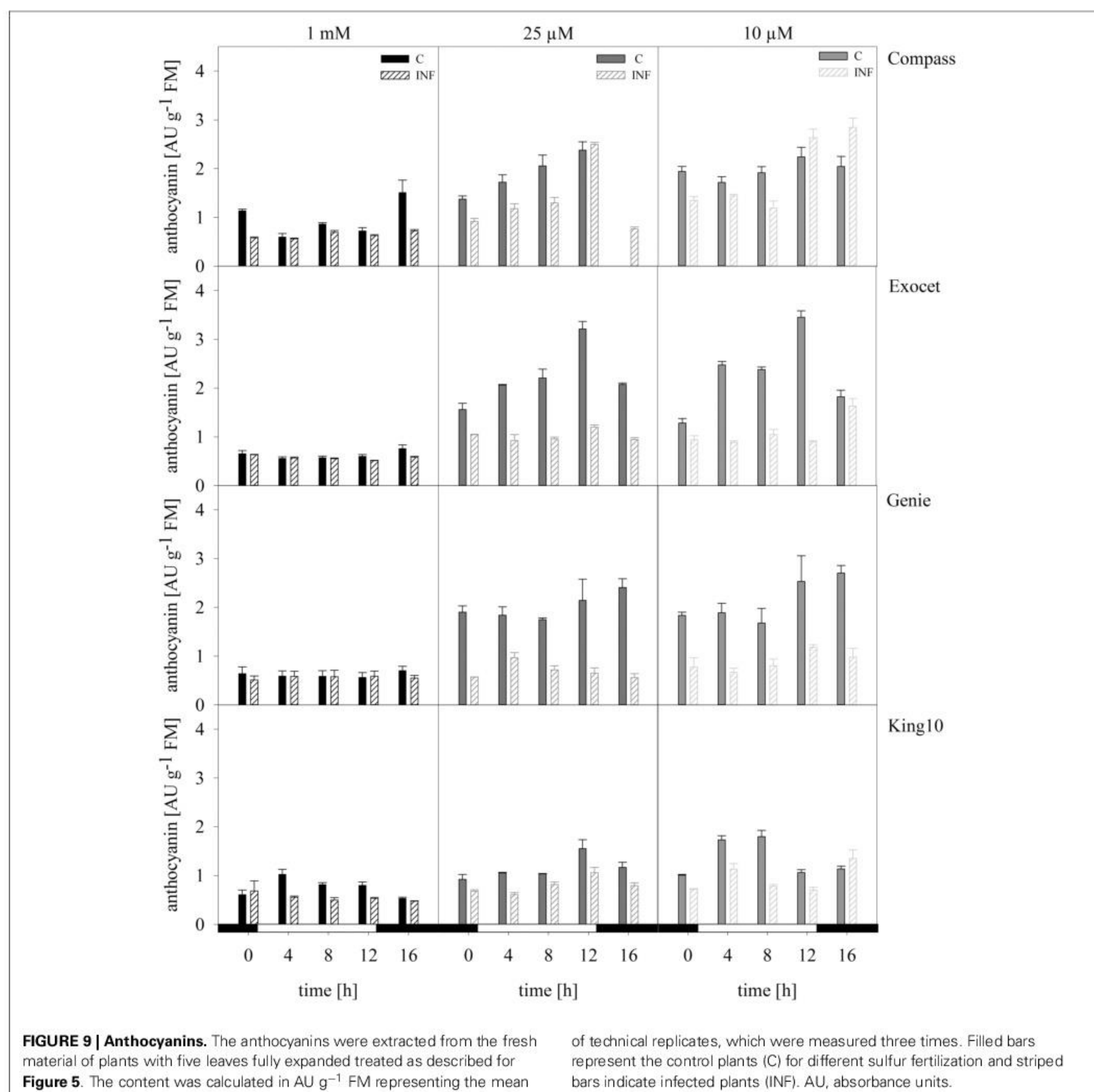

FIGURE 9 | Anthocyanins. The anthocyanins were extracted from the fresh material of plants with five leaves fully expanded treated as described for **Figure 5**. The content was calculated in AU g^{-1} FM representing the mean of technical replicates, which were measured three times. Filled bars represent the control plants (C) for different sulfur fertilization and striped bars indicate infected plants (INF). AU, absorbance units.

both sulfur limitation and infection by *V. longisporum*, indicating general plant health. High biomass is accompanied by comparatively low leaf temperature indicating intact stomatal closure reaction and an intact water status. The photosynthetic parameters determined should be close to the range of the controls revealing intactness of the photosynthetic apparatus. Unfortunately, quantification of the infection rate is currently not possible because fungal DNA concentrations seem to be below the detection limit, as was also reported by Enyck et al. (2009). However, biometrical parameters such as biomass reduction and PAM data clearly indicate successful infection and spreading of the fungus in the infected plants.

Compass and Exocet have the largest root system (**Table 1**). Our results show that Compass and Exocet have significantly longer root hairs (**Figure 1**). Schröder (2013) advised that high-performing plants root very deeply with a production of up to 5 mm long root hairs. The root hairs are essential for a better uptake of nutrients. In greenhouse experiments, a sufficient sulfur supply was added to an intact root system, resulted in a reduced fading away of roots and led to an increased efficiency of nutrient and water use (Schröder, 2013; Grierson et al., 2014). The biomass

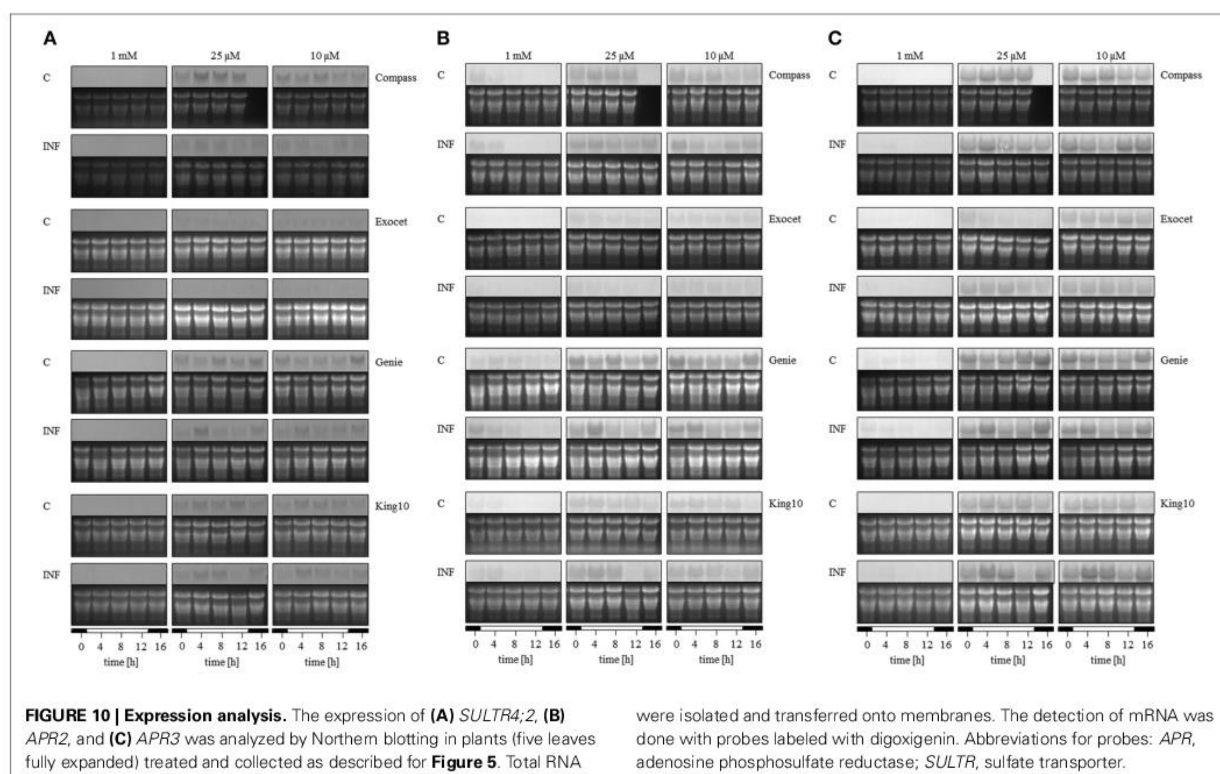

FIGURE 10 | Expression analysis. The expression of **(A)** *SULTR4;2*, **(B)** *APR2*, and **(C)** *APR3* was analyzed by Northern blotting in plants (five leaves fully expanded) treated and collected as described for **Figure 5**. Total RNA were isolated and transferred onto membranes. The detection of mRNA was done with probes labeled with digoxigenin. Abbreviations for probes: *APR*, adenosine phosphosulfate reductase; *SULTR*, sulfate transporter.

measurements in this study are in agreement with these observations (**Figure 2**). With full sulfur supply, cultivars Compass and Exocet showed the highest leaf biomass. Under sulfur limitation, non-infected plants of both cultivars showed also the highest leaf biomass (data not shown). However, for all cultivars a significant reduction of DM and shoot to root ratio was obtained under sulfur limiting conditions. Interestingly, in previous experiments, it was shown that the fungus had no influence on the shoot to root ratio but only on the total DM of the plants after 14 dpi as described in Enyck et al. (2009). In experiments done with *B. napus* and *V. longisporum*, the fungus did not overcome the hypocotyl barrier until 21 dpi, although the plants showed massive stunting of the stem and mild leaf chlorosis. A significant decrease of stem biomass was observed after 28 dpi (Floerl et al., 2008). In experiments done with more than 30 high-performance oilseed cultivars, it also was shown that *V. longisporum* influenced the plant growth. Infected cultivars showed reduced plant growth compared with control plants (Burlacu et al., 2012). However, in experiments we performed with plants grown for 21 day under sulfur deficiency and infection (21 dpi) showed very strong stress symptoms; therefore we disregarded these measurements and decided to analyze under the conditions described in this study. According to our results, Exocet seems to be more susceptible to fungal infections, whereas the biomass of Compass suggests to be the best performing cultivar.

Baker and Rosenqvist (2004) concluded that the reduction of sulfur levels in sugar beet had to reach sulfur starvation level before any changes were detectable in chlorophyll fluorescence parameters, e.g., in F_v/F_m. That leads us to the conclusion that our plants were grown only under sulfur limitation, and not sulfur starvation, and the influence on the maximum quantum yield remained low. Interestingly, the photosynthetic performance of King10 is significantly reduced under sulfur limitation. In contrast, the general fitness of Genie was influenced less by sulfur limitation as demonstrated by steady activities of the photosystems (**Figure 4**). However, after fungal infection the quantum yield in photosystem II was significantly reduced under sulfur limitation in all cultivars. It was shown before that the quantum yield of the photosystems decreased in plants infected with *V. longisporum*, but neither the nitrogen nor the sulfur or phosphorus amounts accumulated differently in non-infected and infected plants (Floerl et al., 2008). In conclusion, the fungus *V. longisporum* did not influence the photosynthetic activity of the plants under full sulfur supply. These results confirm the principle idea of SED (Rausch and Wachter, 2005). The sulfur status did not influence the leaf temperature significantly. Compass, Exocet and Genie, have a lower leaf temperature than the control plants when additionally infected with *V. longisporum*. At decreased applied sulfur amounts, the temperature was slightly higher, at least at 0.025 mM $MgSO_4$. In experiments performed by Muneer et al. (2014) with 8-week-old *B. napus* plants grown under different sulfur supply for 5 or 10 day, the stomata were closed under sulfur limitation. Photosynthesis rates and stomatal conductance were decreased. Previously, it was observed that plants suffering from (abiotic) stress have a higher leaf temperature than non-stressed control plants (Guretzki and Papenbrock, 2013). Obviously, the

thermographic analysis reveals different results, depending on stress and environmental conditions, but often leads to a clear differentiation between control and treatment.

WHAT IS THE ROLE OF SULFUR, SULFATE AND SULFUR-CONTAINING COMPOUNDS IN *VERTICILLIUM* DEFENSE?

Interestingly, the sulfur content itself was generally higher in infected plants than in non-infected plants, independent on the cultivar, indicating a specific increase of sulfur uptake and accumulation induced by pathogen attack. Looking at the accumulated total sulfur, line King10 incorporated the highest amounts of sulfur, in particular, after infection and under low sulfur application (**Figure 5**). Although the variety Genie performed worst compared to the other cultivars, it incorporated high amounts of sulfur, especially in non-infected plants, and in the same range as King10. All cultivars showed fluctuations of the sulfur content, especially Compass with full sulfur supply. Results from Huseby et al. (2013) showed a diurnal regulation of the sulfate uptake and reduction which corresponds with our results. Especially, the large increase in total sulfur contents at the beginning of the light phase is remarkable. The contents were analyzed several times in various experimental set ups and also by other experts (data not shown), resulting in consistent results. One could speculate that sulfate is taken up at the beginning of the light phase, reduced, and then later superfluously reduced sulfur is released as volatile compounds, such as sulfide, via the leaves and also via the soil. However, these assumptions need further investigation. An accumulation of elemental sulfur in the xylem vessels of tomato plants infected by *V. dahliae* was reported by Williams et al. (2002). Our results indicate an accumulation of total sulfur in *B. napus* leaves after infection with *V. longisporum*. Are these accumulations of total sulfur an indication for comparable defense strategies in both plant-pathogen systems? These results further support the hypothesis of SED.

Results from Huseby et al. (2013) showed a diurnal regulation of the sulfate uptake and reduction which corresponds with our results. In our results, the sulfate content was higher in light than in the darkness. The factor light outweighs the factor infection (**Figure 6**). In parallel, the expression of the sulfate transporter *BnSultr4;2* was increased during sulfur-limiting conditions with a maximum degree of expression during the light period (**Figure 10A**). It is clear that sulfate was taken up during periods of active photosynthesis. It was shown previously in experiments done with 4-weeks-old *B. napus* plants, that due to the sulfur limitation, the sulfate concentrations decreased at variable rates, at first in roots and young leaves, then in the middle leaves, and later in the oldest leaves. In parallel the sulfate transporter *BnSultr4;2* was first expressed in roots and in young leaves (Parmar et al., 2007). Interestingly, in our system, the expression of the sulfate transporter *BnSultr4;2* was influenced by the pathogen. However, in contrast to our expectations in all cultivars the mRNA levels of *BnSultr4;2* were lower in infected plants than in non-infected plants. In the context of SED higher mRNA levels of *BnSultr4;2* were expected due to a higher demand for sulfate and subsequent biosynthesis of sulfur-containing defense compounds. One could postulate that *V. longisporum* directly influences the plant's gene expression to prevent availability of sulfate, a prerequisite for the induction of SED. That needs to be investigated in future experiments.

Even though we do not know the full sequence of the isoforms of *APR* in *B. napus*, our results implied that the mRNA levels of *BnAPR2* are more strongly regulated by light than the levels of *BnAPR3*, especially visible in Genie under sulfur-limiting conditions. These results are in agreement with results obtained by Kopriva et al. (1999) and Huseby et al. (2013): Both the expression of three *APR* isoforms and of APR enzyme activity are diurnally regulated and by sulfate availability. In our experiments, the expression of both *APR* isoforms are differently regulated by sulfate and light, and in addition *V. longisporum* infection influences expression either positively or negatively. As was shown by Wang et al. (2011) bacterial infection leads to a reprogramming of the diurnal rhythm and even the circadian clock on expression level. Effects of fungal infections on the genes involved in continuance of diurnal and circadian rhythms need to be further investigated.

With the sulfate assimilation, a co-regulation of the GSL was described (Huseby et al., 2013). GSL act as typical sulfur-containing compounds in Brassicaceae against herbivores and insects occurring in the soil (Halkier and Gershenzon, 2006). Interestingly, experiments done with a fungal pathogen and a Brassicaceae as host generated indication that crude GSL extracts or detached leaf material act defensively (Buxdorf et al., 2013; Witzel et al., 2013). In the *A. thaliana* accessions investigated by Witzel et al. (2013), the total GSL concentrations were about 10 times higher than in our *B. napus* cultivars, where the maximum concentration of total GSL in the leaves was less than 4 μmol g^{-1} TM (data not shown). The most effective GSL with respect to fungal growth inhibition in *A. thaliana* was 2-propenyl GSL that is not present in *B. napus* or can at least not be detected in leaves. In addition, the inhibiting concentration reported by Witzel et al. (2013) of single GSLs would probably not be high enough to reduce the fungal growth rate in our *B. napus* cultivars. Therefore, for the situation in *B. napus* the results by Witzel et al. (2013) are not applicable and relevant. In summary, based on these results and calculations we assume that even if the GSL contents and their composition would differ in the *B. napus* 00 cultivars in high and low sulfur cultivation, we would not see any influence on fungal growth. The role of GSL and their breakdown products in the defense against fungal pathogens needs to be investigated in more detail *in vivo*.

The sulfur-containing glutathione is an important stress indicating metabolite. There are numerous examples that oxidative stress reduces the overall concentration of glutathione, but particularly the concentration of reduced glutathione (GSH). One would expect that after a pathogen attack, the concentration of total glutathione and GSH is drastically reduced. However, there are several examples that this is not the case. For example, an increase of the cysteine and glutathione content was measured in field grown *B. napus* plants due to infection with *Pyrenopeziza brassicae* (Bloem et al., 2004). There is a reduction in GSH observed, but it is not as drastic as one would expect from abiotic stress effects (Cooper et al., 1996; Bloem et al., 2004; Bollig et al., 2013). These results are in agreement with our results. Furthermore, the cysteine content was measured. As a precursor of defense compounds (Smith and Kirkegaard, 2002; Van Wees

et al., 2003; Rausch and Wachter, 2005), a decrease of the cysteine content in infected plants is expected. According to results of infected *A. thaliana* plants (Álvarez et al., 2011), a decrease of about 25% in the cysteine content was measured. In comparison to our results, this observation is not supported. In contrast, the content seems to be slightly increased in infected plants. Fluctuations in the content over the day for glutathione and cysteine make an evaluation even more difficult. In conclusion, our results show that the cysteine and glutathione content is more influenced and increased by the light conditions than by pathogen attack.

In experiments of Blake-Kalff et al. (1998), the glutathione concentration decreased in plants when grown under sulfur deficiency during the whole course of the experiment and the glutathione content decreased more rapidly grown on either 0.02 or 0.100 mM sulfate. In leaves of plants grown under sulfur limitation, the sulfur content was strongly negatively affected. Alternatively, the sulfur content increased in roots. This could also be observed under full sulfur supply. About 50% of the energy obtained by photosynthetic activity is transported into the *B. napus* root systems (Agrios, 2005).

WHICH CULTIVAR PERFORMED BEST UNDER SULFUR LIMITATION AND PATHOGEN INFECTION? RECOMMENDATION FOR CHOOSING A ROBUST CULTIVAR

The phosphorus contents were always higher in infected plants (about 15%). The results indicate that phosphorus, as one of the macronutrients of the plants with 0.2–0.6% of DM and necessary for the energy metabolism in organisms, plays an important role in pathogen defense, probably due to increased energy demand. Based on the fact that increased phosphorus or nitrogen concentrations have also been reported in other *Verticillium*-infected plants like tomato and *Arabidopsis*, the authors suggested that there is a yet unknown interference of *Verticillium* with the phosphorous or nitrogen metabolism (Floerl et al., 2008). High phosphorus amounts could lead to a decrease in disease but also to an increase in disease after fungal infection (Prabhu et al., 2007). The influence of phosphorus remains unexplained, therefore, high phosphorus values, especially in Exocet after infection, are not discussible.

Iron, essential for biological activity of many proteins mediating electron transfer and redox reactions, is influenced due to infection. Experiments with different pathogens and crop plants revealed an influence of the iron status on the host-pathogen relationships in different ways by affecting the pathogen's virulence as well as the host's defense. Arabidopsis plants, infected with *Erwinia chrysanthemi*, developed an iron-withholding response that involved a change of the iron distribution and trafficking (summarized in Expert et al., 2012). The infection could lead to the release of root exudates for iron mobilization. These observations could be also an explanation for our results especially for the line King10.

In experiments done by D'Hooghe et al. (2013) 2-month-old oilseed rape plants were transferred to sulfur limiting conditions. In these experiments, the total shoot biomass was not significantly reduced, but the growth and the photosynthesis rate were inhibited. The increase of the anthocyanins and the H_2O_2 content in sulfur insufficient plants were explained with means of oxidative stress. No significant differences in the chlorophyll and flavonol contents were detectable (D'Hooghe et al., 2013). Prabhu et al. (2007) described that fungistatic substances like phenolic compounds and flavonoids accumulate in epidermal cells of leaves, stems, and roots after infection. We could observe this phenomenon only in line King10.

The accumulation of phytoalexins settled 48 h after inoculation and was accompanied by a more rapid increase in the rate of anthocyanin accumulation. The results suggest that the plant represses less essential metabolic activities, such as anthocyanin synthesis, in order to compensate the immediate biochemical and physiological needs for the defense response (Lo and Nicholson, 1998). According to our results King10 seems to be unaffected by sulfur limitation and infection.

Oilseed rape hybrids have up to 10% higher yields than conventional lines such as King10. The first hybrid cultivars were market-launched in Germany in 1995. Since that time, there has been a discussion which plant performs best: hybrids or bred-lines (Alpmann, 2009). Actually, in our experiments under highly controlled conditions, the hybrid Genie and the line King10 performed equally well with respect to all conditions chosen: to sulfur limitation, reaction to infection, and uptake of nutrients (**Figure S1**).

In 2010, the cultivars Compass and King10 were mentioned as best performing cultivars with high oil contents (DSV). The oil content has a major influence on the market performance, which depends on the cultivar. Cultivars with high oil content are more attractive with increasing prices for oilseed rape. Already one percent more oil content is paid with a 1.5% higher fee (Alpmann, 2009). Therefore, already in early phases of development general plant fitness and plant health need to be carefully checked to obtain finally high seed yields. In the future, the performance in later phases of the development and finally the seed yield, oil content, and composition need to be studied. Then a recommendation with respect to moderate sulfur limitations and pathogens such as *V. longisporum* could be given. In conclusion, our results indicate that line King10 is the most promising cultivar: Under sulfur deficiency and after infection, line King10 had higher contents of flavonoids and accumulated more sulfur. However, one has to keep in mind that the determination of the biomass of 3-weeks-old plants may not have any correlation to the final yield but early stage changes could be predictive of the further development and the following loss in yield.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00009/abstract

Figure S1 | Measurement of iron and phosphorus by ICP-OES. The elements iron **(A)** and phosphorus **(B)** were measured in dried plant material by ICP-OES. Dried material from plants with five leaves fully expanded and treated as described for **Figure 5** were used. Data calculated as mg g^{-1} DM represent the mean of three dependently technical replicates ± SD. Filled dots represent the mock-inoculated plants **(C)** and open dots show the corresponding results of infected plants (INF).

Figure S2 | Total flavonoids. The total flavonoid content was photometrically measured in the cultivars with five fully expanded leaves treated as described for **Figure 5**. The results are given in μg g^{-1} FM as mean of three technical replicates ± SD. For quantification catechin was used as a standard. Filled bars represent the control plants (C) for different sulfur fertilization and striped bars indicate infected plants (INF). CE, catechin equivalent.

Figure S3 | Expression analysis. Results of Northern Blot analysis (**Figure 10**) were evaluated with the program GelAnalyzer (GelAnalyzer.com). Intensity for each band detected on the membrane was calculated in reference to the first sample (0 h). Data represent the relative expression in %. Values of non-infected plants with 1 mM $MgSO_4$ at 0 h were used as 100% for control and infected plants for each cultivar. **(A)** *SULTR4;2*, **(B)** *APR2*, and **(C)** *APR3*. Filled dots represent the mock-inoculated plants (C) and open dots show the corresponding results of infected plants (INF).

REFERENCES

Agrios, G. N. (2005). *Plant Pathology*. Waltham: Academic Press.

Ahmad, A., and Abdin, M. Z. (2000). Photosynthesis and its related physiological variables in the leaves of *Brassica* genotypes as influenced by sulphur fertilization. *Physiol. Plant.* 110, 144–149. doi: 10.1034/j.1399-3054.2000.110119.x

Allègre, M., Hamilton, J. G., Resti, J. P., Zangerl, A. R., Berenbaum, M. R., and DeLucia, E. H. (2007). Stomatal deregulation in *Plasmopara viticola*-infected grapevine leaves. *New Phytol.* 173, 832–840. doi: 10.1111/j.1469-8137.2006.01959.x

Alpmann, L. (2009). Hybride oder liniensorte? Deutsche saatveredelung, lippstadt. *Innovation* 3, 22–23.

Álvarez, C., Bermúdez, M. Á., Romero, L. C., Gotor, C., and García, I. (2011). Cysteine homeostasis plays an essential role in plant immunity. *New Phytol.* 193, 165–177. doi: 10.1111/j.1469-8137.2011.03889.x

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Ann. Rev. Plant Biol.* 59, 9–113. doi: 10.1146/annurev.arplant.59.032607.092759

Baker, N. R., and Rosenqvist, E. (2004). Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *J. Exp. Bot.* 55, 1607–1621. doi: 10.1093/jxb/erh196

Blake-Kalff, M., Harrison, K., Hawkesford, M., Zhao, F., and McGrath, S. (1998). Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. *Plant Physiol.* 118, 1337–1344. doi: 10.1104/pp.118.4.1337

Bloem, E., and Haneklaus, S. (2002). Warum und wieviel Schwefel düngen? Institut für Pflanzenernährung und Bodenkunde, FAL Braunschweig. *Innovation* 1, 1–4.

Bloem, E., Haneklaus, S., Kesselmeier, J., and Schnug, E. (2012). Sulfur fertilization and fungal infections affect the exchange of H_2S and COS from agricultural crops. *J. Agr. Food Chem.* 60, 7588–7596. doi: 10.1021/jf301912h

Bloem, E., Riemenschneider, A., Volker, J., Papenbrock, J., Schmidt, A., Salac, I., et al. (2004). Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. *J. Exp. Bot.* 55, 2305–2312. doi: 10.1093/jxb/erh236

Bollig, K., Specht, A., Myint, S. S., Zahn, M., and Horst, W. J. (2013). Sulphur supply impairs spread of *Verticillium dahliae* in tomato. *Eur. J. Plant Pathol.* 135, 81–96. doi: 10.1007/s10658-012-0067-5

Buchner, P., Stuiver, C. E., Westerman, S., Wirtz, M., Hell, R., Hawkesford, M. J., et al. (2004a). Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H(2)S and pedospheric sulfate nutrition. *Plant Physiol.* 136, 3396–3408. doi: 10.1104/pp.104.046441

Buchner, P., Takahashi, H., and Hawkesford, M. J. (2004b). Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. *J. Exp. Bot.* 55, 1765–1773. doi: 10.1093/jxb/erh206

Burandt, P., Papenbrock, J., Schmidt, A., Bloem, E., Haneklaus, S., and Schnug, E. (2001). Genotypical differences in total sulfur contents and cysteine desulfhydrase activities in *Brassica napus* L. *Phyton* 41, 75–86.

Burlacu, M. C., Leonte, C., Lipsa, F., Simioniuc, D. P., and Lazarescu, E. (2012). Identification of some cultivras of *Brassica napus* with resistence at *Verticillium longisporum*. *Res. J. Agric. Sci.* 44, 14–18.

Buxdorf, K., Yaffe, H., Barda, O., and Levy, M. (2013). The effects of glucosinolates and their breakdown products on necrotrophic fungi. *PLoS ONE* 8:e70771. doi: 10.1371/journal.pone.0070771

Chaerle, L., Hagenbeek, D., Vanrobaeys, X., and Van Der Straeten, D. (2007). Early detection of nutrient and biotic stress in *Phaseolus vulgaris*. *Int. J. Remote Sens.* 28, 3479–3492. doi: 10.1080/01431160601024259

Computational Biology and Functional Genomics Laboratory. (2014). Available online at: http://compbio.dfci.harvard.edu/ [Accessed 15.06.2014].

Cooper, R. M., Resende, M. L. V., Flood, J., Rowan, M. G., Beale, M. H., and Potter, U. (1996). Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao*. *Nature* 379, 159–162. doi: 10.1038/379159a0

Dämmgen, U., Walker, K., Grünhage, L., and Jäger, H.-J. (1998). "The atmospheric sulphur cycle," in *Sulphur in Agroecosystems*, eds E. Schnug, R. F. Hüttl, and H. Beringer (Dordrecht: Kluwer), 75–114.

Dewanto, V., Wu, X., Adom, K. K., and Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem.* 50, 3010–3014. doi: 10.1021/jf0115589

D'Hooghe, P., Escamez, S., Trouverie, J., and Avice, J. C. (2013). Sulphur limitation provokes physiological and leaf proteome changes in oilseed rape that lead to perturbation of sulphur, carbon and oxidative metabolisms. *BMC Plant Biol.* 13:23. doi: 10.1186/1471-2229-13-23

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., and Mérillon, J. M. (2009). Comparative study of the antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assay. *J. Agric. Food Chem.* 57, 1768–1774. doi: 10.1021/jf803011r

Dunker, S., Keunecke, H., Steinbach, P., and von Tiedemann, A. (2008). Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in plant. *J. Phytopathol.* 156, 698–707. doi: 10.1111/j.1439-0434.2008.01429.x

Eberlein, C. V., Morra, M. J., Guttieri, M. J., Brown, P. D., and Brown, J. (1998). Glucosinolate production by five field-grown *Brassica napus* cultivars used as green manures. *Weed Technol.* 12, 712–718.

Enyck, C., Koopmann, B., and von Tiedemann, A. (2009). Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *J. Plant Dis. Protect.* 116, 63–72.

Expert, D., Franza, T., and Dellagi, A. (2012). "Iron in plant-pathogen interactions," in *Molecular Aspects of Iron Metabolism in Pathogenic and Symbiotic Plant-Microbe Assiciations*, eds D. Expert and M. R. O'Brian (New York, NY: Springer Briefs in Molecular Science), 7–40.

Floerl, S., Druebert, C., Majcherczyk, A., Karlovsky, P., Kües, U., and Polle, A. (2008). Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biol.* 8:129. doi: 10.1186/1471-2229-8-129

Friedt, W., and Snowdon, R. (2009). "Oilseed rape," in *Handbook of Plant Breeding*, Vol. 4: *Oil Crops Breeding*, eds J. Vollmann and R. Istvan (New York, NY: Springer), 91–126.

Grant, C., and Kovar, J. (2012). Don't overlook sulfur in crop management. *Fluid J.* 20, 10–12.

Grant, O. M., Chaves, M. M., and Jones, H. G. (2006). Optimizing thermal imaging as a technique for detecting stomatal closure induced by drought stress

under greenhouse conditions. *Physiol. Plant*. 127, 507–518. doi: 10.1111/j.1399-3054.2006.00686.x

Grierson, C., Nielsen, E., Ketelaarc, T., and Schiefelbein, J. (2014). Root hairs. *Arabidopsis Book* 12:e0172. doi: 10.1199/tab.0172

Guretzki, S., and Papenbrock, J. (2013). Comparative analysis of methods analyzing effects of drought on herbaceous plants. *J. Appl. Bot. Food Chem*. 86, 47–54. doi: 10.5073/JABFQ.2013.086.007

Halkier, B. A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol*. 57, 303–333. doi: 10.1146/annurev.arplant.57.032905.105228

Haneklaus, S., Bloem, E., Schnug, E., de Kok, L., and Stulen, I. (2007). "Sulfur," in *Handbook of Plant Nutrient*, eds A. V. Barker and D. J. Pilbeam (New York, NY: CRC Press), 183–238.

Heale, J. B., and Karapapa, K. V. (1999). The *Verticillium* threat to Canada's major oilseed crop: Canola. *Can. J. Plant Pathol*. 21, 1–7. doi: 10.1080/07060661.1999.10600114

Holmes, M. R. J. (1980). *Nutrition of the Oilseed Rape Crop*. London: Applied Science Publishers.

Huseby, S., Koprivova, A., Lee, B.-R., Saha, S., Mithen, R., Wold, A.-B., et al. (2013). Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in *Arabidopsis*. *J. Exp. Bot*. 64, 1039–1048. doi: 10.1093/jxb/ers378

Iñiguez-Luy, F. L., and Federico, M. L. (2011). "The genetics of Brassica napus L.," in *Genetics and Genomics of the Brassicaceae*, eds I. Bancroft and R. Schmidt (New York, NY: Springer), 291–322.

James, R. A., and Sirault, X. R. R. (2012). Infrared thermography in plant phenotyping for salinity tolerance. *Methods Mol. Biol*. 913, 173–189. doi: 10.1007/978-1-61779-986-0_11

Jones, H. G. (2007). Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. *J. Exp. Bot*. 58, 119–130. doi: 10.1093/jxb/erl118

Kitajima, M., and Butler, W. L. (1975). Excitation spectra for photosystem I and photosystem II in chloroplasts and the spectral characteristics of the distributions of quanta between the two photosystems. *Biochim. Biophys. Acta* 408, 297–305. doi: 10.1016/0005-2728(75)90131-0

Klepacka, J., Gujska, E., and Michalak, J. (2011). Phenolic compounds as cultivar- and variety-distinguishing factors in some plant products. *Plant Foods Hum. Nutr*. 66, 64–69. doi: 10.1007/s11130-010-0205-1

Kopriva, S., Muheim, R., Koprivova, A., Trachsel, N., Catalano, C., Suter, M., et al. (1999). Light regulation of assimilatory sulphate reduction in *Arabidopsis thaliana*. *Plant J*. 20, 37–44. doi: 10.1046/j.1365-313X.1999.00573.x

Kruse, C., Haas, F. H., Jost, R., Reiser, B., Reichelt, M., Wirtz, M., et al. (2012). Improved sulfur nutrition provides the basis for enhanced production of sulfur-containing defense compounds in *Arabidopsis thaliana* upon inoculation with *Alternaria brassicicola*. *J. Plant Physiol*. 169, 740–743. doi: 10.1016/j.jplph.2011.12.017

Kruse, C., Jost, R., Lipschis, M., Kopp, B., Hartmann, M., and Hell, R. (2007). Sulfur-enhanced defence: effects of sulfur metabolism, nitrogen supply, and pathogen lifestyle. *Plant Biol*. 9, 608–619. doi: 10.1055/s-2007-965432

Lewandowska, M., and Sirko, A. (2008). Recent advances in understanding plant response to sulfur-deficiency stress. *Acta. Biochim. Pol*. 55, 457–471.

Lo, S. C., and Nicholson, R. L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. Implications for a compensatory role in the defense response. *Plant Physiol*. 116, 979–989. doi: 10.1104/pp.116.3.979

Muneer, S., Lee, B. R., Kim, K. Y., Park, S. H., Zhang, Q., and Kim, T. H. (2014). Involvement of sulphur nutrition in modulating iron deficiency responses in photosynthetic organelles of oilseed rape (*Brassica napus* L.). *Photosynth. Res*. 119, 319–329. doi: 10.1007/s11120-013-9953-8

Ogura, H. (1968). Studies of the new male sterility in Japanese radish with special reference to the utilization of this sterility towards the practical raising of hybrid seeds. *Mem. Fac. Agric. Kagoshima Univ*. 6, 39–78.

Parmar, S., Buchner, P., and Hawkesford, M. J. (2007). Leaf developmental stage affects sulfate depletion and specific sulfate transporter expression during sulfur limitation in *Brassica napus* L. *Plant Biol*. 9, 647–653. doi: 10.1055/s-2007-965428

Prabhu, A. S., Frageria, N. K., and Berni, R. F. (2007). "Phosphorus and plant disease," in *Mineral Nutrition and Plant Disease*, eds L. E. Datnoff, W. H. Elmer, and D. M. Huber (Minnesota: APS press), 45–56.

Quackenbush, J., Liang, F., Holt, I., Pertea, G., and Upton, J. (2000). The TIGR Gene Indices: reconstruction and representation of expressed gene sequences. *Nucl. Acids Res*. 28, 141–145. doi: 10.1093/nar/28.1.141

Rabino, I., and Mancinelli, A. L. (1986). Light, temperature, and anthocyanin production. *Plant Physiol*. 81, 922–924. doi: 10.1104/pp.81.3.922

Rausch, T., and Wachter, A. (2005). Sulfur metabolism: a versatile platform for launching defence operations. *Trends Plant Sci*. 10, 503–509. doi: 10.1016/j.tplants.2005.08.006

R Core Team. (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org

Riemenschneider, A., Nikiforova, V., Hoefgen, R., De Kok, L. J., and Papenbrock, J. (2005). Impact of elevated H_2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. *Plant Physiol. Biochem*. 43, 473–483. doi: 10.1016/j.plaphy.2005.04.001

Scherer, H. W. (2001). Sulphur in crop production—invited paper. *Eur. J. Agron*. 14, 81–111. doi: 10.1016/S1161-0301(00)00082-4

Schnug, E., Booth, E., Haneklaus, S., and Walker, K. C. (1995). "Sulphur supply and stress resistance in oilseed rape," in *Proceedings of the 9th International Rapeseed Congress, Cambridge* 1995, 229–231.

Schröder, C. (2013). Hohe Erträge wurzeln tief. Deutsche Saatveredelung AG,·Schinkel. *Innovation* 4, 20–22.

Smith, B. J., and Kirkegaard, J. A. (2002). *In vitro* inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathol*. 51, 585–593. doi: 10.1046/j.1365-3059.2002.00744.x

Sokolowsky, V., Kaldenhoff, R., Ricci, M., and Russo, V. E. A. (1990). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. *Fungal Genet. Newslett*. 36, 41–43.

Sperdouli, I., and Moustakas, M. (2012). Spatio-temporal heterogeneity in *Arabidopsis thaliana* leaves under drought stress. *Plant Biol*. 14, 118–128. doi: 10.1111/j.1438-8677.2011.00473.x

Van Wees, S. C. M., Chang, H. S., Zhu, T., and Glazebrook, J. (2003). Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. *Plant Physiol*. 132, 606–617. doi: 10.1104/pp.103.022186

Vollmann, J., and Rajcan, I. (2009). "Oil crop breeding and genetics," in *Oil Crops. Series: Handbook of Plant Breeding*, Vol. 4, eds J. Vollmann and I. Rajcan (New York, NY: Springer), 1–30.

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., et al. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi: 10.1038/nature09766

Williams, J. S., Hall, S. A., Hawkesford, M. J., Beale, M. H., and Cooper, R. M. (2002). Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiol*. 128, 150–159. doi: 10.1104/pp.010687

Witzel, K., Hanschen, F. S., Schreiner, M., Krumbein, A., Ruppel, S., and Grosch, R. (2013). *Verticillium* suppression is associated with the glucosinolate composition of *Arabidopsis thaliana* leaves. *PLoS ONE* 8:e71877. doi: 10.1371/journal.pone.0071877

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Supplementary Information

Figure S1.

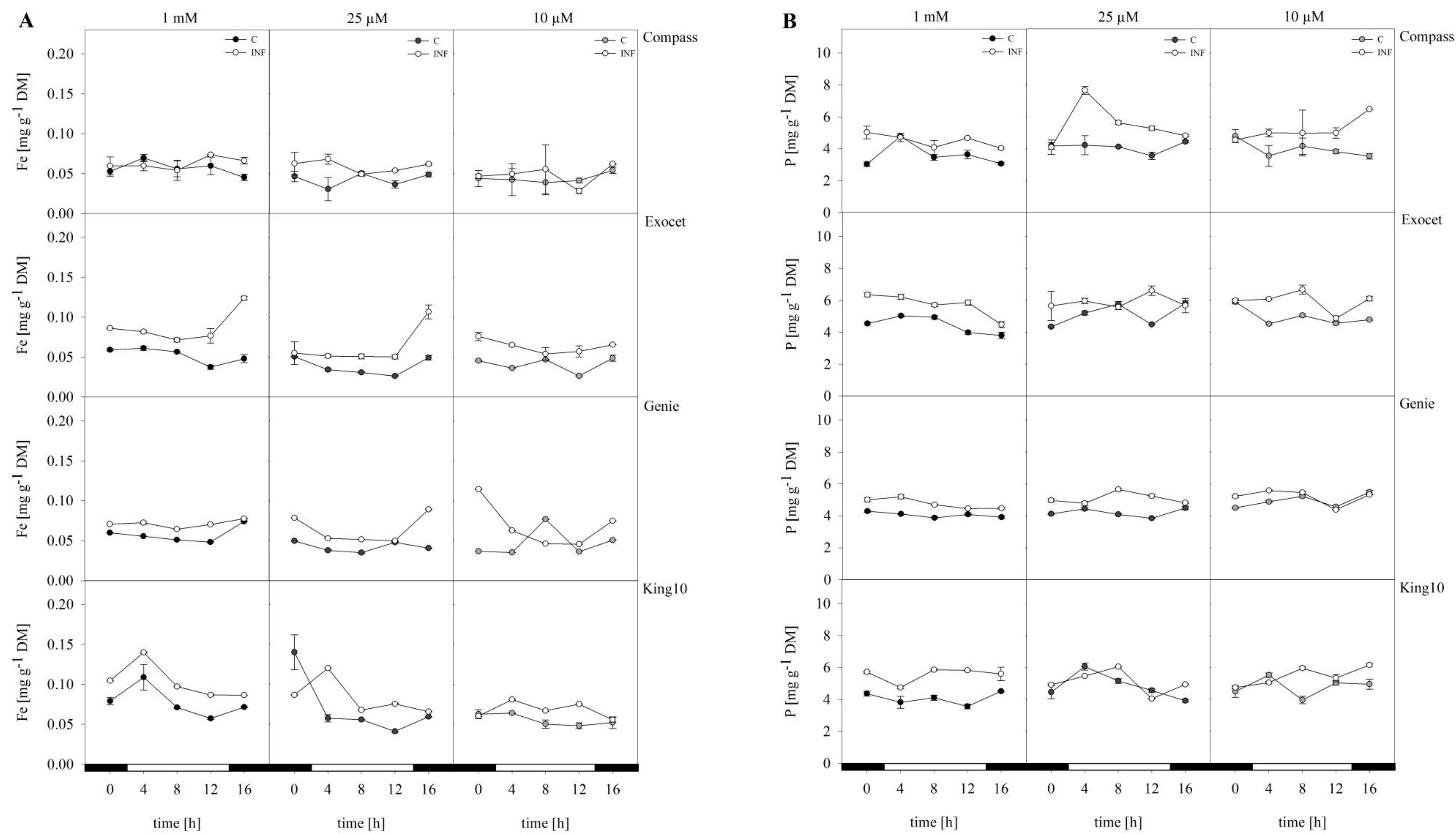


Figure S2.

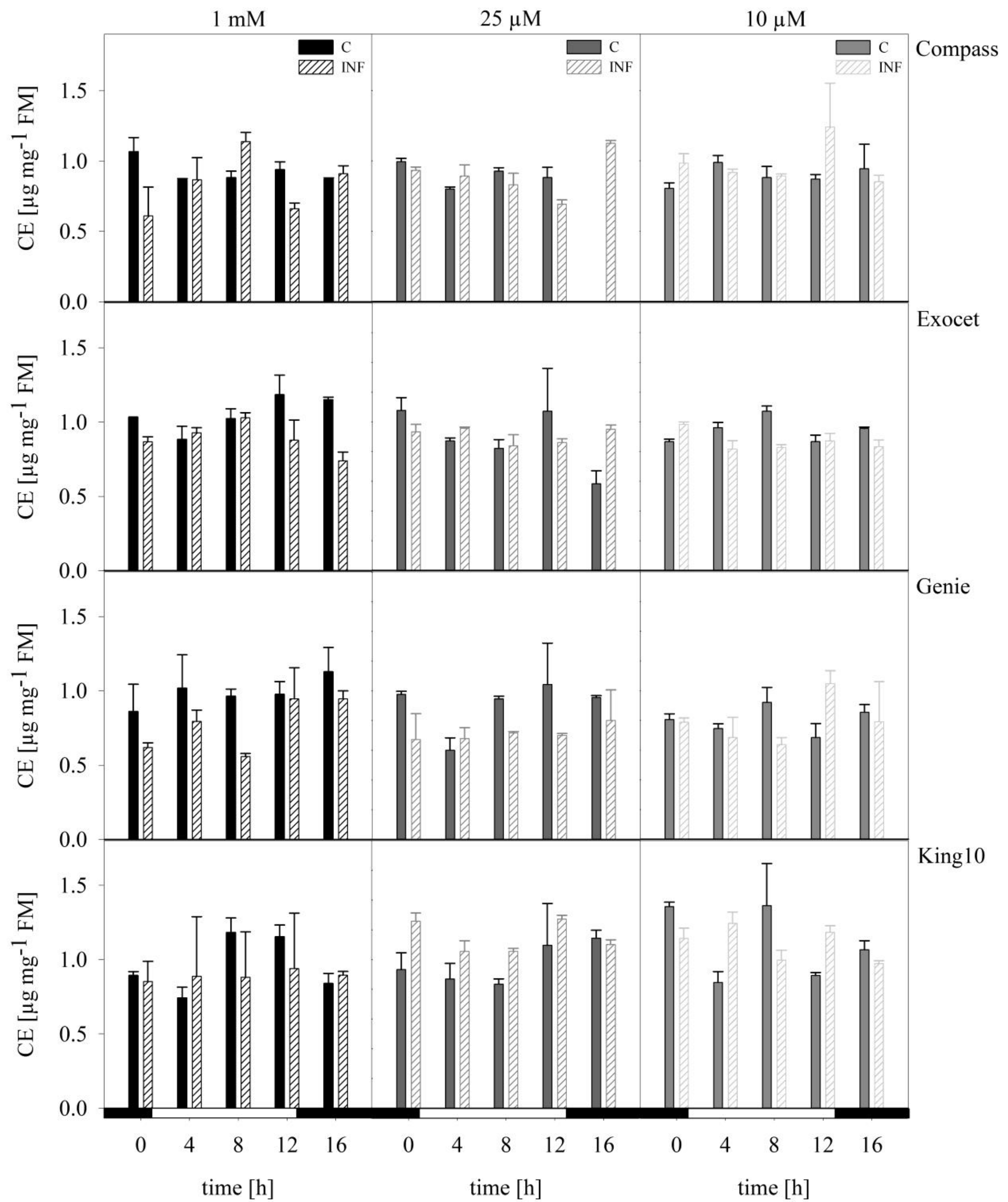
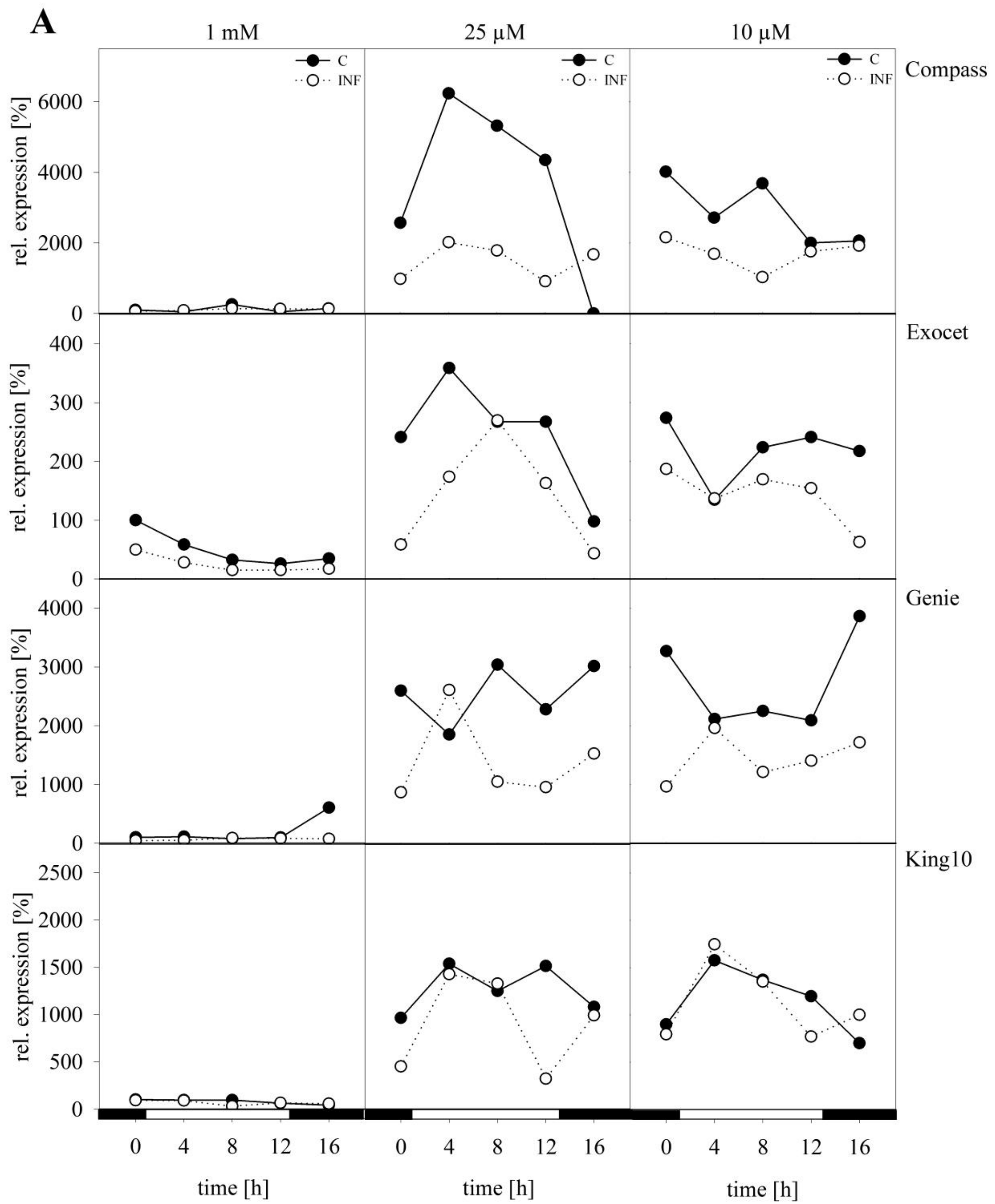


Figure S3.



B

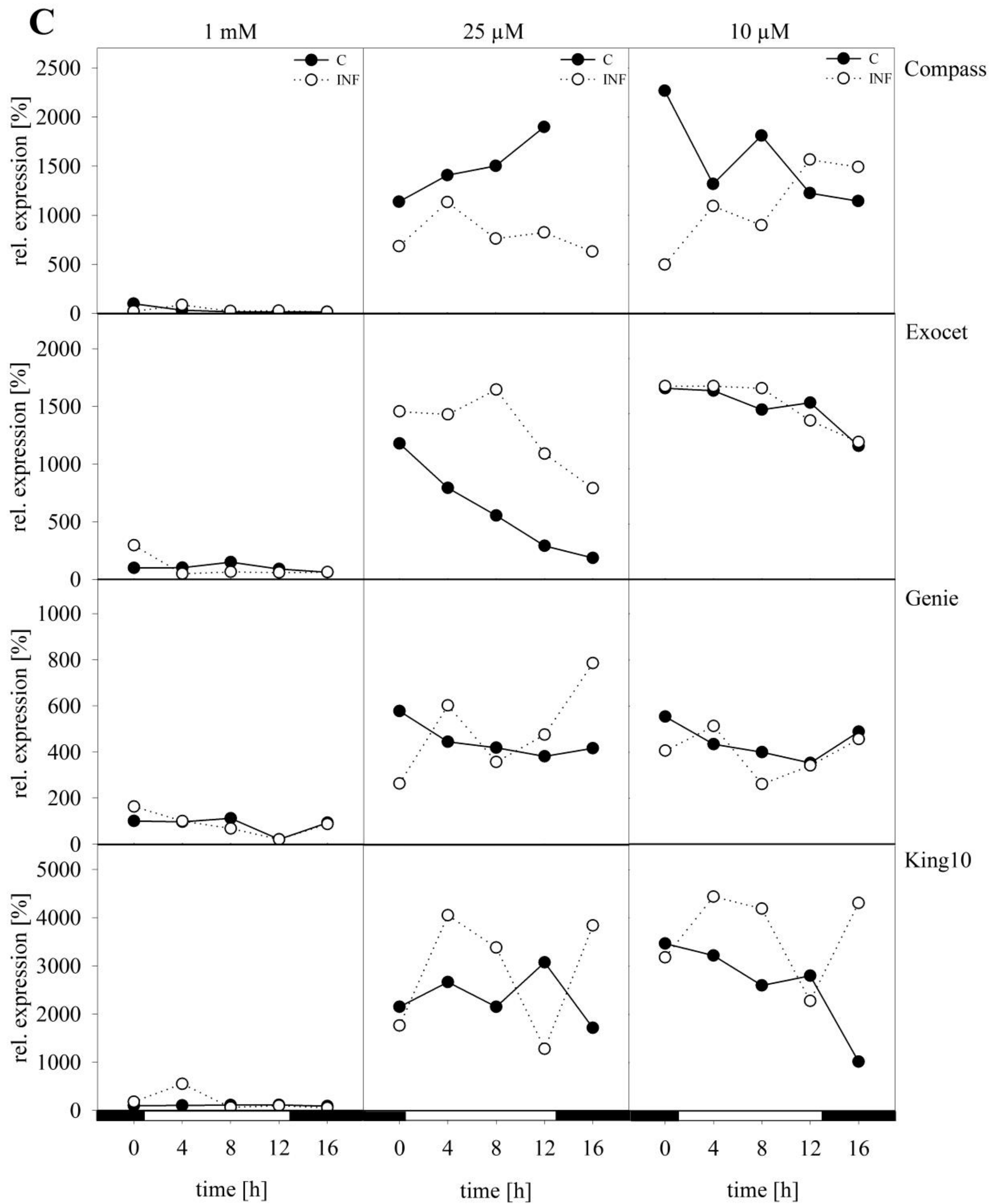

C
1 mM
25 µM
10 µM
C
INF
Compass
Exocet
Genie
King10
rel. expression [%]
time [h]

Table S1. Differences in root hair length among the varieties. Statistical tests were performed using Sigma Plot. Mean values of five plants ± SD are shown along with letters indicating significant differences for pairwise comparisons (Tukey test) among the cultivars.

Root hair length [cm]			
Compass	Exocet	Genie	King10
1.166±0.282[a]	1.14±0.287[a]	0.582±0.079[b]	0.698±0.085[b]

Table S2. Influence of S-fertilization, infection and cultivar on the total dry mass. Statistical analysis was performed using R. The p-values refer to pairwise comparisons (Tukey test) of the data shown in Figure 2A. Significances: p<0.05*, p<0.01**,p<0.001***.

variable	comparison	p-value	significance
S-fertilization	1 mM – 0.025 mM	<0.001	***
	1 mM – 0.01 mM	<0.001	***
	0.025 mM – 0.01 mM	0.556	ns
infection	Compass: INF - C	0.064	ns
	Exocet: INF - C	<0.001	***
	Genie: INF - C	<0.001	***
	King10: INF - C	0.100	ns
cultivar	C: Compass - Exocet	0.639	ns
	C: Compass - Genie	0.015	*
	C: Compass - King10	<0.001	***
	C: Exocet - Genie	0.260	ns
	C: Exocet - King10	<0.001	**
	C: Genie - King10	0.546	ns
	INF: Compass - Exocet	<0.001	***
	INF: Compass - Genie	<0.001	***
	INF: Compass - King10	<0.001	***
	INF: Exocet - Genie	0.367	ns
	INF: Exocet - King10	0.989	ns
	INF: Genie - King10	0.206	ns

Table S3. Influence of S-fertilization, infection and cultivar on the shoot-to-root ratio. Statistical tests were performed using R. The p-values refer to pairwise comparisons (Tukey tests) of the data shown in Figure 2B. Significances: p<0.05*, p<0.01**, p<0.001***.

variable	comparison	p-value	significance
S-fertilization	1 mM – 0.025 mM	<0.001	**
	1 mM – 0.010 mM	0.082	ns
	0.025 mM – 0.010 mM	0.510	ns
infection	INF- C	0.220	ns
cultivar	Compass - Exocet	<0.001	***
	Compass - Genie	<0.001	***
	Compass - King10	<0.001	***
	Exocet - Genie	0.918	ns
	Exocet - King10	0.033	*
	Genie - King10	0.154	ns

Table S4. Influence of the interaction of S-fertilization, infection and cultivar on the leaf temperature. Statistical analysis was performed using R. The p-values refer to pairwise comparisons (Tukey tests) of the data shown in Figure 3. Since all interactions were significant in the F-test, comparisons were carried out separately at all factor levels. Significances: p<0.05*, p<0.01**, p<0.001***.

variable	comparison	p-value	significance
S-fertilization	C: Compass: 1 mM – 0.025 mM	0.413	ns
	C: Compass: 1 mM – 0.01 mM	0.999	ns
	C: Compass: 0.025 mM – 0.01 mM	0.387	ns
	C: Exocet: 1 mM - 0.025 mM	0.858	ns
	C: Exocet: 1 mM - 0.01 mM	0.747	ns
	C: Exocet: 0.025 mM - 0.01 mM	0.422	ns
	C: Genie: 1 mM - 0.025 mM	0.945	ns
	C: Genie: 1 mM - 0.01 mM	0.122	ns
	C: Genie: 0.025 mM - 0.01 mM	0.058	ns
	C: King10: 1 mM - 0.025 mM	0.339	ns
	C: King10: 1 mM - 0.01 mM	0.859	ns
	C: King10: 0.025 mM - 0.01 mM	0.654	ns
	INF: Compass: 1 mM - 0.025 mM	<0.001	***
	INF: Compass: 1 mM - 0.01 mM	0.729	ns
	INF: Compass: 0.025 mM - 0.01 mM	<0.001	***
	INF: Exocet: 1 mM - 0.025 mM	0.673	ns
	INF: Exocet: 1 mM - 0.01 mM	0.686	ns
	INF: Exocet: 0.025 mM - 0.01 mM	0.999	ns

	INF: Genie: 1 mM - 0.025 mM	0.851	ns
	INF: Genie: 1 mM - 0.01 mM	0.971	ns
	INF: Genie: 0.025 mM - 0.01 mM	0.948	ns
	INF: King10: 1 mM - 0.025 mM	<0.001	***
	INF: King10: 1 mM - 0.01 mM	0.004	**
	INF: King10: 0.025 mM - 0.01 mM	0.006	**
infection	1 mM: Compass: INF - C	0.869	ns
	1 mM: Exocet: INF - C	0.251	ns
	1 mM: Genie: INF - C	0.537	ns
	1 mM: King10: INF - C	0.516	ns
	0.025 mM: Compass: INF - C	<0.001	***
	0.025 mM: Exocet: INF - C	0.012	*
	0.025 mM: Genie: INF - C	0.402	ns
	0.025 mM: King10: INF - C	<0.001	***
	0.01 mM: Compass: INF - C	0.333	ns
	0.01 mM: Exocet: INF - C	0.212	ns
	0.01 mM: Genie: INF - C	0.005	**
	0.01 mM: King10: INF - C	<0.001	***
cultivar	C: 1 mM: Compass - Exocet	0.999	ns
	C: 1 mM: Compass - Genie	0.573	ns
	C: 1 mM: Compass - King10	0.675	ns
	C: 1 mM: Exocet - Genie	0.637	ns
	C: 1 mM: Exocet - King10	0.736	ns
	C: 1 mM: Genie - King10	0.999	ns
	C: 0.025 mM: Compass - Exocet	0.326	ns
	C: 0.025 mM: Compass - Genie	0.987	ns
	C: 0.025 mM: Compass - King10	0.589	ns
	C: 0.025 mM: Exocet - Genie	0.177	ns
	C: 0.025 mM: Exocet - King10	0.016	*
	C: 0.025 mM: Genie - King10	0.793	ns
	C: 0.01 mM: Compass - Exocet	0.819	ns
	C: 0.01 mM: Compass - Genie	0.922	ns
	C: 0.01 mM: Compass - King10	0.326	ns
	C: 0.01 mM: Exocet - Genie	0.435	ns
	C: 0.01 mM: Exocet - King10	0.844	ns
	C: 0.01 mM: Genie - King10	0.092	ns
	INF: 1 mM: Compass - Exocet	0.700	ns
	INF: 1 mM: Compass - Genie	0.305	ns
	INF: 1 mM: Compass - King10	0.374	ns
	INF: 1 mM: Exocet - Genie	0.915	ns

	INF: 1 mM: Exocet - King10	0.953	ns
	INF: 1 mM: Genie - King10	0.999	ns
	INF: 0.025 mM: Compass - Exocet	<0.001	***
	INF: 0.025 mM: Compass - Genie	<0.001	***
	INF: 0.025 mM: Compass - King10	0.816	ns
	INF: 0.025 mM: Exocet - Genie	0.986	ns
	INF: 0.025 mM: Exocet - King10	<0.001	***
	INF: 0.025 mM: Genie - King10	<0.001	***
	INF: 0.01 mM: Compass - Exocet	0.656	ns
	INF: 0.01 mM: Compass - Genie	0.621	ns
	INF: 0.01 mM: Compass - King10	<0.001	***
	INF: 0.01 mM: Exocet - Genie	0.999	ns
	INF: 0.01 mM: Exocet - King10	0.019	*
	INF: 0.01 mM: Genie - King10	0.022	*

Table S5. **Influence of the interaction of S-fertilization, infection and cultivar on the cholorophyll fluorescence.** Statistical analysis was performed using R. The p-values refer to pairwise comparisons (Tukey test) of the data shown in Figure 4. Since all the interactions were significant in the F-test, comparisons were carried out separately at all factor levels. Significances: p<0.05*, p<0.01**, p<0.001***.

variable	comparison	p-value	significance
S-fertilization	C: Compass: 1 mM - 0.025 mM	<0.001	***
	C: Compass: 1 mM - 0.01 mM	<0.001	***
	C: Compass: 0.025 mM - 0.01 mM	0.633	ns
	C: Exocet: 1 mM - 0.025 mM	<0.001	***
	C: Exocet: 1 mM - 0.01 mM	0.999	ns
	C: Exocet: 0.025 mM - 0.01 mM	<0.001	***
	C: Genie: 1 mM - 0.025 mM	0.964	ns
	C: Genie: 1 mM - 0.01 mM	0.861	ns
	C: Genie: 0.025 mM - 0.01 mM	0.962	ns
	C: King0.01: 1 mM - 0.025 mM	<0.001	***
	C: King0.01: 1 mM - 0.01 mM	<0.001	***
	C: King0.01: 0.025 mM - 0.01 mM	0.569	ns
	Inf: Compass: 1 mM - 0.025 mM	<0.001	***
	Inf: Compass: 1 mM - 0.01 mM	<0.001	***
	Inf: Compass: 0.025 mM - 0.01 mM	0.964	ns
	Inf: Exocet: 1 mM - 0.025 mM	<0.001	***
	Inf: Exocet: 1 mM - 0.01 mM	<0.001	***
	Inf: Exocet: 0.025 mM - 0.01 mM	0.500	ns
	Inf: Genie: 1 mM - 0.025 mM	<0.001	***

	Inf: Genie: 1 mM - 0.01 mM	<0.001	***
	Inf: Genie: 0.025 mM - 0.01 mM	0.781	ns
	Inf: King0.01: 1 mM - 0.025 mM	<0.001	***
	Inf: King0.01: 1 mM - 0.01 mM	<0.001	***
	Inf: King0.01: 0.025 mM - 0.01 mM	0.845	ns
infection	1 mM: Compass: Inf - C	0.474	ns
	1 mM: Exocet: Inf - C	0.360	ns
	1 mM: Genie: Inf - C	0.681	ns
	1 mM: King0.01: Inf - C	0.706	ns
	0.025 mM: Compass: Inf - C	<0.001	***
	0.025 mM: Exocet: Inf - C	0.001	**
	0.025 mM: Genie: Inf - C	<0.001	***
	0.025 mM: King0.01: Inf - C	0.896	ns
	0.01 mM: Compass: Inf - C	0.030	*
	0.01 mM: Exocet: Inf - C	<0.001	***
	0.01 mM: Genie: Inf - C	<0.001	***
	0.01 mM: King0.01: Inf - C	0.555	ns
cultivar	C: 1 mM: Compass - Exocet	0.812	ns
	C: 1 mM: Compass - Genie	0.702	ns
	C: 1 mM: Compass - King0.01	0.997	ns
	C: 1 mM: Exocet - Genie	0.997	ns
	C: 1 mM: Exocet - King0.01	0.899	ns
	C: 1 mM: Genie - King0.01	0.812	ns
	C: 0.025 mM: Compass - Exocet	0.748	ns
	C: 0.025 mM: Compass - Genie	0.091	ns
	C: 0.025 mM: Compass - King0.01	0.002	**
	C: 0.025 mM: Exocet - Genie	0.005	**
	C: 0.025 mM: Exocet - King0.01	0.044	*
	C: 0.025 mM: Genie - King0.01	0.	***
	C: 0.01 mM: Compass - Exocet	0.001	**
	C: 0.01 mM: Compass - Genie	0.016	*
	C: 0.01 mM: Compass - King0.01	0.324	ns
	C: 0.01 mM: Exocet - Genie	0.894	ns
	C: 0.01 mM: Exocet - King0.01	0.	***
	C: 0.01 mM: Genie - King0.01	0.	***
	Inf: 1 mM: Compass - Exocet	0.698	ns
	Inf: 1 mM: Compass - Genie	0.866	ns
	Inf: 1 mM: Compass - King0.01	0.999	ns
	Inf: 1 mM: Exocet - Genie	0.989	ns
	Inf: 1 mM: Exocet - King0.01	0.606	ns

Inf: 1 mM: Genie - King0.01	0.794	ns
Inf: 0.025 mM: Compass - Exocet	0.817	ns
Inf: 0.025 mM: Compass - Genie	0.961	ns
Inf: 0.025 mM: Compass - King0.01	0.976	ns
Inf: 0.025 mM: Exocet - Genie	0.519	ns
Inf: 0.025 mM: Exocet - King0.01	0.967	ns
Inf: 0.025 mM: Genie - King0.01	0.802	ns
Inf: 0.01 mM: Compass - Exocet	0.108	ns
Inf: 0.01 mM: Compass - Genie	0.972	ns
Inf: 0.01 mM: Compass - King0.01	0.999	ns
Inf: 0.01 mM: Exocet - Genie	0.263	ns
Inf: 0.01 mM: Exocet - King0.01	0.141	ns
Inf: 0.01 mM: Genie - King0.01	0.989	ns

CHAPTER 3

Quantitative expression analysis in *Brassica napus* by Northern blot analysis and reverse transcription-quantitative PCR in a complex experimental setting

Rumlow, A., Keunen, E., Klein, J., Pallmann, P., Riemenschneider, A., Cuypers, A., Papenbrock, J. (2016) Quantitative expression analysis in Brassica napus by Northern blot analysis and reverse transcription-quantitative PCR in a complex experimental setting. *PLoS ONE* 11(9): e0163679. doi:10.1371/journal.pone.0163679

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RESEARCH ARTICLE

Quantitative Expression Analysis in *Brassica napus* by Northern Blot Analysis and Reverse Transcription-Quantitative PCR in a Complex Experimental Setting

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Abstract

Analysis of gene expression is one of the major ways to better understand plant reactions to changes in environmental conditions. The comparison of many different factors influencing plant growth challenges the gene expression analysis for specific gene-targeted experiments, especially with regard to the choice of suitable reference genes. The aim of this study is to compare expression results obtained by Northern blot, semi-quantitative PCR and RT-qPCR, and to identify a reliable set of reference genes for oilseed rape (*Brassica napus* L.) suitable for comparing gene expression under complex experimental conditions. We investigated the influence of several factors such as sulfur deficiency, different time points during the day, varying light conditions, and their interaction on gene expression in oilseed rape plants. The expression of selected reference genes was indeed influenced under these conditions in different ways. Therefore, a recently developed algorithm, called GrayNorm, was applied to validate a set of reference genes for normalizing results obtained by Northern blot analysis. After careful comparison of the three methods mentioned above, Northern blot analysis seems to be a reliable and cost-effective alternative for gene expression analysis under a complex growth regime. For using this method in a quantitative way a number of references was validated revealing that for our experiment a set of three references provides an appropriate normalization. Semi-quantitative PCR was prone to many handling errors and difficult to control while RT-qPCR was very sensitive to expression fluctuations of the reference genes.

Introduction

In recent years, different techniques and new methods have been developed to investigate the reaction of plants and their underlying regulating mechanisms to different environmental

changes at the level of gene expression. Depending on the research question, different methods have been applied to analyze the expression levels of genes and in this way measure the abundance of specific mRNA transcripts. Northern blot analysis [1] was often used as the gold standard to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. During the hybridization step, a labeled probe is required to form a double-stranded RNA-DNA or RNA-RNA molecule that can be detected by using an antibody-assisted technology in a colorimetric or a luminescent reaction. The resulting intensity of the color or light signal is proportional to the enzyme activity, which in turn is correlated to the transcript level of the gene of interest (GOI). Thus, expression analysis with this method is focused on an endpoint signal. Northern blot analysis is the only method providing information on transcript size and integrity of the isolated RNA. Furthermore, it is a very versatile method including different labeling and detection methods and acceptance of different lengths of probes. One advantage is that RNA quantity and quality can be verified after gel electrophoresis, which makes it possible to evaluate the progress. On the other hand, there are a number of disadvantages such as the need for high quality RNA in large amounts. In addition, intensive washing steps, followed by a detection step of hybridization products make the Northern blot technique time-consuming.

Due to the discovery of the reverse transcriptase (RT) enzyme converting RNA into DNA, very low GOI transcript levels can be detected with a high specificity using PCR-based methods. To quantify the transcript levels of a target gene, semi-quantitative non-real-time PCR (sqPCR) has been applied. After a defined number of cycles in the range of the exponential phase, the reaction is stopped and the product visualized by electrophoretic separation and staining. By measuring the intensity of the band corresponding to the amplified product, the GOI expression level can be determined. However, this method has a number of pitfalls such as having to determine the cycle number for all samples, potentially leading to false interpretation of results. Therefore, this technique is suitable for investigating differences in the linear phase, e.g. in knockout studies to illustrate expression versus no expression (for an overview, see [2]). Although, this method is generally only accepted in knockout studies it is still in use for analyzing gene expression in plants treated by different treatments [3,4].

Nowadays, quantitative or real-time PCR (RT-qPCR) seems to be the method of choice for a rapid and reliable quantification of mRNA transcripts (for an overview, see [2,5]). This technique combines the PCR chemistry with the use of fluorescent reporter molecules to assess the rate of amplicon accumulation during the exponential phase in the course of the reaction cycles. RT-qPCR constitutes an excellent combination of sensitivity, specificity and reproducibility in a relatively short period of time [6]. A number of published RT-qPCR data showed a lack of experimental details such as RNA quality and integrity or PCR efficiencies, making a critical evaluation of the quality of the results by the reader difficult [7]. Therefore, a guideline was established called Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), providing authors with a list of information that should be reported for RT-qPCR experiments [5]. However, the accuracy of the expression analysis—whether performed by Northern blot analysis or RT-qPCR—crucially depends on the use of stably expressed reference genes. This is very important when it comes to RT-qPCR analysis where the transcript level of each target gene is normalized to the expression of a combination of at least three reference genes ideally correcting for technical errors, e.g. introduced during the RT step and affecting all genes similarly [8]. Algorithms such as geNorm [9] and Normfinder [10] can be used to identify a combination of stable reference genes under specific experimental conditions. Based on expression levels of the best performing reference genes, a sample-specific normalization factor is calculated. Evaluating candidate genes as suitable reference genes using such algorithms increased the accuracy of gene expression analysis in mammals, yeast and

bacteria. However, a number of publications surveyed from 2009–2011 and 2012–2013 showed inadequate normalization leading to results the reader cannot rely on properly [7].

Regarding expression analysis in plants a lack of an appropriate validation of reference genes in the past led to the use of candidate genes for normalization turning out to be not a good choice either (reviewed in [11]). To avoid such incidents, a number of reference genes in plants were tested under different stress conditions and in different tissues, resulting in different recommendations of reference genes for each condition [12–16]. Depending on the material and experimental settings, one has to search for an appropriate combination of reference genes whose expression is minimally affected under the given conditions [17,18]. However, candidate reference genes should be (re)validated in each subsequent experiment [19]. Furthermore, not all variability can be removed by reference genes, even when appropriate procedures are applied. Indeed, reference genes always show small or larger expression changes between tissues or treatments, which potentially cause differences in the calculation of GOI transcript levels leading to false biological conclusions. With the GrayNorm algorithm, a method was developed to maximize data accuracy by selecting the optimal combination of reference genes for each particular experiment [19].

We describe the technical prerequisites to quantitatively analyze the expression of specific genes in experiments where oilseed rape (*Brassica napus* L.) plants were grown at different sulfur (S) supplies and analyzed over a day/night cycle and continuous light as well. When the plants are grown under constant conditions, the circadian rhythms persist and oscillate with an endogenous period close to 24 h. In their natural environment, the plants are exposed to environmental "zeitgebers" such as light and temperature. It entrains the endogenous organismal clock in each cell with the local time (reviewed in [20,21]). Since S is an essential macronutrient required to synthesize the amino acids cysteine and methionine as well as glutathione, phytochelatins, vitamins and cofactors, the dependence of the circadian clock on S supply is evaluated in this study. Expression levels of genes involved in the circadian rhythm and the S assimilation were therefore determined using Northern blot analysis. In the first part of this study, its reliability was verified by comparing it to PCR-based expression analyses. In addition to a detailed statistical comparison, factors like working hours, ease of handling and costs for each sample are taken into account when evaluating our results. In the second part, we show the importance of selecting reliable reference genes under complex experimental settings affecting the plants in multiple ways. Therefore, a suitable combination of reference genes was determined by the GrayNorm algorithm to normalize expression levels obtained by Northern blot analysis. Results were additionally compared to data normalized with only one reference gene to highlight the importance of an adequate normalization.

Material and Methods

Plant growth

Oilseed rape (*Brassica napus* L.) seeds of cultivar Genie were obtained from the Deutsche Saatveredelung AG (DSV) (Lippstadt, Germany). For experiments under circadian and diurnal conditions, the seeds were germinated in a pot with a diameter of 8 cm containing sand (0–2 mm grain size, Hornbach, Hannover, Germany) in a climate chamber [22°C, 70% humidity, 12 h light/12 h dark, 480 µmol m^{-2} s^{-1} (lamp type CMT 360LS/W/BH-E40, Eye Lighting Europe Ltd, Uxbridge, UK)]. A total of 102 plants were grown, one plant per pot, for 19 d and watered once per week using 150 ml Blake-Kalff medium [22] containing 1 mM $MgSO_4$. After a washing step with deionized water one half of the plants were transferred to "plus S" conditions with 1 mM $MgSO_4$. The other half of the plants were transferred to "minus S" conditions using Blake-Kalff medium with only 10 µM $MgSO_4$. Plants were grown under these conditions for 4

days. One hour before the light was switched on three plants of each treatment were then harvested every 4 h over a time period of 36 h. The material was pooled and immediately frozen in liquid nitrogen. Additionally 42 plants under "plus S" and "minus S" conditions were transferred to continuous light. These were then harvested at the same time as the plants grown under 12 h light/12 h dark every 4 h beginning after 16 h representing the beginning of the subjective night. For a 24 h cycle plants under continuous light were additionally harvested at 40 h. The complete experiment was performed twice.

Sequence analysis

Sequences homologous to *Arabidopsis thaliana* DNA sequences for the genes *18S ribosomal RNA (18S rRNA)*, *Actin2 (ACT2)*, *ELONGATION FACTOR 1α (EF1α)*, *TATA BOX BINDING PROTEIN 2* (*TBP2*) and *TIP41-LIKE PROTEIN* (*TIP41)* as reference genes, as well as the *CIRCADIAN CLOCK ASSOCIATED1 (CCA1*) and *ADENOSINE 5'-PHOSPHOSULFATE REDUCTASE 3* (*APR3*) sequences as the GOIs were searched in the *B. napus* database (http://compbio.dfci.harvard.edu/compbio) [23] using BLAST. The data bank uses parts of short homologous sequences (high-fidelity virtual transcripts; TC-sequences, tentative consensus sequences) to generate EST sequences [24] that were used for primer design (http://www.dosbox.com) [25]. For the other reference genes *ADENINE PHOSPHORIBOSYL TRANSFERASE 1 (APT1)*, *GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1 (GDI1)*, *SERINE/THREONINE PROTEIN PHOSPHATASE 2A (PP2A)*, and *UBIQUITINE-CONJUGATING ENZYME 21 (UBC21)* the database NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used due to the availability of *B. napus* sequences [26]. Primers were used to amplify cDNA fragments with a size of about 300 bp for Northern blot analysis and about 100 bp for RT-qPCR analysis (Table 1).

To identify homologous genes in *B. napus*, the known sequences from *A. thaliana* were blasted against the *B. napus* databases [23,26]. for: forward; rev: reverse; *CCA1*: *CIRCADIAN CLOCK ASSOCIATED1; APR3*: *ADENOSINE 5' PHOSPHOSULFATE REDUCTASE 3; EF1α*: *ELONGATION FACTOR 1α; TIP41*: *TIP41-LIKE PROTEIN; ACT2*:*ACTIN2; 18S rRNA*: *18S RIBOSOMAL RNA; TBP2*: *TATA BOX BINDING PROTEIN 2; PP2A*: *SERINE/THREONINE PROTEIN PHOSPHATASE 2A; APT1*: *ADENINE PHOSPHORIBOSYL TRANSFERASE 1; UBC21*: *UBIQUITINE-CONJUGATING ENZYME 21; GDI1*: *GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1.*

RNA extraction and Northern blot analysis

Total RNA was extracted from ground plant material according to [27] and spectrophotometrically quantified. Fifteen μg RNA was separated on 1% denaturing agarose-formaldehyde gels. Equal loading was examined by staining the gels with ethidium bromide. After RNA transfer onto nylon membranes, they were probed with digoxigenin (DIG)-labeled cDNA probes obtained by PCR (PCR DIG probe synthesis kit, Roche, Mannheim, Germany). To amplify the respective probes, the sequence-specific primers listed in Table 1 were used. Colorimetric detection was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates for alkaline phosphatase. Quantitative analysis of the Northern blot results was done by GelAnalyzer 2010a (www.GelAnalyzer.com) [28].

cDNA synthesis

Isolated RNA was spectrophotometrically quantified and afterwards, 250 ng of the total RNA was utilized for reverse transcription. As a first step, the remaining DNA was degraded by

Table 1. Primer pairs used in this study.

Primer pairs	*A. thaliana* AGI	Sequences
P741_Bn_CCA1_for	At2g46830	5'-TTCTTGTGGCTCGAACACTCCT-3'
P742_Bn_CCA1_rev		5'-GGATTGGTGTTGCTGATGACTC-3'
P745_Bn_APR3_for	At4g21990	5'-CATCAAGGAGAACAGCAACGCA-3'
P746_Bn_APR3_rev		5'-TCGGGAACACTAGTATCGTCGG-3'
P747_Bn_EF1α_for	At5g60390	5'-GCTTGGTTGGAGTCATCTTCAC-3'
P748_Bn_EF1α_rev		5'-TCTCCTTGAGGCTCTTGACCAG-3'
P768A_Bn_TIP41_for	At4g34270	5'-GGCTTACGAATCCATGACTG-3'
P769B_Bn_TIP41_rev		5'-GAGGAGGAACCATGAACTTG-3'
P780_Bn_ACT2_for	At3g18780	5'-AACCTTCAACTCTCCAGCTA-3'
P781_Bn_ACT2_rev		5'-GAGTTGTAAGTCGTCTCGTG-3'
P782_Bn_18S rRNA_for	X16077.1	5'-ATGAACGAATTCAGACTGTG-3'
P783_Bn_18S rRNA_rev		5'-ACTCATTCCAATTACCAGAC-3'
P784_Bn_TBP2_for	At1g55520	5'-GGCTGAACAAGGAATGGAAG-3'
P785_Bn_TBP2_rev		5'-TCTCTCATCTTGGCTCCGGT-3'
P816_Bn_Act2_qPCR_f	At3g18780	5’-ACTCTCCAGCTATGTATGTCGCC-3’
P817_Bn_Act2_qPCR_r		5’-GAGACACACCATCACCAGAATCC-3’
P818_Bn_CCA1_qPCR_f	At2g46830	5’-GTCATCATCATCCTTGTGCAGCG-3’
P819_Bn_CCA1_qPCR_r		5’-GTGTTCGAGCCACAAGAAGACCT-3’
P822_Bn_APR3_qPCR_f	At4g21990	5’-AACGGCTAATGTCAATGGGACG-3’
P823_Bn_APR3_qPCR_r		5’-AAGCACAACGATCCAAGCCTCT-32019
P824_Bn_EF1α_qPCR_f	At5g60390	5'-GCAGATTGGTAACGGTTACG-3'
P825_Bn_EF1α_qPCR_r		5'-CTCCTTACCAGAACGCCTGT-3'
P960_Bn_q18SrRNA_f	X16077.1	5'-TGCAACAAACCCCGACTTCT-3'
P961_Bn_q18SrRNA_r		5'-TGCGATCCGTCGAGTTATCA-3'
P962_Bn_qTIP41_f	At4g34270	5'-GCGGCACGATTCTCACTTCT-3'
P963_Bn_qTIP41_r		5'-CACTAACGCATTCTCGCCAA-3'
P968_Bn_PP2A_f	At1g69960	5'-ACGAGGACGGATTTGGTTCC-3'
P969_Bn_PP2A_r		5'-GCTCCGAGCTTGTCATCGAA-3'
P970_Bn_qPP2A_f	At1g69960	5'-GTCAACAATCCGCACTACCTACA-3'
P971_Bn_qPP2A_r		5'-ACCACAGGAAGAAACTTAGAGCA-3'
P976_Bn_APT1_f	At1g27450	5'-TTCTTCTCGACACAGAGGCG-3'
P977_Bn_APT1_r		5'-TTCTCCCTGCCCTTAAGCTCT-3'
P978_Bn_qAPT1_f	At1g27450	5'-CATTGCTACGGGTGGGACTC-3'
P979_Bn_qAPT1_r		5'-CCCTTAAGCTCTGGTAACTCAATCA-3'
P980_Bn_UBC21_f	At5g25760	5'-ATCACGAGCGAGACTGTTCA-3'
P981_Bn_UBC21_r		5'-CCTCAGGATGAGCCATCAGT-3'
P982_Bn_qUBC21_f	At5g25760	5'-GACTGCACTTATCAAGGGACCG-3'
P983_Bn_qUBC21_r		5'-ACGGTTCGGGAACAGCGAAT-3'
P984_Bn_GDI1_f	At2g44100	5'-TGCACGTTTCCAAGGAGGTT-3'
P986_Bn_GDI1_r		5'-CGGTCTGAGGGTTGTCAGTC-3'
P987_Bn_qGDI1_f	At2g44100	5'-CGAGCCTGTCAACGAACCCA-3'
P988_Bn_qGDI1_r		5'-ATCCAGTTCCTTGCCGGTGA-3'

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DNaseI (Thermo Fisher Scientific Inc., Waltham, USA). For the cDNA synthesis, oligo-(dT)$_{18}$-primers from the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) were used. The addition of 1 μL RiboLock RNase inhibitor (Roche) enhanced the quality of the cDNA.

Semi-quantitative qPCR

Semi-quantitative PCR (sqPCR) assays were performed with a Thermocycler cyclone 25 (Peq-Lab, Erlangen, Germany) using Dream *Taq*™ DNA Polymerase (Thermo Fisher Scientific Inc.) and the primers listed in Table 1. For each primer pair, we determined the number of cycles with differently diluted cDNA where the amplification was still exponential. Products of sqPCR were separated on a 2% agarose gel and quantified by the intensity of the bands relative to the first band using the program GelAnalyzer 2010a [28].

Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was realized with SYBR Green fluorescence and a ROX reference dye (Platinum SYBR Green RT-qPCR Mix; Thermo Fisher Scientific Inc.) on an ABI PRISM 7300 sequence detection system (Thermo Fisher Scientific Inc.) with the primers listed in Table 1. Raw data were converted into expression data by the ΔCt method [29].

GrayNorm algorithm

Expression data of selected reference genes obtained by Northern blot analysis was evaluated with the GrayNorm algorithm according to [19] to maximize data accuracy by selecting the right combination of reference genes for each particular experiment.

Statistical analysis

Six biological samples each with three technical replicates were used to determine the technical and biological variability. A restricted maximum likelihood (REML) variance component estimation in a linear mixed-effects model (for more information see [30]) was performed, where the technical variability within the biological replicates was determined by calculating the ratio of the biological to the technical variance component. Furthermore, the costs to perform each method per sample were individually calculated. Additionally data were compared in a Bland Altman Plot [31]. Using this method, agreements between individual measurements can be quantified whereas the correlation factor only measures the strength of a relation between two variables [32]. Each data point for every sample obtained using both methods is directly compared by calculating the difference and the average of each individual data point. The difference of each data point was then plotted against the average of each data point from both methods. Ideally, the data points lie very close to the mean of the difference indicating a high agreement between both methods. Analyzing the data obtained by both methods in a proper way the data were standardized first by centralizing the mean for both methods to zero and then divided by the standard deviation to achieve unit variance. The 95% limits of agreement were calculated with ±1.96xSD for the two or three technical replicates respectively ([29], chapter 5.2).

The relative expression with non-normalized and normalized data were evaluated using a Two-Way ANOVA with the relative band intensities as dependent variable and S concentration and time point of harvest as independent factors. For the independent factors S concentration, time point of harvest and light a Three-Way ANOVA was performed with the relative band intensities as dependent variable. Significance of factors and their interactions was assessed by means of F-tests.

Results

Comparison of the methods reveals the same trends of expression

To compare the technical variability of the three methods, an expression analysis of *ACT2* as a reference gene with a low biological variability was performed. Therefore, samples from plants

grown under "plus S" conditions and 12 h light/12 h dark harvested every 4 h starting 1 h before the light was switched on were used. The total RNA was isolated out of three technical replicates for each sample and quantified. This RNA was then used as the initial point for all three methods.

Both Northern blot analysis and PCR-based methods showed a number of advantages and disadvantages. For Northern blot analysis, the isolated RNA was directly used for expression analysis, whereas for the PCR-based methods it was necessary to first perform reverse transcription. However, high amounts of RNA (15 μg per sample) were needed for Northern blot analysis. In contrast, 250 ng was sufficient to perform adequate cDNA synthesis. Evaluation of the success of cDNA synthesis was only possible by using control reactions, which were processed later on as samples in the PCR. For Northern blot analysis, the process was evaluated after the electrophoretic separation as well as after the blotting by visualizing the RNA under UV light. For the sqPCR pre-experiments, it was necessary to identify the correct cycle number and amount of template for every primer pair separately. On the other hand, these remained constant for each primer pair used in Northern blot analysis and RT-qPCR. The results obtained by Northern blot analysis and sqPCR are based on band intensities on the membrane or in the gel. Therefore, careful documentation was necessary as well as suitable software to measure band intensity. Inadequate membrane or gel quality can lead to false results that in turn provoke false biological conclusions. For RT-qPCR, quantification is more precise by measuring the amount of synthesized DNA in real-time. Nevertheless, all three methods are highly dependent on high-quality non-degraded RNA, precise pipetting to guarantee the same total amount of mRNA and a good documentation of results. Especially for the PCR-based methods, a number of steps during the process were dependent on precise pipetting indicated by less standard deviation after training, making this a big source of possible errors during sample preparation.

Based on the first results of *ACT2* expression analysis, sqPCR showed less agreement with the other two methods. Using sqPCR, the transcript level of *ACT2* was higher in the light phase, whereas its transcript levels were decreased in the light phase for Northern blot analysis and RT-qPCR (data not shown). Furthermore, the technical variability was about 44% higher than the biological variability (Table 2). With Northern blot the lowest technical variability of about 14% was shown. Investigating the suitable cycle number for the sqPCR was very time consuming and may vary for each primer pair used. Therefore, this method was excluded in further experiments.

Northern blot and RT-qPCR analysis were performed to compare the expression of additional genes (Fig 1). Besides *ACT2*, *EF1α* and *18S rRNA* were chosen as other commonly used

Table 2. Overview of the costs, duration and technical to biological ratio of the methods.

	Costs (€ per sample)	Costs for 34 samples and 10 genes*	Duration (18 samples)	Technical:biolo-gical variation (%)
Northern blot analysis	2.01	~700	3 d	14.9
Semi-quantitative PCR (sqPCR)	3.29	~600	8 h	44.5
RT-qPCR	6.04	~1500	6 h	21.4

* The costs were calculated based on the whole data set consisting of 34 samples in total and 10 genes for expression analysis. For the calculation 3 repetitions per sample were taken into consideration. The cDNA synthesis for every sample was calculated for the PCR-based methods only once due to reuse of the cDNA for each primer system. The costs to perform the methods for one sample excluding RNA isolation were estimated in the same way for all three methods. Only the costs for the required materials were included in this calculation. Duration describes the time needed to perform each method after the RNA was isolated. For calculating the ratio between the biological to technical variation, a REML variance component estimation was performed.

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reference genes. In addition, one of the key genes in sulfate assimilation, *APR3*, and *CCA1* as part of the circadian oscillator were included in the expression analysis. The mean of two to three technical replicates showed the same trends of expression over the day for all five genes using both methods. However, the replicates of RT-qPCR analysis showed higher variations in their relative expression as compared to those in the Northern blot analysis. Moreover, some replicates for RT-qPCR analysis could not be used for the evaluation due to a missing fluorescence signal for these samples and were therefore omitted.

For *ACT2*, a higher expression level was measured 1 h before the light was switched on, as well as for plants harvested 1 h before the light was switched off as compared to the other time points (Fig 1). Interestingly, *ACT2* relative expression obtained by Northern blot analysis showed a decrease of about 30% after 3 h of light, which was not the case with RT-qPCR analysis. However, for both methods, lower *ACT2* transcript levels were observed in the middle of the light phase followed by an increase at the end of the light phase. Moreover, higher oscillations of the relative expression occurred due to high variations between the technical replicates when using RT-qPCR analysis.

As another reference gene, the expression of *EF1α* was analyzed (Fig 1). For both methods, a high degree of upregulation was observed in the middle of the light phase, remaining high at the end of the light phase and increasing slightly again at the beginning of the dark phase. Although the trend of expression was in agreement for both methods again high variations between the three replicates for RT-qPCR analysis were observed.

As a third reference gene the expression of *18S rRNA* was analyzed as well. First Northern blot analyses resulted in high band intensities indicating a possible saturated signal which might lead to underestimation of differences in the transcript amount. Therefore, the probe was tested for different RNA concentrations ranging from 1 to 24 μg revealing a saturated signal already at low RNA concentration. According to the results the *18S rRNA* probe was diluted 1:10 for further experiments (S1 Fig). Comparing the expression of *18S rRNA* with both methods higher oscillations for the RT-qPCR analysis were shown compared to the Northern blot analysis (Fig 1).

Relative expression levels of *APR3* showed for both methods an upregulation in the middle of the light phase as well as for the beginning of the night phase. Oscillations of transcript amounts were in this case higher when using Northern blot analysis (Fig 1). Based on its circadian regulation, *CCA1* showed a typical expression pattern during the course of the day. In the morning, before the light was switched on, relatively high *CCA1* transcript levels were detected. Three hours after the light was switched on, the highest degree of upregulation appeared. Afterwards, transcript levels decreased and reached almost undetectable levels at the end of the light phase. In the middle of the night phase, *CCA1* expression increased again. Both methods led to similar expression patterns. However, using RT-qPCR analysis, *CCA1* relative expression increased up to 150% compared to the first time point, whereas with Northern blot analysis expression was only 25% higher (Fig 1).

Additionally, the methods were compared in a Bland Altman plot (Fig 2). For all genes, most of the two or three replicates for each harvesting time point were located around the mean of the difference. Furthermore, limits of agreement based on the standard deviation of the mean were added as well. Data points within these limits indicated a reliable agreement between both methods. Especially with *CCA1* expression analysis, most of the data points lie very close to zero. The lowest/worst agreement of the results was observed for *ACT2* and *18S rRNA*, indicated by higher variability around zero and wider limits of agreement.

Again all three methods were additionally compared by estimating the costs for one sample as well as for performing the methods with a big data set, working hours and the ratio of technical to biological variability (Table 2). The Northern blot analysis showed a low technical

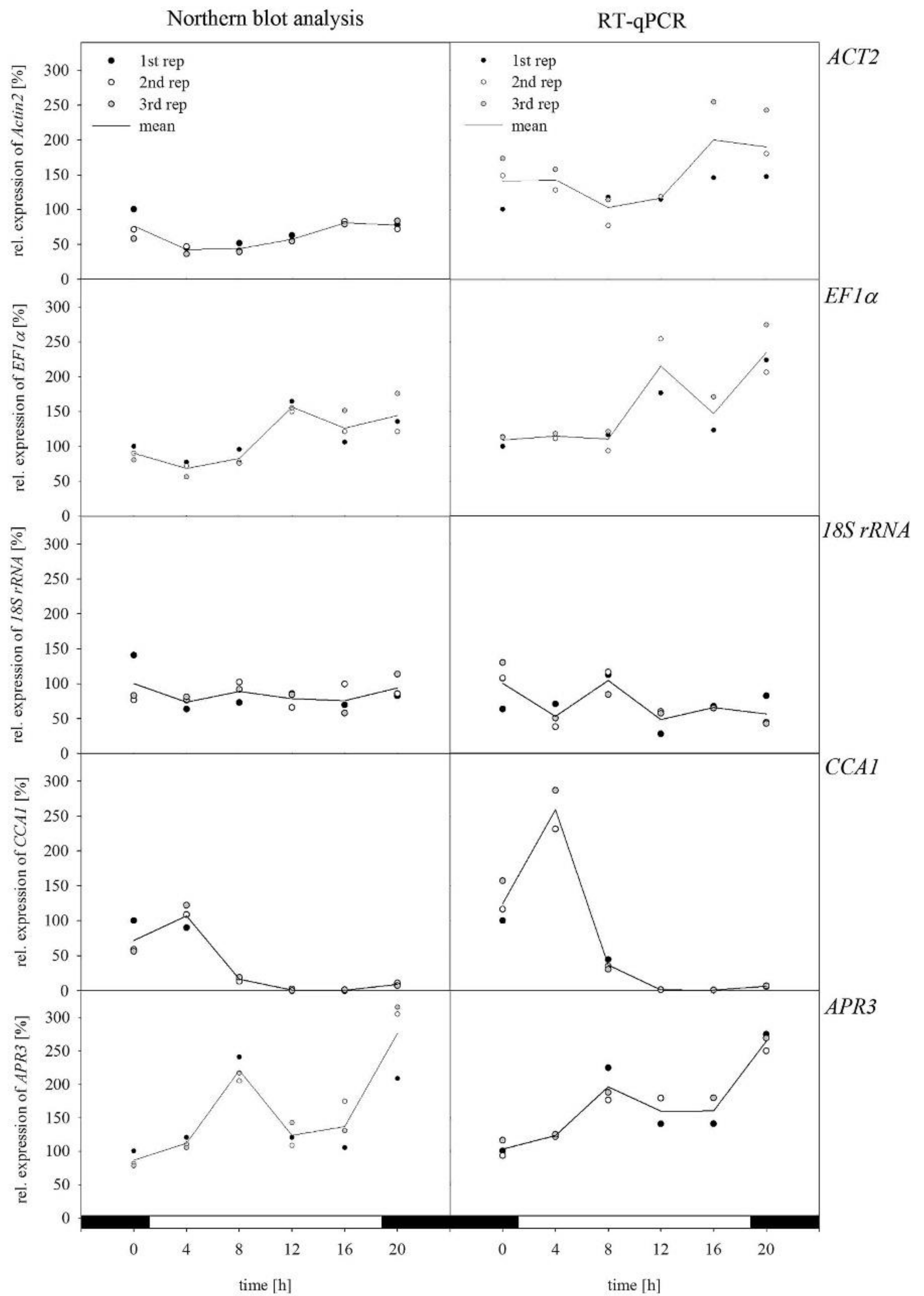
Northern blot analysis
RT-qPCR
ACT2
EF1α
18S rRNA
CCA1
APR3
1st rep
2nd rep
3rd rep
mean
rel. expression of Actin2 [%]
rel. expression of EF1α [%]
rel. expression of 18S rRNA [%]
rel. expression of CCA1 [%]
rel. expression of APR3 [%]
time [h]
time [h]

Fig 1. Comparison of Northern blot analysis and RT-qPCR analysis. Plants with five fully expanded leaves were harvested over a period of 20 h every 4 h, starting 1 h before the onset of light. Leaves from three plants were harvested and pooled. The relative expression for two to three technical replicates of each sample and the resulting mean is shown. Relative expression for Northern blot analysis was calculated based on the band intensity. Percentages refer to the first mean of the three technical replicates as 100% for both methods separately.

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variability with about 14.9% and low costs per sample as well (Table 2). However, expression analysis by PCR-based methods is less time-consuming but results in much higher costs. The RT-qPCR analysis results revealed a lower technical variability than the sqPCR. Regarding the costs for performing these methods with a large sample set the RT-qPCR analysis would be the most expensive method to use. Taking the technical to biological variation and the costs into consideration, Northern blot analysis would be the method of choice for expression analysis with a large sample set. Moreover, comparison of the trend of expression of selected genes obtained by Northern blot and RT-qPCR as well as the comparison of both methods in a Bland Altman Plot supported the use of a traditional method as an alternative even more.

Constitutive expression of reference genes should not be taken for granted

After choosing the method for a quantitative expression analysis the normalization had to be optimized under the given experimental conditions by finding suitable reference genes. To analyze the possible influence of the circadian rhythm on gene expression levels, plants were harvested every 4 h under diurnal and circadian conditions (Fig 3). Furthermore, half of the plants were grown under S deficiency to investigate a possible influence on gene expression as well. In this experiment, *ACT2*, *EF1α* and *18S rRNA* expression levels were analyzed again. However, first results showed an unstable expression of these commonly used genes. Therefore, the experiment was complemented with transcript levels of six additional reference genes *APT1*, *GDI1*, *PP2A*, *TBP2*, *TIP41*, and *UBC21* (Figs 2 and S2). The expression of *ACT2* was upregulated during the dark under diurnal conditions (Fig 2). This trend was unaffected by continuous light. Moreover, the expression was also unaffected by S-limiting conditions. On the contrary *APT1* showed a relatively constant expression level under continuous light whereas under diurnal conditions higher transcript levels were detected at the end of the light phase. The expression of *APT1* was not influenced by the sulfur limiting conditions. *GDI1* showed a relatively stable expression in the course of a day. However, under sulfur deficiency transcript levels showed more oscillations in the course of the day. Under constant light the expression of *GDI1* was unaffected. The transcript level of *EF1α* was affected differently under the given conditions. In plants grown under full sulfur supply and diurnal conditions *EF1α* was upregulated during the dark phase. Except for 20 h this was also the case in plants exposed to continuous light. However, here after the subjected night the degree of downregulation in the light phase was not as high as under diurnal conditions. In plants grown under sulfur-limiting conditions the expression of *EF1α* was regulated in different ways. Here under diurnal conditions transcript amounts were higher in the first light phase than with full sulfur supply. Moreover, expression of *EF1α* was unaffected after the subjective night compared to diurnal conditions. Furthermore, expression of *EF1α* was slightly upregulated under S-limiting conditions. Analysis of *PP2A* under the given conditions showed only slight oscillations in the course of the day were detected. Under the growth conditions the plants were grown under expression of *PP2A* was only influenced slightly. For the expression of *TIP41*, only slight differences in the transcript amount in the course of the day were detected. Under circadian conditions, the expression was at a more stable level. Sulfur-limiting conditions led to a slight down-regulation of

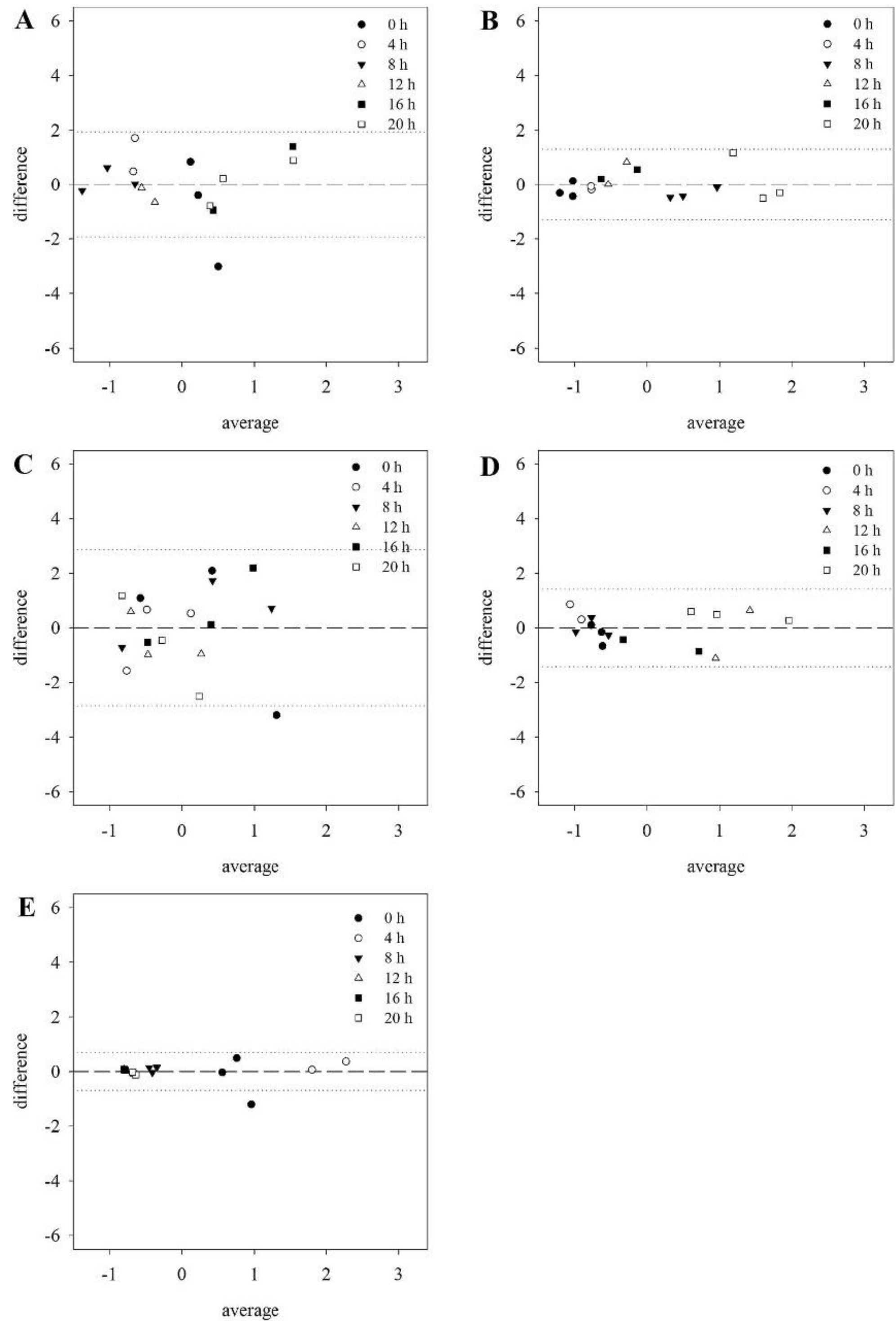
A
B
C
D
E
0 h
4 h
8 h
12 h
16 h
20 h
difference
average

Fig 2. Bland Altman plot for method comparison. Data from Fig 1 were used to generate all graphs. For each data point from both methods, the value was standardized and the difference between the measurements by the two methods was plotted against their mean. Data are shown with the mean of the difference (dashed line) and the 95% limits of agreement ± 1.96xSD (dotted lines). A) *ACT2* B) *EF1α* C) *18S rRNA* D) *APR3* and E) *CCA1*.

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TIP41. On the contrary, the reference gene *TBP2* was slightly upregulated under sulfur-limiting conditions. Moreover, a more constant transcript amount was measured in plants grown under sulfur-limiting conditions in the course of the day and expression was unaffected by the continuous light. Under sufficient sulfur supply only a low level of *TBP2* in the beginning of the light phase was detected increasing at the end of the light phase. For the second light phase higher transcript amounts were detected. In plants exposed to continuous light *TBP2* was upregulated and showed less oscillation in the transcript amount. Analyzing the expression of *UBC21* an unstable expression in the course of a day was shown which was further influenced under sulfur-limiting conditions. The expression of *18S rRNA* was influenced neither by the diurnal or circadian, nor by the S-limiting conditions. Except for *18S rRNA*, reference genes did not show steady expression levels under the given conditions. The two biological repetitions of the entire experiment revealed principally the same results within a variation less than 15%.

Choosing the right reference genes is indispensable for a reliable expression analysis of the target genes

Expression data for the reference genes used in this study were analyzed by the GrayNorm algorithm [19] in consideration of the given plant growth conditions. Based on these results, a set of reference genes yielding the lowest level of uncertainty was validated for normalization purposes. In addition, single commonly used reference genes were chosen to compare the effect of normalization when using a set of reference genes and a single gene. Samples from plants grown under diurnal and circadian conditions were used for analyzing changes in the expression pattern and were measured for three dependent technical replicates.

According to the GrayNorm analysis the lowest coefficient of variation can be achieved when using the combination of *18S rRNA*, *PP2A*, and *GDI1* for normalization. These results indicated that a set of three genes is sufficient for a proper normalization of the expression data for the GOIs. In this study the expression of *CCA1* and *APR3* was analyzed. The expression of the gene *CCA1* regulated in a circadian way was normalized with the validated set of reference genes (Fig 4). Without normalization, *CCA1* showed a high degree of upregulation in the morning followed by a downregulation in the course of the day, again followed by an upregulation in the morning (Fig 4A). Based on Two-way-ANOVA these oscillations were statistically significant. Under S limitation, the expression pattern was similar, although relative expression was significantly higher than in plants grown under full S supply in the beginning of the light phase. When normalized using the validated set of the reference genes *18S rRNA*, *PP2A*, and *GDI1*, the same trend of expression was observed. However, there was a higher expression of *CCA1* at 24 h when normalized. The two-way ANOVA of the non-normalized data revealed a significant effect of the harvesting time point and S status on the expression of *CCA1* (S1 Table). Moreover, there was a significant interaction between both parameters. Interestingly, for the normalized data, the effect of the S status on the expression of *CCA1* was not significant. However, there was a significant interaction between the S status and the point of time when analyzing the normalized data (S1 Table).

Additionally to plants grown in a light dark cycle, some plants were exposed to continuous light resulting in the same expression trend (Fig 4B). However, under continuous light the

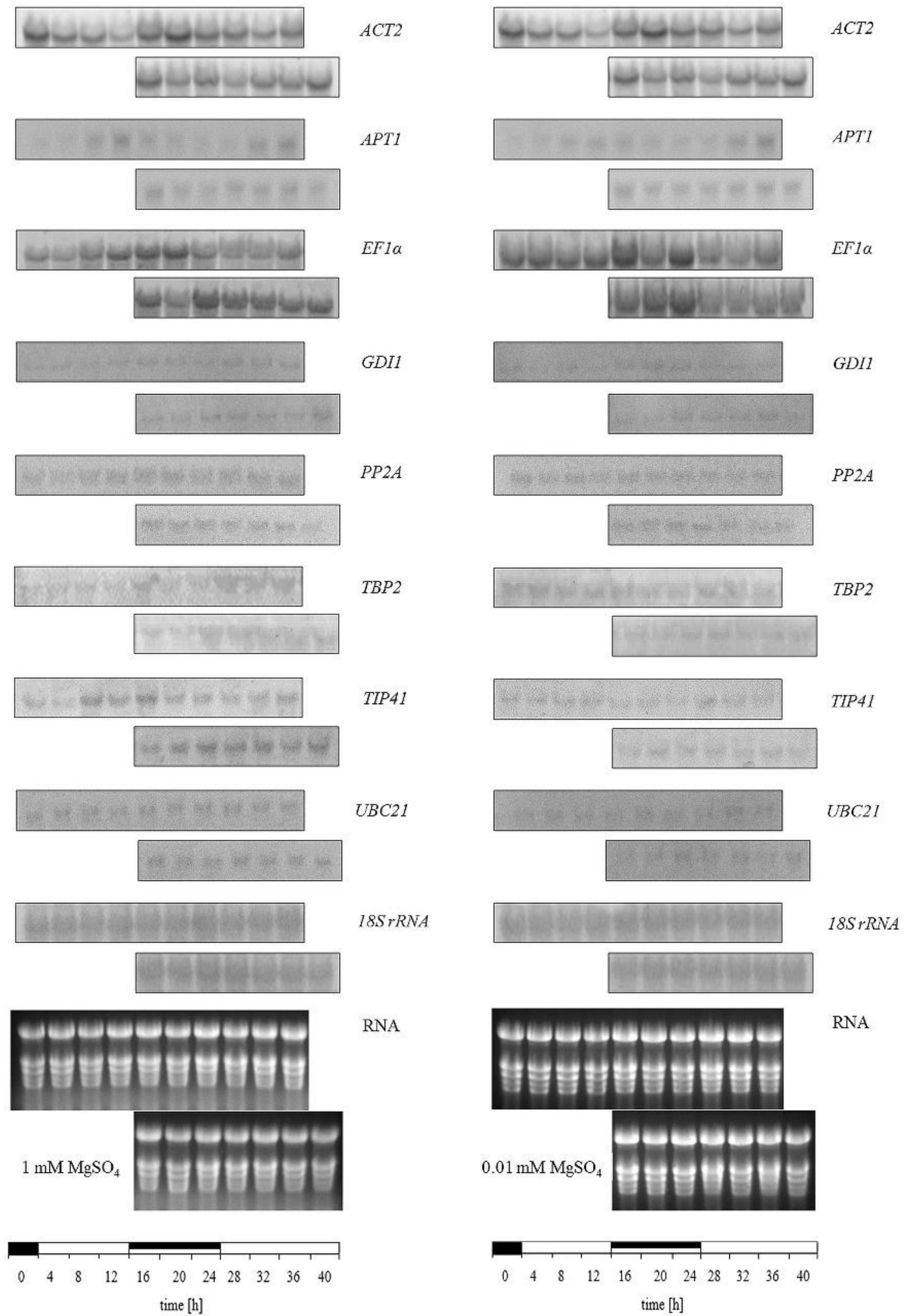
ACT2
APT1
EF1α
GDI1
PP2A
TBP2
TIP41
UBC21
18S rRNA
RNA
1 mM $MgSO_4$
0.01 mM $MgSO_4$
0 4 8 12 16 20 24 28 32 36 40
time [h]

Fig 3. Expression of selected reference genes under circadian and S limiting conditions. Expression levels were analyzed in plants (with five fully expanded leaves) grown using 1 mM $MgSO_4$ as a control and using 0.01 mM $MgSO_4$ for four days to obtain S-limiting conditions. Plants were harvested over a period of 40 h every 4 h starting 1 h before the onset of light (left blot per gene). In addition, plants grown in a chamber with continuous light were harvested after 16 h (right blot per gene). Total RNA was isolated, and for Northern blot analysis 15 μg RNA was electrophoretically separated and transferred onto membranes. The detection of mRNA was done with probes labeled with DIG. Abbreviations: see legend of Table 1. The first row for the genes represents the 12 h light/12 h dark conditions and the second row represents the 24 h light conditions.

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transcript amount of *CCA1* was decreased. There were no differences for the expression pattern when normalizing data with the validated set of reference genes. Including the third parameter light a three-way ANOVA was performed. The effect of light on the expression was significant whereas, the effect of S is independent of the light for non-normalized and normalized expression data (S2 Table). Moreover, ANOVA revealed a significant interaction between the time point and the light. Combining all three conditions the plants were grown under, there was a statistical interaction between these factors, which was independent of normalization.

For normalization with *ACT2* only, the expression pattern of *CCA1* compared to the non-normalized data remained the same (Fig 5). However, compared to the normalization with the

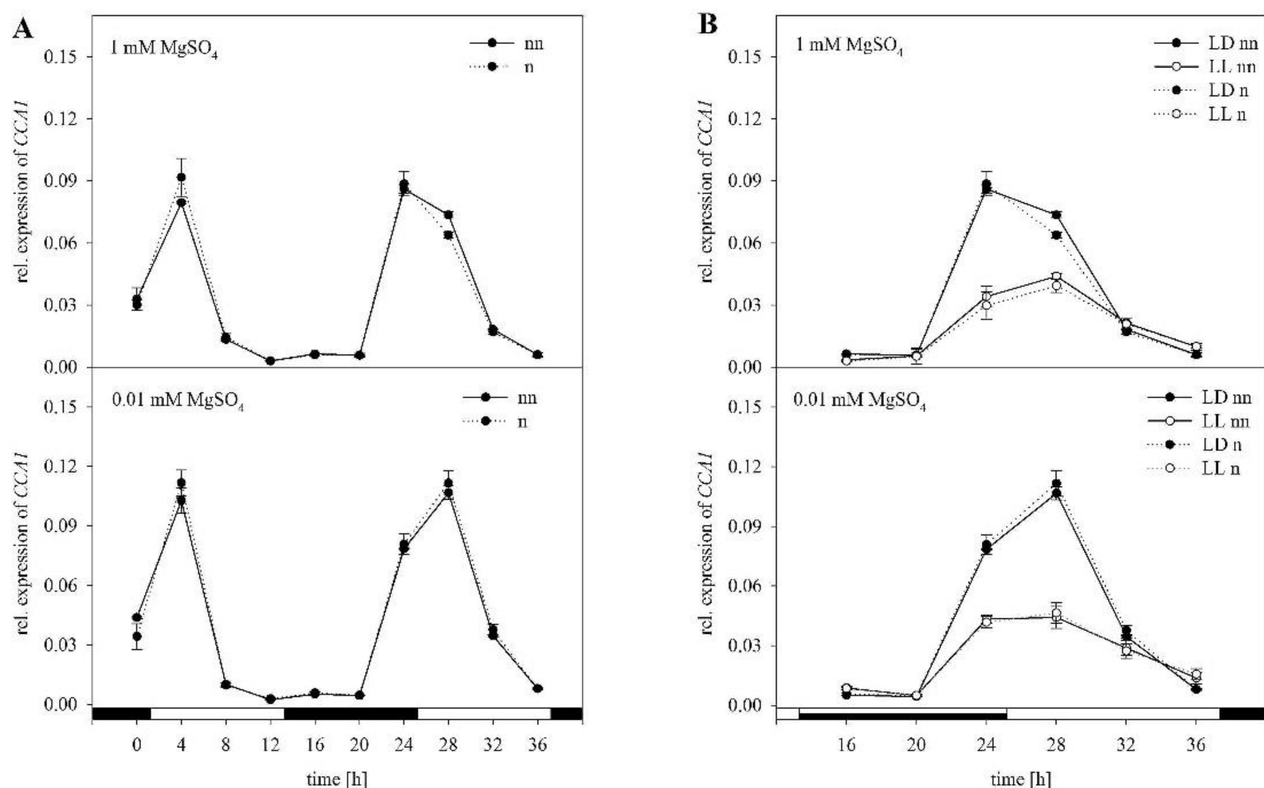

Fig 4. Normalization of *CCA1* expression with the validated set of reference genes *18S rRNA*, *PP2A*, and *GDI1*. Expression analysis for *CCA1* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using three reference genes according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free-running conditions with continuous light (LL) in comparison to the light-dark (LD) conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity.

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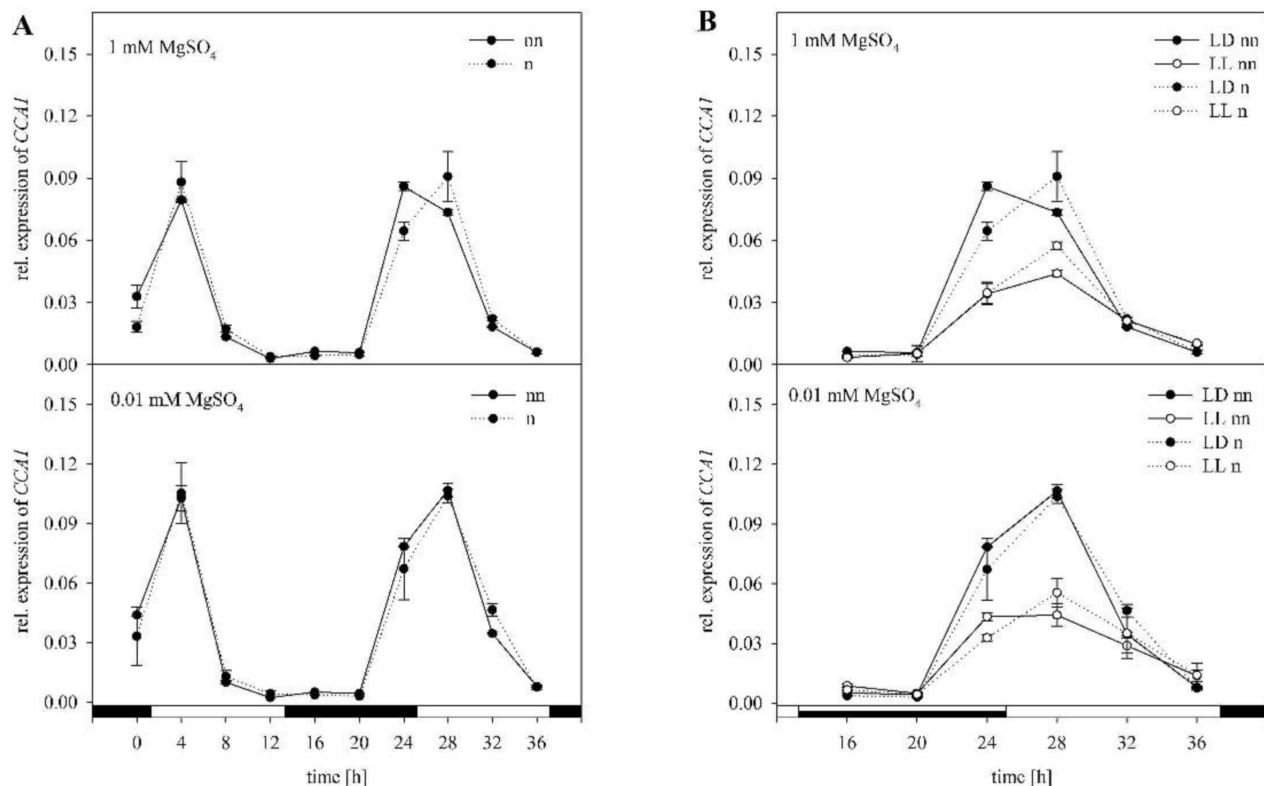

Fig 5. Normalization of *CCA1* with *ACT2*. Expression analysis for *CCA1* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using *ACT2* according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free running conditions with continuous light (LL) in comparison to the light dark (LD) conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity.

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set of reference genes the graphs are not as much in congruence especially when it comes to the continuous light (Fig 5B). In contrast to the set of reference genes there was a significant effect of the S status when analyzing the normalized data in a two-way ANOVA (S1 Table). However, the interaction between the S status and the time of harvesting was not significant. For the expression data in plants exposed to continuous light (Fig 5B), normalization with *ACT2* led to the same expression trend. However, there was a difference in the results of three-way ANOVA. When normalizing with *ACT2*, there was no significant interaction of the three factors S status, light and time.

As *EF1α* was most affected under the experimental conditions, normalization with it was performed as well (Fig 6). Comparing the resulting graphs to non-normalized data there were a number of disagreements. Interestingly, for normalization with *EF1α* the relative expression at 4 h under full S supply was about one third higher than for the non-normalized data (Fig 6A). Furthermore, under constant free-running conditions normalization with *EF1α* led to a lower level of expression in the morning. Under S-limiting and diurnal conditions, in contrast the amplitude was lower at 0 and 4 h after normalization. Furthermore, the relative expression of *CCA1* at 24 h was nearly 50% lower in comparison to the non-normalized data. Whereas after 32 h normalization led to a doubling of the expression level compared to non-normalized data. Under free-running conditions the expression level at 32 h was nearly the same as for 28

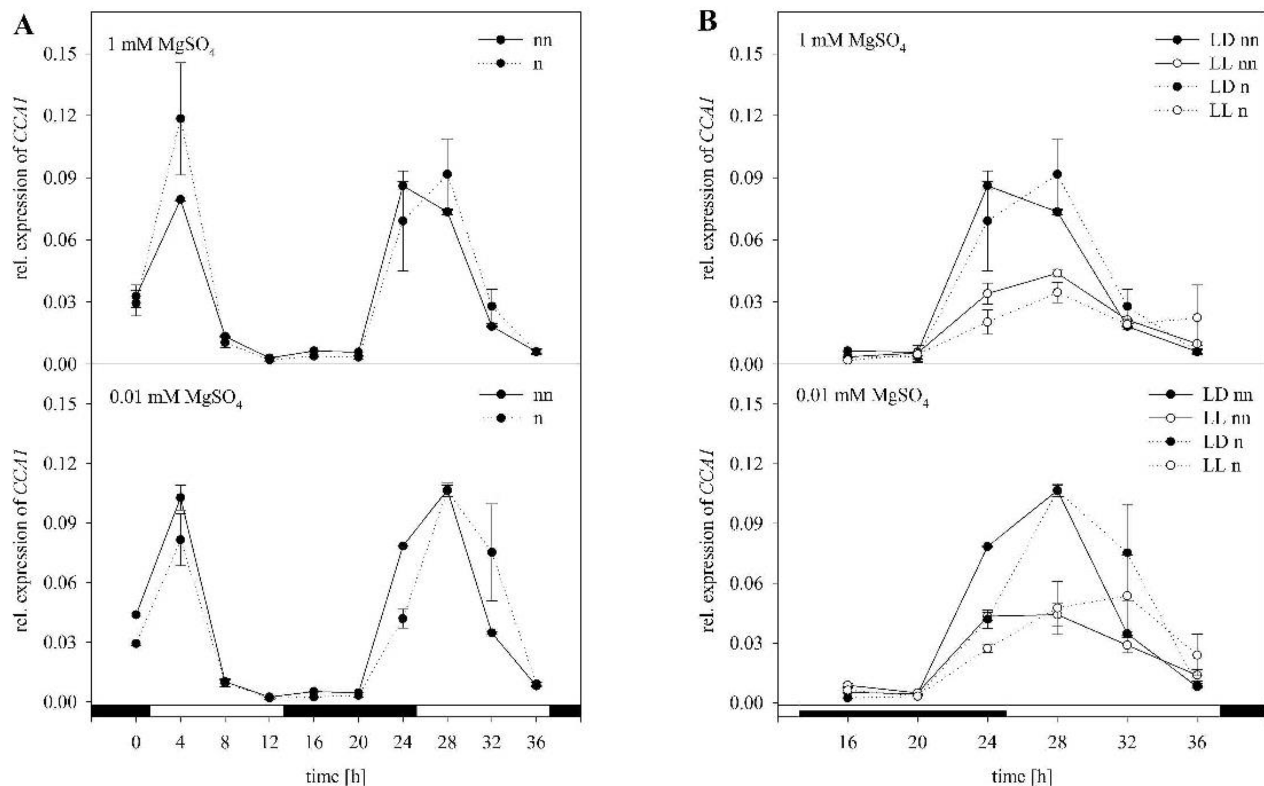

Fig 6. Normalization of *CCA1* with *EF1α*. Expression analysis for *CCA1* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using *EF1α* according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free running conditions with continuous light (LL) in comparison to the light dark (LD) conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity.

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h when normalizing with *EF1α* (Fig 6B). The two-way ANOVA revealed a significant interaction between the S status and the time point of harvest for the non-normalized as well for the normalized data (S1 Table). There was no significant difference between normalizing with *EF1α* or the suggested set of reference genes. Including the light factor and performing a three-way ANOVA there is a significant interaction between the S status, the time of harvesting and the light condition when analyzing the non-normalized data (S2 Table). Normalizing with the suggested set of reference genes led to the same result for statistical analysis. However, normalized data with *EF1α* showed a non-significant interaction between these three factors.

The relative expression of *APR3* as part of the S assimilation pathway was normalized as well (Fig 7). In plants grown under full S supply, transcript levels of *APR3* were significantly higher in the middle of the light phase as well as after 16 h at the end of the night phase (Fig 7A). In plants grown under S-limiting conditions, *APR3* was upregulated only after 20 h again. Moreover, a significantly higher upregulation was shown under S deficiency. A significant interaction between the S status and the harvesting time of the plants was revealed by performing a two-way ANOVA (S3 Table). Normalizing the expression data with the set of reference genes had no effect on the expression trend. However, there was a higher upregulation in the beginning of the first light phase and in the end of the dark phase with 1 mM $MgSO_4$. Under S-limiting conditions, a slightly lower degree of upregulation was observed. However, statistical

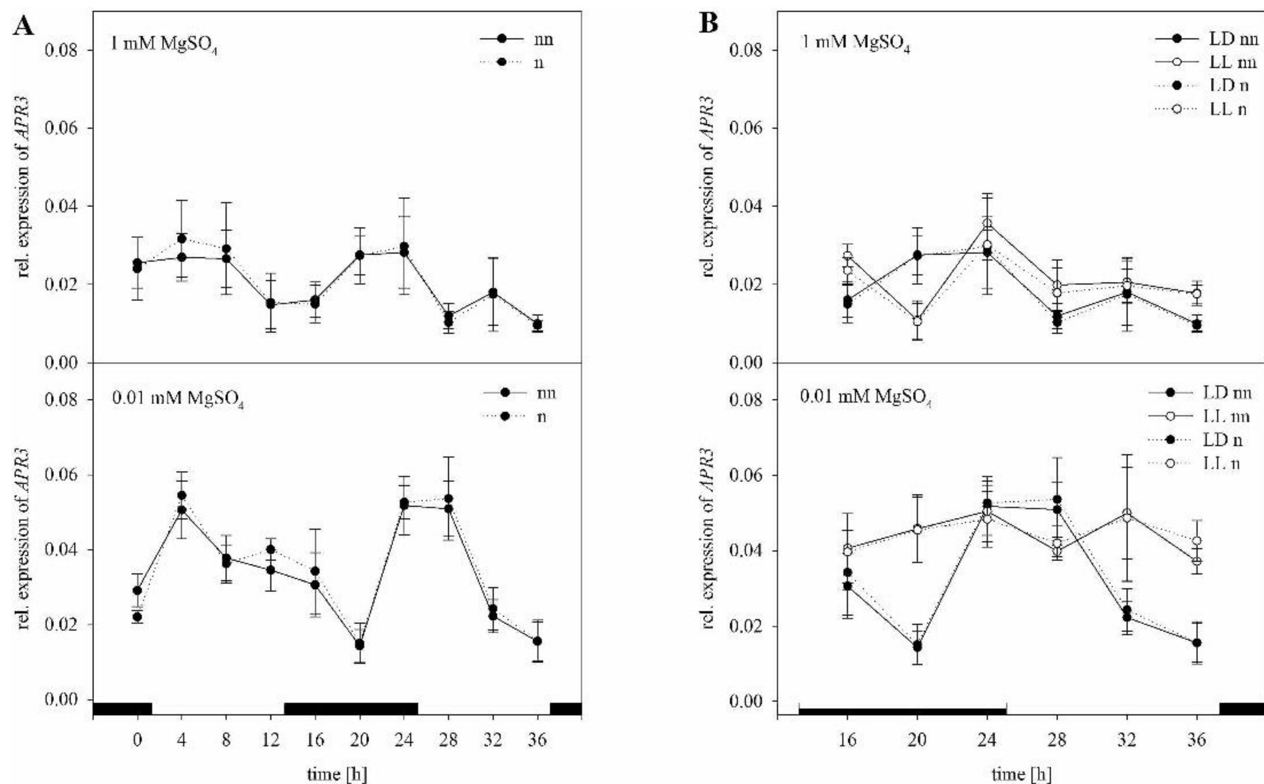

Fig 7. Normalization of *APR3* with the validated set of reference genes *18S rRNA*, *PP2A*, and *GDI1*. Expression analysis for *APR3* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using three reference genes according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free running conditions with continuous light (LL) in comparison to the light dark (LD) conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity.

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analysis of the normalized data revealed the same results as for non-normalized data. In plants exposed to continuous light, upregulation started at the end of the subjective night and was kept at a nearly steady transcript level (Fig 7B). Under S-limiting conditions, a significantly higher degree of *APR3* upregulation was measured at the beginning of the light phase. Interestingly, under free-running conditions, no oscillations for the relative expression could be determined. For the non-normalized as well as for the normalized data, there was a significant interaction in the three-way ANOVA between the S status and light (S4 Table). Interestingly, no significant interaction was observed between the time and light without normalization, whereas analyzing the normalized data showed a significant interaction. In agreement with the non-normalized data, the combination of all three factors showed a significant interaction when normalized using the validated set of reference genes.

Normalization of the *APR3* expression with *ACT2* as a single reference resulted in a different shape of the curve compared to the non-normalized data (Fig 8). Here a higher degree of upregulation in the light phase and a lower degree of upregulation in the night phase after normalization was shown (Fig 8A). Under S-limiting conditions, *APR3* expression was about one third higher than non-normalized expression data at 12 h. Despite the deviations in the results, they were similar to the normalized data with the set of reference genes after statistical analysis

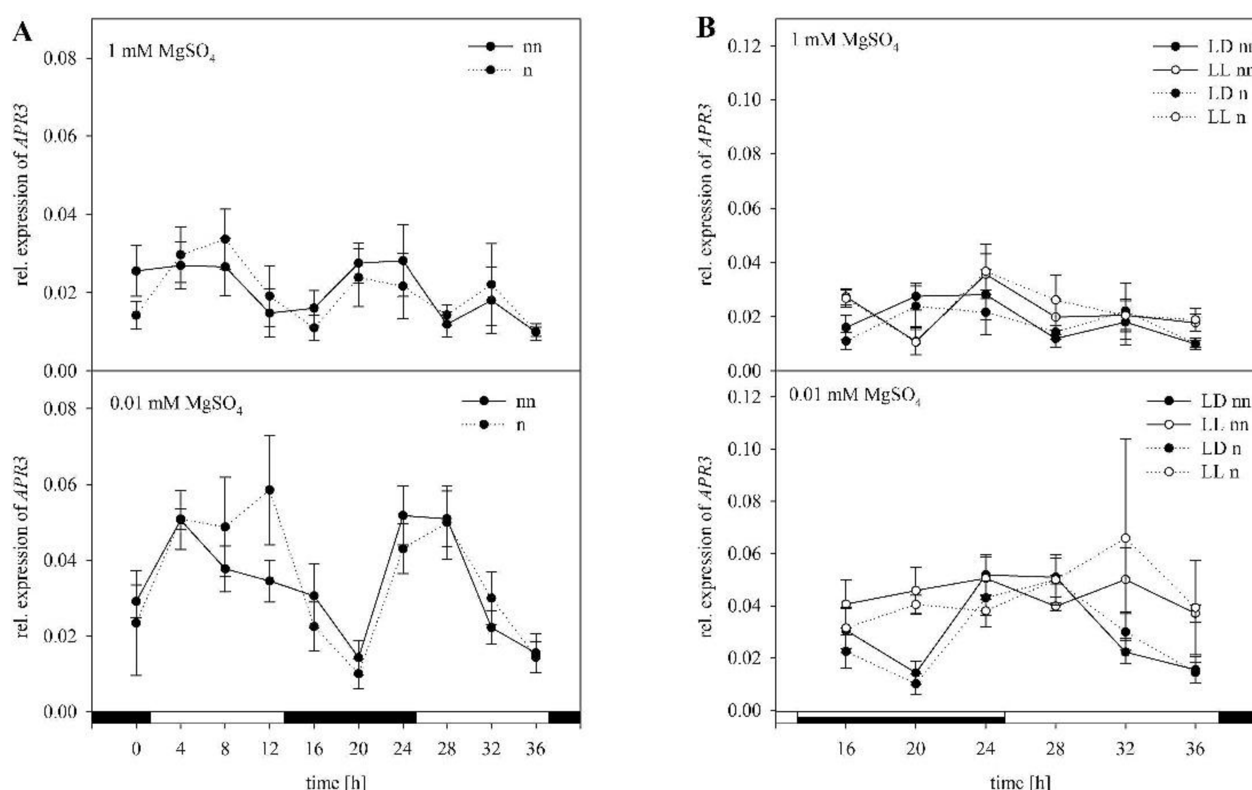

Fig 8. Normalization of *APR3* with *ACT2*. Expression analysis for *APR3* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using *ACT2* according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free running conditions with continuous light (LL) in comparison to the light dark (LD) conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity.

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(S3 Table). Under free-running conditions (Fig 8B), there was a lower expression level in the subjective night and a higher expression level in the light phase at 28 h. Results of statistical analysis with the normalized data using *ACT2* were with one exception comparable to the normalized data with the set of reference gene (S4 Table).

Using *EF1α* as a reference gene more differences in the expression level after normalization occurred (Fig 9). Under full sulfur supply and diurnal conditions expression of *APR3* was nearly one third higher three hours after onset of light compared to the non-normalized data (Fig 9A). Furthermore, here the expression was slightly lower in the night and higher again in the middle of the day. Under sulfur-limiting conditions the expression level of the normalized data was slightly decreased for the first day and slightly increased for the second day compared to non-normalized data. Furthermore, normalization led to a shift of the expression maxima from 24 and 28 h to 28 and 32 h. Normalizing the expression data obtained under free running conditions led to major differences compared to the non-normalized data (Fig 9B). In the subjective night a much lower expression level was shown. Furthermore the expression level was nearly doubled after 32 h and 36 h. Statistical analysis showed that there is a significant interaction between the sulfur status and the time point of harvest for the non-normalized data (S3 Table). This was also the case when normalizing with the suggested best choice. However, analyzing the *EF1α* normalized data there was no significant interaction between these two factors.

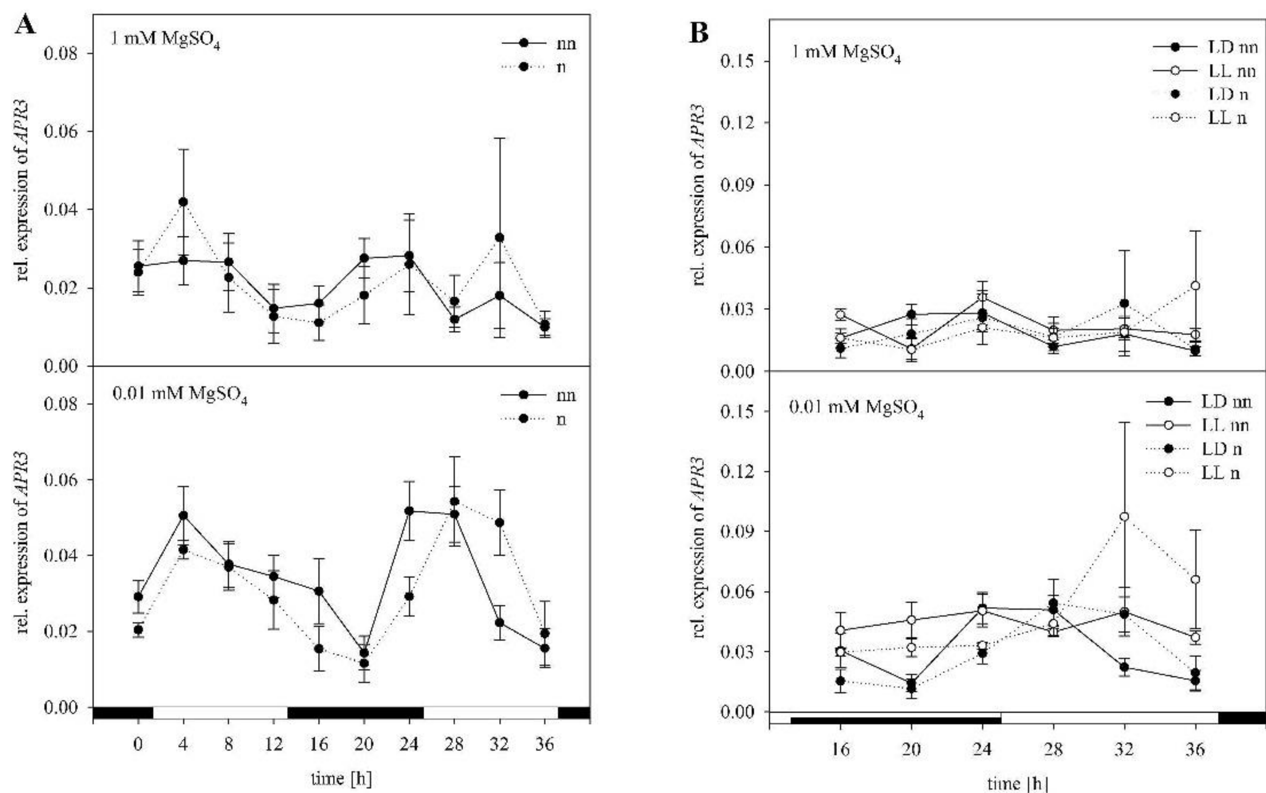

Fig 9. Normalization of *APR3* with *EF1α*. Expression analysis for *APR3* was performed in plants treated as previously described in Fig 3 by Northern blot analysis. Results were normalized using *EF1α* according to Fig 3. The normalized (n) and non-normalized (nn) data are presented as the relative expression under (A) diurnal conditions over a period of 36 h and (B) free running conditions with continuous light (LL) in comparison to the light dark (LD) conditions. Data are shown as the mean of three technical replicates ±SD. Relative expression calculation was based on band intensity.

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When it comes to factor light (S4 Table), there was as well a significant interaction between the three factors when analyzing the non-normalized data. In agreement with the suggested best choice this was also the case whereas normalized data with EF1α showed no significant interaction between the three factors.

To summarize, the expression pattern of *CCA1* and *APR3* certainly remained the same when normalizing with the validated set of reference genes, or with *ACT2* or *EF1α* instead. Nevertheless, normalization with a single reference gene led to greater discrepancy between the curves compared to normalization with the set of reference genes, where the curves were more similar. Moreover, analysis based on single reference genes resulted in large differences in expression values among sampling points after normalization.

Discussion

Despite the development of newer methods, such as quantitative (real-time) PCR, nuclease protection assays, microarrays, and RNA-Seq, Northern blot analysis is still a standard technique used in the detection and quantification of mRNA [33,34]. There are a number of studies where Northern blot analysis and PCR-based methods were compared to assess if different methods affect the outcome [35–37]. Furthermore, it was suggested to verify gene expression

by different methods whenever possible [36]. Regarding expression analysis in our study, we compared Northern blot analysis, sqPCR and RT-qPCR to find a suitable method for our experimental setup involving three technical replicates of selected samples. Based on the first results of analyzing the expression of *ACT2*, sqPCR was omitted due to a high technical variability within the biological replicates. With the two remaining methods, further experiments were performed to verify the reliability of the results. If results generated with Northern blot analysis were similar to those obtained with RT-qPCR, the reliability of both methods was confirmed. This was verified by comparing the trend of expression of selected genes by means of Northern blot and RT-qPCR analysis (Fig 1). This was also observed in previous studies [35–37]. However, in the end, RT-qPCR remained the method of choice in these studies due to its high sensitivity and speed. Despite such advantages, various technical parameters like pipetting and efficient reverse transcriptase can influence the accuracy and precision of the results during RT-qPCR [19].

In addition to comparing the trend of expression, each sample of each technical replicate was compared between the two methods in a Bland Altman Plot. In our study, nearly all data points lay within the 95% limits of agreement indicating an agreement between the measurements obtained from the two methods (Fig 2).

Taking the high sample amount of this complex experiment into consideration using RT-qPCR analysis would yield in higher costs compared to Northern blot analysis (Table 2). Based on these results, we selected Northern blot analysis to perform further experiments due to its simplicity, low costs, direct visibility and sufficient sensitivity for our purposes. However, the reliability of Northern blot analysis in a quantitative way needed to be further improved by optimizing the normalization with suitable reference genes.

Although they are commonly used as reference genes, neither *ACT2* nor *EF1α* showed a steady expression over the day, which makes their only use as reference genes inappropriate whereas the third commonly used reference *18S rRNA* showed only slight oscillations in the course of the day. However, using only one reference gene for normalization would be inappropriate. In further experiments, additional conditions like nutrition (S)-induced stress, continuous light and the time points of harvesting might also influence the expression of these reference genes. Therefore, we also analyzed six additional reference genes by Northern blot analysis under the conditions mentioned above. As a typical reference gene used for expression analysis, *ACT2* is present and constitutively expressed in all vegetative tissues in *A. thaliana* [38]. However, under abiotic stress like salt and cold, *ACT2* was not stably expressed anymore [13]. In our study (Fig 3), its expression was slightly reduced under S-limiting conditions. Moreover, in the course of a day oscillations of the transcript level of *ACT2* were detected.

According to literature *APT1* was stably expressed under a number of abiotic stresses [39]. In agreement with our results same transcript amounts were detected in plants grown under sulfur deficiency. However, expression was strongly affected under diurnal and circadian conditions due to its role in the cytokinin metabolic processes [40].

As part of the protein synthesis *EF1α* is also a commonly used reference gene, but its transcript amounts oscillated over the course of a day as well. Its expression opposed to *ACT2* was upregulated in plants grown under S-limiting conditions. In *B. napus* the expression of *EF1α* was affected in different directions under abiotic stress and only showed a stable expression under heavy metal stress [15].

As a novel reference gene *GDI1*, which is part of the membrane vesicular traffic, was validated as a stable expressed gene in *B. napus* under a number of stress conditions [41]. This is in agreement with our study when analyzing the expression in plants grown under diurnal or circadian conditions where the transcript amount was nearly not affected. However, higher

oscillations occurred under these conditions when plants were exposed to sulfur limiting conditions.

In a study where reference genes were validated for a diurnal time course in lettuce the enzyme PP2A that removes phosphatase groups from the given substrate showed a stable expression. In agreement with our results under diurnal conditions *PP2A* showed in *B*. *napus* a relatively stable expression [42]. Furthermore the expression of *PP2A* analyzed under various abiotic stresses in *B*. *napus* could be determined as stably expressed for a number of treatments such as drought and salt stress [15]. Based on our results a constant expression is provided under sulfur limiting conditions as well. Even in plants exposed to continuous light the transcript amount of *PP2A* was nearly unaffected making it a promising candidate for a suitable reference.

The gene coding for the membrane protein channel TIP41 is recommended as an internal control due to a very stable expression in vegetative samples [43,44] and *TIP41* was stably expressed under biotic and abiotic stress as well [15]. In our study however, transcript levels of *TIP41* fluctuated in the course of the day. Interestingly, in lettuce *TIP41* was the most stably expressed reference gene under diurnal conditions [42]. Nevertheless fluctuations were not as high as for *ACT2* and *EF1α*. Furthermore the expression was only slightly down-regulated under S-limiting conditions.

As a non-traditional reference gene, expression of a gene encoding for a TATA box binding protein (*TBP2)* was analyzed as well. It was recommended as a reference gene because of its function as a transcription factor that binds DNA and therefore has a steady state level of expression [45]. In this study, transcript levels of *TBP2* only showed slight oscillations in the course of the day. Expression seemed to be influenced by the S status and light as well.

In previous studies a stable expression of *UBC21* in *B*. *napus* under various stress conditions was already verified [15,44]. According to our analysis in this study transcript amounts were indeed affected by the sulfur limiting conditions plants were grown under. Furthermore expression was influenced under diurnal and circadian conditions.

The use of *18S* and *28S rRNA* as an internal standard for expression analysis was recommended in 1999 by Thellin and colleagues [46], and *18S rRNA* was commonly used as a reference for expression analysis [47–49]. However, the use of *18S rRNA* as an internal control was often criticized due to its high transcript abundance compared to the GOI. Furthermore, for a number of plant species it was not recommended as a suitable reference gene (reviewed in [50]). Nevertheless, based on our results for our experimental design *18S rRNA* was the most stably expressed reference gene in *B*. *napus* among the other tested reference genes.

In agreement with our results, recent studies revealed that continuous expression under biotic and abiotic stress is not always provided and strongly depends on the used reference genes and experimental setup [13,15]. However, in these studies, plants were always exposed to one condition to reveal the best reference gene. In our study, the challenge was to analyze selected reference genes under combined experimental conditions such as the S status, time point of harvesting and free-running conditions like 24 h of light. According to our results of the Northern blot analysis *18S rRNA* would be the reference gene of choice. However, normalizing the expression level of a target gene with only one of the reference genes tested in this study would be inappropriate. Therefore, by using the GrayNorm algorithm, a combination of reference genes yielding the lowest level of uncertainty can be determined, ensuring a more reliable normalization [19]. This algorithm was actually developed evaluating RT-qPCR data. In this study data obtained by Northern blot analysis based on band intensities was used for GrayNorm analysis verifying suitable references confirming a proper normalization of the GOIs. This is actually the first time the GrayNorm algorithm was used for evaluating data generated by Northern Blot analysis. Regarding our study, the combination of *18S rRNA*, *PP2A*,

and *GDI1* was verified as the best combination of reference genes with the lowest CV. This result indicates that normalization with three references is sufficient for normalization and also verifies the stability of the references in their expression under the given circumstances. In agreement with our results obtained by Northern blot analysis, these were the genes that were least affected in their expression. Demonstrating how the outcome was affected for our experimental setup, expression data based on Northern blot analysis were normalized with the validated set of reference genes and compared to normalization with single reference genes. Therefore, expression levels of *CCA1* and *APR3* were analyzed under the same conditions as described before (Figs 4–9). To demonstrate the effect of normalization, two- and three-way ANOVAs were performed to determine the influence of S status, time of harvest and light and possible interactions between the factors using non-normalized and normalized data respectively (S1–S4 Tables). For *CCA1*, normalization with the validated set of reference genes resulted in nearly the same trend of expression as compared to non-normalized expression data (Fig 4). However, normalized data revealed no significant effect of the S status on the expression of *CCA1* as opposed to non-normalized data (S1 Table). Based on these results, one would reason that the S status in the plants did not affect the circadian rhythm. In contrast, there was a significant influence of the S status on *CCA1* expression when non-normalized and normalized data with *ACT2* were considered (Fig 5, S1 Table). Whereas it is known that *CCA1* indirectly affects the nutrition metabolism [51], the opposite effect is not yet clearly demonstrated. Therefore, our results need to be interpreted with caution. Furthermore, as for the normalized data with the set of reference genes, three-way ANOVA revealed a significant interaction of the three conditions affecting the expression of *CCA1* (S2 Table). In contrast, there was no significant interaction when normalizing with *ACT2* or *EF1α* alone. Our results demonstrated clearly how normalization with only one reference gene can distort/invalidate the outcome and lead to false conclusions.

Normalizing *APR3* with *ACT2* or *EF1α* also led to disagreements compared to normalization with the set of reference genes (Figs 8 and 9). Here normalization with *ACT2* or *EF1α* seemed to have a stronger influence on the trend of expression compared to normalizing *CCA1*. However, within the analysis of *APR3* expression, investigating the influence of S and the time point of harvest, there was a clear interaction between both variables according to normalization with a suitable set of reference genes or *ACT2* whereas this was not significant when normalizing with *EF1α* (S3 Table). Furthermore when the third factor continuous light was included, the interaction between all three factors was highly significant for normalization with the selection of reference genes ($p<0.001$), *ACT2* alone ($p<0.05$), but not significant when normalizing with *EF1α* alone (S4 Table). According to our results, using only *ACT2* or *EF1α* as a reference gene would prevent a proper evaluation of the expression analysis. This clearly shows how important a proper validation of the used reference gene is before normalizing, even when those genes are commonly used reference genes such as *ACT2* or *EF1α*.

There are no universal reference genes in plants known so far with a constant transcript amount under different conditions across all plant species [12,52,53]. Therefore, a proper evaluation of references for new experimental set-ups or plants is inevitable for generating results one can rely on.

Conclusion

In this study, it was confirmed that under several conditions like the S status in plants, the harvesting time point as well as light regime the expression of commonly selected reference genes can be strongly influenced. Using only one of them as a single reference gene to quantify the relative expression of GOIs under the circumstances used in this study, normalization would

be inappropriate whereas a combination out of these genes determined by the GrayNorm algorithm seems to be the best choice. Whereas RT-qPCR is the standard to perform gene expression analysis, our results demonstrate that similar results can be obtained using Northern Blot analysis. However, also here a set of suitable reference is essential to guarantee a confident evaluation of gene expression analysis. To our best knowledge, this is the first time that data obtained with Northern blot analysis were normalized with a set of reference genes maximizing the accuracy of normalization and hence data interpretation in a complex experimental set up.

Supporting Information

S1 Fig. Northern blot analysis of *18S rRNA* with different RNA concentrations. Different concentrations (1 to 24 μg) of pooled RNA samples as described in the legend to Fig 1 were electrophoretically separated on a 1% agarose gel. The probe of *18S rRNA* was also diluted 1:10 before hybridization (in the middle).
(TIF)

S2 Fig. Comparison of Northern blot analysis and RT-qPCR analysis for other tested reference genes. Plants with five fully expanded leaves were harvested over a period of 20 h every 4 h, starting 1 h before the onset of light. Leaves from three plants were harvested and pooled. The relative expression for two to three technical replicates of each sample and the resulting mean are shown. Relative expression for Northern blot analysis was calculated based on the band intensity. Percentages refer to the first mean of the three technical replicates set at 100% for both methods separately.
(TIF)

S1 Table. Two-way ANOVA analysis of the expression data obtained for *CCA1*.
(DOCX)

S2 Table. Three-way ANOVA analysis of the expression data obtained for *CCA1*.
(DOCX)

S3 Table. Two-way ANOVA analysis of the expression data obtained for *APR3*.
(DOCX)

S4 Table. Three-way ANOVA analysis of the expression data obtained for *APR3*.
(DOCX)

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References

1. Alwine JC, Kemp DJ, Stark GR. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci U S A. 1977; 74: 5350–4. doi: 10.1073/pnas.74.12.5350 PMID: 414220
2. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques. 2008; 44: 619–626. doi: 10.2144/000112776 PMID: 18474036
3. Huang SQ, Xiang AL, Che LL, Chen S, Li H, Song JB, et al. A set of miRNAs from *Brassica napus* in response to sulphate deficiency and cadmium stress. Plant Biotechnol J. 2010; 8: 887–899. doi: 10.1111/j.1467-7652.2010.00517.x PMID: 20444207
4. Parkash J, Kashyap S, Kirti S, Singh AK, Dutt S. Cathepsin B cysteine protease gene is upregulated during leaf senescence and exhibits differential expression behavior in response to phytohormones in *Picrorhiza kurrooa* Royle ex Benth. Plant Gene. 2015; 3: 11–19. doi: 10.1016/j.plgene.2015.07.001
5. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines:Minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55: 611–622. doi: 10.1373/clinchem.2008.112797 PMID: 19246619
6. Navarro E, Serrano-Heras G, Castaño MJ, Solera J. Real-time PCR detection chemistry. Clin Chim Acta. 2015; 439: 231–50. doi: 10.1016/j.cca.2014.10.017 PMID: 25451956
7. Bustin SA, Benes V, Garson J, Hellemans J, Huggett J, Kubista M, et al. The need for transparency and good practices in the RT-qPCR literature. Nat Methods. 2013; 10: 1063–1067. doi: 10.1038/nmeth.2697 PMID: 24173381
8. Bustin SA. Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol. 2005; 34: 597–601. doi: 10.1677/jme.1.01755 PMID: 15956331
9. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002; 3: RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034 PMID: 12184808
10. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004; 64: 5245–50. doi: 10.1158/0008-5472.CAN-04-0496 PMID: 15289330
11. Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre J-F, Louvet R, et al. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J. 2008; 6: 609–18. doi: 10.1111/j.1467-7652.2008.00346.x PMID: 18433420
12. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol. 2005; 139: 5–17. doi: 10.1104/pp.105.063743 PMID: 16166256
13. Nicot N, Hausman J-F, Hoffmann L, Evers D. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot. 2005; 56: 2907–14. doi: 10.1093/jxb/eri285 PMID: 16188960
14. Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. Planta. 2008; 227: 1343–1349. doi: 10.1007/s00425-008-0706-4 PMID: 18273637

15. Wang Z, Chen Y, Fang H, Shi H, Chen K, Zhang Z, et al. Selection of reference genes for quantitative reverse-transcription polymerase chain reaction normalization in *Brassica napus* under various stress conditions. Mol Genet Genomics. 2014; 289: 1023–35. doi: 10.1007/s00438-014-0853-1 PMID: 24770781

16. Machado RD, Christoff AP, Loss-Morais G, Margis-Pinheiro M, Margis R, Körbes AP. Comprehensive selection of reference genes for quantitative gene expression analysis during seed development in *Brassica napus*. Plant Cell Rep. 2015; 34: 1139–1149. doi: 10.1007/s00299-015-1773-1 PMID: 25721200

17. Hruz T, Wyss M, Docquier M, Pfaffl MW, Masanetz S, Borghi L, et al. RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genomics. BioMed Central; 2011; 12: 156. doi: 10.1186/1471-2164-12-156 PMID: 21418615

18. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun. 2004; 313: 856–862. doi: 10.1016/j.bbrc.2003.11.177 PMID: 14706621

19. Remans T, Keunen E, Bex GJ, Smeets K, Vangronsveld J, Cuypers A. Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. Plant Cell. 2014; 26: 3829–37. doi: 10.1105/tpc.114.130641 PMID: 25361954

20. Mcclung CR. Plant Circadian Rhythms. Plant Cell. 2006; 18: 792–803. doi: 10.1017/CBO9781107415324.004 PMID: 16595397

21. Harmer SL. The circadian system in higher plants. Annu Rev Plant Biol. 2009; 60: 357–77. doi: 10.1146/annurev.arplant.043008.092054 PMID: 19575587

22. Blake-Kalff MM, Harrison KR, Hawkesford MJ, Zhao FJ, McGrath SP. Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. Plant Physiol. 1998; 118: 1337–1344. doi: 10.1104/pp.118.4.1337 PMID: 9847107

23. Computational Biology and Functional Genomics Laboratory—Home. Available: http://compbio.dfci.harvard.edu/compbio. Accessed 25 April 2016

24. Quackenbush J, Liang F, Holt I, Pertea G, Upton J. The TIGR gene indices: reconstruction and representation of expressed gene sequences. Nucleic Acids Res. 2000; 28: 141–5. doi: 10.1093/nar/28.1.141 PMID: 10592205

25. DOSBox. Available: http://www.dosbox.com/. Accessed 25 April 2016

26. BLAST: Basic Local Alignment Search Tool. Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed 27 July 2016.

27. Sokolovsky V, Kaldenhoff R, Ricci M, Russo V. Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. Fungal Genet Newslett. 1990;

28. GelAnalyzer.com–Welcome. Available: http://gelanalyzer.com/. Accessed 25 April 2016

29. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45. doi: 10.1093/nar/29.9.e45 PMID: 11328886

30. Searle SR, Casella G, McCulloch CE. Variance Components. 2009.

31. Bland JM, Altman DG. Statistical Methods for AssessingAgreement Between Two Methods ofClinical Measurement.: 307–310. doi: 10.1016/S0140-6736(86)90837-8

32. Bland JM, Altman DG. Statistical Methods in Medical Research. Stat Methods Med Res. 1999; 8: 161–179.

33. Streit S, Michalski CW, Erkan M, Kleeff J, Friess H. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat Protoc. Nature Publishing Group; 2009; 4: 37–43. doi: 10.1038/nprot.2008.216 PMID: 19131955

34. Keren I, Abudraham S, Ostersetzer-biran O. An optimized method for the analysis of plant mitochondria RNAs by Northern-blotting. J Endocytobiosis Cell Res. 2011; 21: 34–42.

35. Dean JD, Goodwin PH, Hsiang T. Comparison of relative RT-PCR and Northern blot analyses to measure expression of β-1,3-glucanase in *Nicotiana benthamiana* infected with *Colltotrichum destructivum*. Plant Mol Biol Report. 2002; 20: 347–356. doi: 10.1007/BF02772122

36. Ding Y, Xu L, Jovanovic BD, Helenowski IB, Kelly DL, Catalona WJ, et al. The methodology used to measure differential gene expression affects the outcome. J Biomol Tech. 2007; 18: 321–30. PMID: 18166675

37. Gan Y, Zhou Z, An L, Bao S, Forde BG. A Comparison cetween Northern blotting and quantitative real-time PCR as a means of detecting the nutritional regulation of genes expressed in roots of *Arabidopsis thaliana*. Agric Sci China. 2011; 10: 335–342. doi: 10.1016/S1671-2927(11)60012-6

38. Slajcherová K, Fišerová J, Fischer L, Schwarzerová K. Multiple actin isotypes in plants: diverse genes for diverse roles? Front Plant Sci. 2012; 3: 226. doi: 10.3389/fpls.2012.00226 PMID: 23091476

39. Borowski JM, Galli V, da Silva Messias R, Perin EC, Buss JH, dos Anjos e Silva SD, et al. Selection of candidate reference genes for real-time PCR studies in lettuce under abiotic stresses. Planta. 2014; 239: 1187–1200. doi: 10.1007/s00425-014-2041-2 PMID: 24573225

40. Zhang X, Chen Y, Lin X, Hong X, Zhu Y, Li W, et al. Adenine phosphoribosyl transferase 1 is a key enzyme catalyzing cytokinin conversion from nucleobases to nucleotides in *Arabidopsis*. Mol Plant. 2013; 6: 1661–1672. doi: 10.1093/mp/sst071 PMID: 23658065

41. Yang H, Liu J, Huang S, Guo T, Deng L, Hua W. Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in *Brassica napus* L. Gene. 2014; 538: 113–122. doi: 10.1016/j.gene.2013.12.057 PMID: 24406618

42. Sgamma T, Pape J, Massiah A, Jackson S. Selection of reference genes for diurnal and developmental time-course real-time PCR expression analyses in lettuce. Plant Methods. BioMed Central; 2016; 12: 21. doi: 10.1186/s13007-016-0121-y PMID: 27011764

43. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 2008; 8: 131. doi: 10.1186/1471-2229-8-131 PMID: 19102748

44. Chen X, Truksa M, Shah S, Weselake RJ. A survey of quantitative real-time polymerase chain reaction internal reference genes for expression studies in *Brassica napus*. Anal Biochem. 2010; 405: 138–40. doi: 10.1016/j.ab.2010.05.032 PMID: 20522329

45. Condori J, Nopo-Olazabal C, Medrano G, Medina-Bolivar F. Selection of reference genes for RT-qPCR in hairy root cultures of peanut. BMC Res Notes. 2011; 4: 392. doi: 10.1186/1756-0500-4-392 PMID: 21985172

46. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. Housekeeping genes as internal standards: use and limits. J Biotechnol. 1999; 75: 291–295. doi: 10.1016/S0168-1656(99)00163-7 PMID: 10617337

47. Burleigh SH. Relative quantitative RT-PCR to study the expression of plant nutrient transporters in arbuscular mycorrhizas. Plant Sci. 2001; 160: 899–904. doi: 10.1016/S0168-9452(00)00460-X PMID: 11297786

48. González M-C, Echevarría C, Vidal J, Cejudo FJ. Isolation and characterisation of a wheat phosphoenolpyruvate carboxylase gene. Modelling of the encoded protein. Plant Sci. 2002; 162: 233–238. doi: 10.1016/S0168-9452(01)00548-9

49. Klok EJ, Wilson IW, Wilson D, Chapman SC, Ewing RM, Somerville SC, et al. Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. Plant Cell. 2002; 14: 2481–94. doi: 10.1105/tpc.004747 PMID: 12368499

50. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013; 54: 391–406. doi: 10.1007/s13353-013-0173-x PMID: 24078518

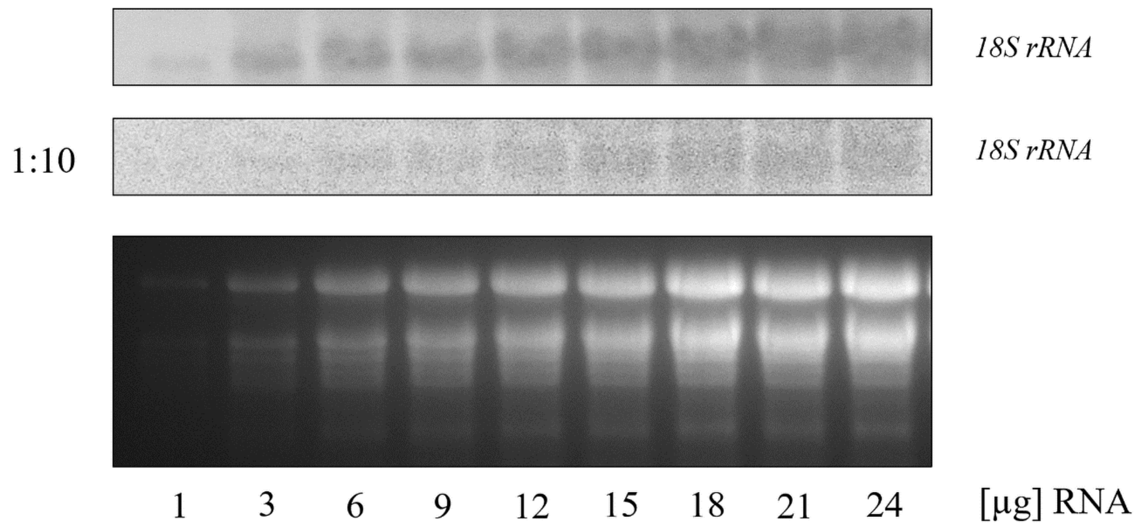
51. Haydon MJ, Román Á, Arshad W. Nutrient homeostasis within the plant circadian network. Front Plant Sci. 2015; 6: 299. doi: 10.3389/fpls.2015.00299 PMID: 25972889

52. Marum L, Miguel A, Ricardo CP, Miguel C. Reference gene selection for quantitative real-time PCR normalization in *Quercus suber*. Wu S-B, editor. PLOS ONE 2012; 7: e35113. doi: 10.1371/journal.pone.0035113 PMID: 22529976

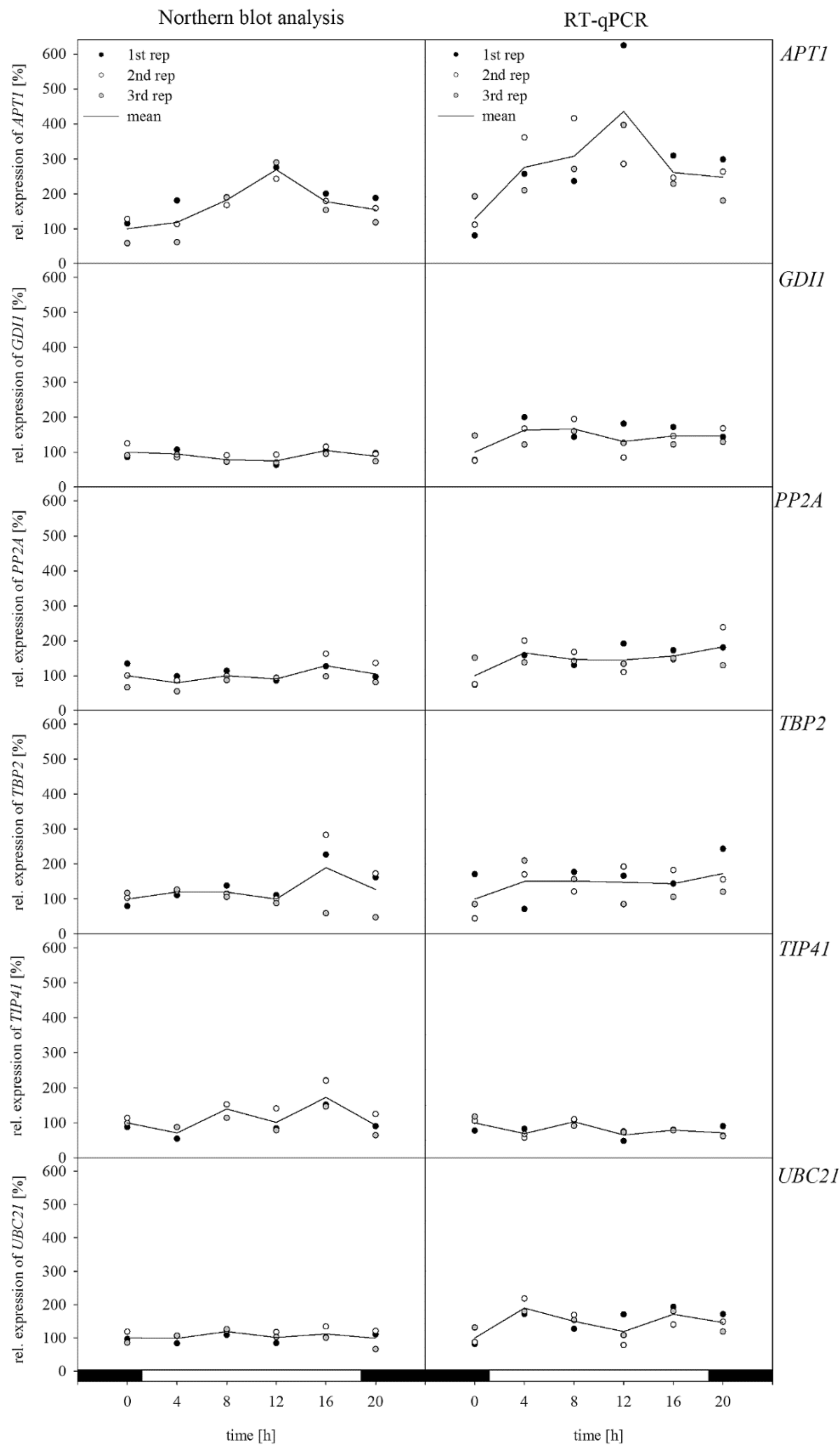
53. Zhu J, Zhang L, Li W, Han S, Yang W, Qi L. Reference gene selection for quantitative real-time PCR normalization in *Caragana intermedia* under different abiotic stress conditions. PLOS ONE 2013; 8: e53196. doi: 10.1371/journal.pone.0053196 PMID: 23301042

Supporting Information

S1 Fig



S2 Fig



S1 Table: Two-way ANOVA analysis of the expression data obtained for *CCA1*.

	p-value		
	Sulfur status (S)	**Time point of harvest (T)**	**SxT**
Non-normalized	<0.001	<0.001	<0.001
Set of reference genes	<0.001	<0.001	<0.001
ACT2	0.004	<0.001	0.075
EF1α	0.950	<0.001	0.001

S2 Table: Three-way ANOVA analysis of the expression data obtained for CCA1.

	p-value						
	Sulfur status (S)	**Time point of harvest (T)**	**Light (L)**	**SxL**	**SxT**	**LxT**	**SxLxT**
Non-normalized	<0.001	<0.001	<0.001	0.054	<0.001	<0.001	<0.001
Set of reference genes	<0.001	<0.001	<0.001	0.029	<0.001	<0.001	<0.001
ACT2	0.005	<0.001	<0.001	0.246	0.006	<0.001	0.457
EF1α	0.012	<0.001	<0.001	0.536	<0.001	<0.001	0.333

S3 Table: Two-way ANOVA analysis of the expression data obtained for *APR3*.

	p-value		
	Sulfur status (S)	**Time point of harvest (T)**	**SxT**
Non-normalized	<0.001	<0.001	<0.001
Set of reference genes	<0.001	<0.001	<0.001
ACT2	<0.001	<0.001	0.002
EF1α	0.005	<0.001	0.085

S4 Table: Three-way ANOVA analysis of the expression data obtained for *APR3*.

	p-value						
	Sulfur status (S)	**Time point of harvest (T)**	**Light**	**SxL**	**SxT**	**LxT**	**SxLxT**
Non-normalized	<0.001	<0.001	<0.001	0.014	0.078	0.089	<0.001
Set of reference genes	<0.001	0.003	<0.001	0.023	0.089	0.070	<0.001
ACT2	<0.001	0.006	<0.001	0.117	0.166	0.773	0.014
EF1α	<0.001	<0.001	0.01	0.026	0.037	0.054	0.221

CHAPTER 4

The circadian clock influences the levels of sulfur-containing metabolites in *Brassica napus* and its defense status against the fungal pathogen *Verticillium longisporum* (in preparation)

Rumlow A., Hornbacher J., Pallmann P., Riemenschneider A., Papenbrock J. (2016). The circadian clock influences the levels of sulfur-containing metabolites in *Brassica napus* and its defense status against the fungal pathogen *Verticillium longisporum. In preparation*

Abstract

The circadian rhythm evolved in plants as an adaption to daily changes in their environment. Adapting biological processes to an endogenous rhythm enable plants to cope with the daily changes in light and temperature in a more predictable way enhancing growth and fitness. A number of biological processes such as metabolic pathways as well as the immunity in plants are under circadian control. Certain time periods of the day might be more suitable for pathogen attack than others. In this study a possible circadian regulation of key enzymes in the sulfur assimilation and the corresponding metabolites with respect to possible circadian oscillations was investigated in the agriculturally important crop plant oilseed rape (*Brassica napus*). Therefore, a commercially available cultivar was harvested in a course of a day under diurnal conditions. In addition, plants were harvested under free-running conditions with constant light. The same cultivar was infected with the fungus *Verticillium longisporum* at different time points verifying the dependence of the host-pathogen interaction on the time of the day. Analyses in this study were focused on sulfur-containing metabolites and expression analysis of enzymes involved in sulfur metabolism. Expression analysis showed that the transcripts amounts of the sulfate transporters *Sultr3;1* and *Sultr4;2* as well as *APR2* and *APR3* oscillated diurnally. Results revealed a periodic rhythm of certain sulfur-containing metabolites such as glutathione (GSH), sulfate and certain glucosinolates (GSLs) in the course of a day which were partly maintained under constant light. In the infection experiments GSH and a small number of GSLs were affected due to infection after 7 dpi.

Keywords: canola, Circadian rhythm, diurnal, oilseed rape, sulfur-containing metabolites, *Verticillium longisporum*.

1. Introduction

Life on earth is exposed to daily changes in light, temperature and other environmental factors due to the rotation of the earth. Most organisms developed endogenous rhythms to be able to react faster to periodic exogenous influences. The metabolism, behavior, and physiology in organisms adapted to theses daily changes differ in the light and in the dark. Those oscillations are known as diurnal rhythms. In the absence of an external cue many of these oscillations persist and free-run with an endogenous period that is close to 24 h. Those rhythms are called circadian (from the Latin *circa,* approximately, and *dies,* day) deprived from an endogenous biological clock. Under natural conditions light and temperature cycles act as environmental

"zeitgeber" and these "zeitgeber" serve to entrain the endogenous organismic clock in each cell with the local time (McClung, 2006; Harmer 2009).

The model of the circadian clock in plants had been so far best described in *Arabidopsis thaliana* (Salome and McClung, 2004, 2005; Harmon et al., 2005; Mizuno and Nakamichi, 2005). The myb domain transcription factors CIRCADIAN AND CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL 1 (LHY1) along with TIMING OF CAB 1 (TOC1) represent the core oscillator of the circadian clock. Linked to this core oscillator further feedback loops are formed regulating the expression of so called morning as well as evening genes (for detailed information see McClung 2006). Up to 80% of the transcriptome in rice, poplar, and *A. thaliana* are regulated by the circadian clock enabling the timing of a number of biological processes and stress responses respectively (Michael et al., 2008; Filichkin et al., 2011; Dawn et al., 2015). Circadian-regulated processes such as water and carbon availability and light and hormone signaling pathways affect the growth of plants. Furthermore, molecular processes like the expression of genes or protein phosphorylation can be circadian-regulated as well (reviewed in Farré et al., 2012). The circadian rhythm influences the movements of leaves, localization of chloroplasts as well as opening and closing of the stomata. The photosynthesis as the primary biological process in this respect shows circadian oscillations in the light harvesting complex and a circadian-regulated CO_2 fixation by Rubisco (reviewed in Dodd et al., 2014). As mentioned before the circadian rhythm is also involved in certain stress responses. It could already be shown that the phytohormone abscisic acid (ABA) as an essential factor in response to drought stress is under circadian regulation. In this connection a clock gene of the core oscillator directly regulates the expression in an antagonistically way (Legnaioli et al., 2009). The involvement of the circadian rhythm in reactive oxygen species (ROS) mediated stress was already shown in *A. thaliana* where the overexpression of the *CCA1* led to an enhanced drought tolerance by increasing the expression of ROS controlling genes (Lai et al., 2012). Furthermore, cold responsive genes are regulated by the circadian clock as well (Fowler et al., 2005). However, the circadian rhythm is not solely involved in abiotic stress responses but also in plant immunity (reviewed in Bolouri Moghaddam and van den Ende, 2013). Here a particularly sensitivity of the clock to sucrose-mediated signaling which is involved in immunity and abiotic stress responses is described. Furthermore, plant immune responses go along with hormones which interact with the circadian clock as well. Dodd et al. (2005) reported an adaptive advantage and increased fitness due to a functional circadian oscillator with a period of approximately 24 h. Plants with a circadian clock period entrained to the environment had a higher chlorophyll content, higher carbon fixation, a faster growth rate, and higher survival

rates compared to plants with an altered circadian clock period. Furthermore, it is well known that many plant pathogens attack plants only during special seasons of the year (reviewed in Roden and Ingle, 2009). The magnitude of the attack depends on the environmental conditions among other factors. Results from Wang et al. (2011) indicate that defense genes are under circadian control by CCA1, allowing plants to estimate infection at dawn when the pathogen normally disperses the spores and time immune responses according to the perception of different pathogenic signals upon infection. Temporal control of the defense genes by CCA1 differentiates their involvement in basal and R-gene-mediated defense. The studies revealed a key functional link between the circadian clock and plant immunity. Mutants overexpressing *CCA1* showed enhanced resistance against downy mildew supporting a direct interaction of the clock with plant immunity (Wang et al., 2011). In recent studies infection experiments with *A. thaliana* at different time points were performed analyzing a clock-mediated variation in resistance (Bhardwaj et al., 2011; Ingle et al., 2015). Plants in these experiments showed a decreased susceptibility when infected at dawn with a bacterial pathogen and fungus respectively. Based on these results the resistance network in plants is indeed influenced by the circadian clock.

The importance of the plant clock due to its role in agriculture is now rising. In recent studies involvement of clock genes homolog to *A. thaliana* in the regulation of photoperiodic flowering in barley, wheat and sorghum was reported (Turner et al., 2005; Murphy et al., 2011; Shaw et al. 2012). Furthermore, alteration in the expression of clock genes influences the yield (Preuss et al., 2011). As an agriculturally important oilseed crop *Brassica napus* is the most closely related species to the crucifer *A. thaliana* with a number of highly conserved genes among both species. Thus findings in *A. thaliana* according to the circadian rhythm might be similar in *B. napus.* Compared to other crops and cereals the requirements for nitrogen, phosphorus and sulfur is higher making it more sensitive against sulfur-deficient conditions (Schnug and Haneklaus, 2005). Enhanced emission controls led to sulfur-deficient soils as well as to the reduction of the atmospheric deposition of sulfur causing a high demand of sulfur supplementation (Dämmgen et al., 1998; Lewandowska and Sirko, 2008).

Sulfur is an essential macroelement for plant growth and has various biological functions (Leustek et al., 2000). Sulfur is taken up into roots from the soil as inorganic sulfate. The rate of uptake and assimilation of sulfur is mainly controlled by the sulfur content of the plant and depends on the requirements for growth, which can be defined as the rate of sulfur uptake and assimilation required per gram plant biomass produced with time (De Kok et al., 2000). The sulfur requirement fluctuates during plant development and may vary between species and even

genotypes differing in sulfur need for growth and the potential sink capacity of secondary sulfur compounds.

The uptake of sulfate by the roots and its transport to the shoot seem to be one major site of regulation of sulfur assimilation (Hawkesford and Wray, 2000). In *A. thaliana* and *B. napus* 14 sulfate transporter genes have been identified (Parmar et al., 2007; Takahashi et al., 2012), which are subdivided into five different groups with different affinities to sulfate and located in different organs and organelles (Yamaguchi and Sano, 2001; Hawkesford and De Kok, 2006). The function and localization of the sulfate transporters of group three was long unknown. A recent study revealed that the transporter 3;1 is located in the chloroplast enabling the sulfate uptake of chloroplasts (Cao et. al, 2013). Furthermore, transcriptome analysis revealed a circadian regulation of this transporter in *A. thaliana* (Covington et al., 2008). Members of group 4 are known to be localized at the tonoplast enabling the efflux of sulfate out of the vacuole. It was already shown that the transporter *Sultr4;2* in *B. napus* was only expressed under sulfur-deficient conditions, thus playing a major role in the response to sulfur deficiency (Parmar et al., 2007).

Feeding experiments using $^{35}SO_4^{2-}$ showed that the incorporation of ^{35}S into reduced sulfur compounds in vivo was significantly higher in light than in the dark (Kopriva et al., 1999) in accordance with investigations on adenosine 5′-phosphosulphate reductase (APR), considered to be a key enzyme of sulfate assimilation in higher plants. The mRNA levels of all three APR isoforms showed a diurnal rhythm, with a maximum at 2 h after the onset of light. Summarizing all results, in higher plants *APR* mRNA, APR activity and *in vivo* sulfate reduction change with a diurnal rhythm, sulfate assimilation also takes place during the dark period, and sucrose feeding positively affects *APR* mRNA expression and APR activity in roots (Kopriva et al., 1999).

The first stable sulfur-containing compound in the sulfur assimilation cysteine acts next to its role in the protein synthesis as a precursor for essential biomolecules such as vitamins and cofactors. A small portion of the cysteine content is used for the biosynthesis of the tripeptide glutathione (GSH). Levels of cysteine and GSH have been suggested as markers for the elevated activity of primary sulfur metabolism after pathogen infection (Kruse et al., 2007). Sulfur is also present in secondary compounds, i.e. glucosinolates (GSLs). As a member of the class of secondary metabolites, mainly found in family of the Brassicaceae, GSLs play an important role in the response to abiotic stresses such as salinity, drought, extreme temperatures, light cycling, and nutritional deficiency (reviewed in Martínez-Ballesta et al., 2013). Previous studies revealed a circadian regulation of genes as part of the biosynthesis of GSLs (Goodspeed et al.,

2013; Kerwin et al., 2011). One of the major roles of the GSL-myrosinase system was thought to be a source for sulfur by degradation of the GSLs (Schnug et al., 1990). Regarding the portion, the GSL comprises of the total sulfur content in single and double low genotypes of *B. napus* this function had to be rejected (Fieldsend et al., 1996). Even if the GSL content can be altered by a number of factors the most accepted function for the role of the GSL-myrosinase system by now is the involvement in the defense against herbivores and pathogens (reviewed in Redovnikovic et al., 2008). Whereas the role of GSLs in defense against herbivores was already well studied, little is known in the role of GSLs against pathogens. Nevertheless, there is evidence that the breakdown products of GSLs in *Brassica* crops affect soil-borne pathogens negatively (Angus et al., 1994; Brown et al., 1996).

In addition to volatile sulfur compounds the accumulation of elemental sulfur in the veins and vascular tissues might also be involved in resistance against pathogens (Cooper et al., 1996; Williams et al., 2002). In *Brassica napus* L. plants it was observed that the GSH and GSL contents in plants are not major sources of sulfur during sulfur deficiency (Blake-Kalff et al., 1998). These results indicate that under sulfur deficiency these compounds are not adequate for a fast and efficient answer to pathogen attack. Therefore, sulfur-enhanced defense (SED) (Rausch and Wachter, 2005) is probably based on other sulfur-containing compounds. For *B. napus* fertilization experiments showed a higher susceptibility towards the fungus *Verticillium longisporum* under sulfur-deficient conditions verifying the importance of a sufficient sulfur supply (Davidson and Goss, 1972; Schnug, 1996).

Verticillium wilt as a cause of infection with the fungus is a relatively novel disease on oilseed rape threatening its production particularly in Northern Europe (Dunker et al., 2008; Friedt and Snowdon, 2009). Because of the circumstances that *Verticillium* spp., especially *V. longisporum,* in oilseed rape cannot be controlled efficiently with fungicides as well as the extended survival of microsclerotia in the soil (Heale et al., 1999), it is required to understand the host-pathogen interaction in more detail to be able to develop alternative control strategies.

There are emerging evidences for a relationship between the nutrient status and circadian rhythm in plants (reviewed in Haydon et al., 2015). For nitrate metabolism and during nitrate deficiency, respectively, interactions with the circadian clock could already be shown (Sweeney and Folli, 1984; Gutiérrez et al., 2008; Chiasson et al., 2014). However, a direct interaction of the circadian clock and the sulfur status was not analyzed in detail so far. A number of key genes in metabolic pathways including the sulfur metabolism have been reported as circadian-regulated in *A. thaliana* (Harmer et al., 2000; Michael and McClung, 2003).

Therefore, *B. napus* plants entrained to a 12 h light/12 h dark cycle were harvested during the course of a day. For analyzing gene expression and metabolites under free-running conditions, plants were exposed to continuous light. As the sulfur status might lead to alterations in the clock period, plants were additionally grown under sulfur-deficient conditions for 4 d. Sulfur-containing metabolites and selected genes as part of the sulfur assimilation pathway as well as sulfate transporters were analyzed under diurnal and circadian conditions. Further on, plants were infected with *V. longisporum* at different time points of the day analyzing the dependence of the immunity on the day time. In this experiment, sulfur-deficient conditions were applied at an earlier developmental stage to increase the susceptibility against the fungus. In these infection experiments GSH and some GSLs were already affected 7 days post inoculation (dpi).

2. Material and Methods

2.1. Plant material and growth conditions

Seeds from the MSL-hybrid (Male Sterility Lembke) winter oilseed rape cultivar Genie were obtained from the Deutsche Saatveredelung AG (DSV) (Lippstadt, Germany). The cultivar is very vital, has a medium-size root system, shows a resistance to Verticillium wilt and has a high oil content in comparison to other cultivars from the DSV (for more information see: http://www.dsv-saaten.de/raps/winterraps/sorten/genie.html). Plants were grown and harvested as described in Rumlow et al. (2016). Infection experiments were conducted as described in Weese et al. (2015). For the production of *Verticillium longisporum* spores, 500 µl of a frozen spore culture (isolate VL43, Eynck et al., 2009) was cultivated in 500 ml potato dextrose liquid medium (Difco PDB, Becton, Dickinson and Company, New Jersey, USA) in 1 L flasks. The flasks were incubated at 23°C in a rotary incubator at 150 rpm in darkness for two weeks until a dense spore suspension was produced. The concentration of the filtered spores per ml suspension was determined using a Thoma chamber and diluted with sterile water (pH 7.0) to $1*10^6$ spores per ml. *Brassica napus* seedlings were mock-inoculated with water or root dip-inoculated for 30 min.

In this study seedlings were infected at four different time points according to the harvesting time points chosen in Rumlow et al. (2016) and harvested after 7, 14, and 21 dpi (Fig. 1). Infection for 0 and 16 h were performed in the dark according to their growth conditions where at this time the light was switched off. After 7, 14 and 21 dpi leaves from plants for each treatment was harvested and directly frozen into liquid nitrogen. Due to the small size of the

plants after 7 dpi six plants were harvested and pooled for each treatment. After 14 and 21 dpi 3 plants were harvested and pooled. This experiment was performed three times.

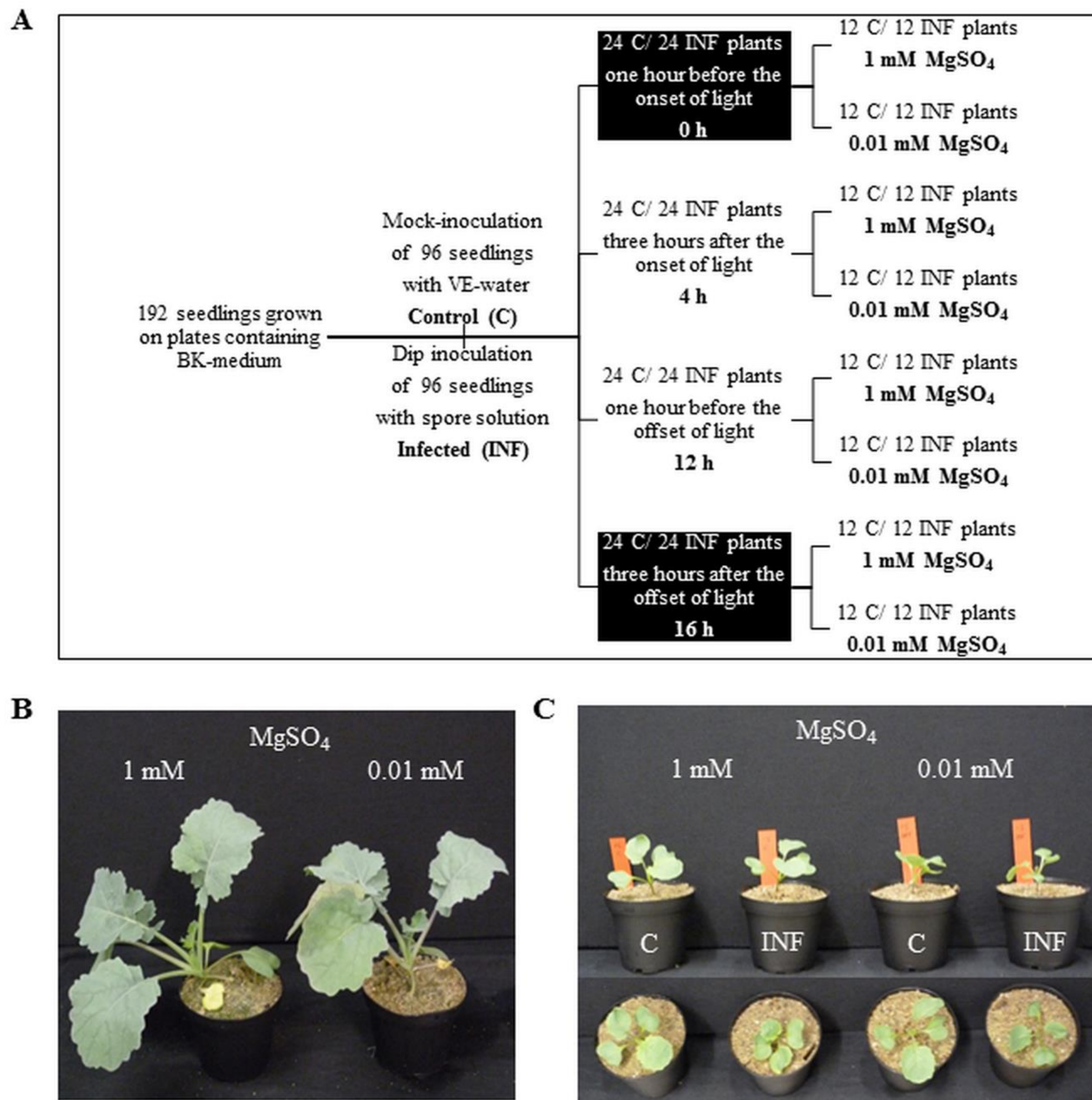


Figure 1. **Experimental design and pictures of the experimental plants under both sulfur regimes.** A) scheme for the infection experiment at different time points, B) plants grown under diurnal conditions, C) plants infected at different time points 7 dpi.

2.2. Sequence analysis and primer design

Sequences homolog to *A. thaliana* DNA sequences for *APR2, APR3* and *CCA1* were searched for the primer design in the recently closed *B. napus* database (http://compbio.dfci.harvard.edu/compbio) using BLAST. The data bank used parts of short

homologous sequences (high-fidelity virtual transcripts and tentative consensus sequences) to generate EST sequences (Quackenbush et al., 2000). For the primer pair design the program Dosbox with the Primer Design version 2.2 (Scientific & Educational Software, Cary, USA) was utilized (http://www.dosbox.com). To design the primer pairs for the amplification of cDNA fragments of sulfate transporters, the respective homologous sequences from *Brassica oleracea* were used (Buchner et al., 2004). The amplification of cDNA with the chosen primers generated fragments between 339 and 973 bp (Table 1). For a molecular proof of infection several genes were tested, either generally used molecular marker like ITS (Eynck et al., 2007) or differentially expressed genes identified by an RNA Sequence (RNA-Seq) analysis of single samples.

For the RNA-Seq analysis isolated RNA from different samples of the cultivar Genie from the experiment described in Weese et al. (2015) were chosen. In addition to samples from non-infected and infected plants, samples from plants grown under sufficient sulfur supply as well as under sulfur-deficient conditions were selected. Consequently, genes could be selected whose expression was only affected by the infection. Total RNA was purified with the RNAeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany) and DNA was digested with DNAseI (Qiagen, Hilden, Germany). An amount of 1 µg purified RNA was used for the transcriptome sequencing. The analysis was performed via Illumina technology generating 50 bp single reads (GATC, Biotech, Konstanz, Germany). After obtaining the results from database of GATC a quality assessment with the program FastQC (http://www.bioinformatics.babraham.ac.uk) was performed. With the program RobiNA (http://mapman.gabipd.org/web/guest/robin) the adapters from the Illumina sequencing were removed. Afterwards the processed sequences were imported into the CLC Genomics Workbench 7.5.1 (http://www.clcbio.com/products/clc-genomics-workbench/; Qiagen). The reference genome and annotation from *B. napus* was taken from the *Brassica napus* Genome Browser Genoscope (http://www.genoscope.cns.fr/brassicanapus/). For identifying a gene involved in the pathogen defense the reads for each gene were chosen as expression values in the different samples and were compared with each other in a heat map.

Table 1. Primer pairs used in this study. To identify homologous genes in *B. napus*, the known sequences from *A. thaliana* genes were used to search the *B. napus* database using the BLAST program. s, sense; as, antisense; for, forward; rev, reverse; f, forward; r, reverse. *BoST: BRASSICA OLERACEA SULFATE TRANSPORTER; CCA1: CIRCADIAN CLOCK ASSOCIATED1; APR: ADEONOSINE 5'-PHOSPHOSULFATE REDUCTASE; 18S rRNA: 18S RIBOSOMAL RNA; PP2A: SERINE/THREONINE PROTEIN PHOSPHATASE 2A; GDI1:*

GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1; *PR2: PATHOGEN RELATED PROTEIN 2.*

Primer pairs	***A. thaliana* AGI**	**Sequences**
P216BoST3;1s	At3g51895	5’-TTCTTGTGGCTCGAACACTCCT-3’
P217BoST3;1as		5’-GCCTTACATGTCAACAGCTCTC-3’
P226BoST4;2s	At3g12520	5’-GGTCTTTGACGTGTGAAGCATG-3’
P227BoST4;2as		5’-GTGTACGCTTCTGGATACTGC-3’
P741_Bn_CCA1_for	At2g46830	5'-TTCTTGTGGCTCGAACACTCCT-3'
P742_Bn_CCA1_rev		5'-GGATTGGTGTTGCTGATGACTC-3'
P743_BnAPR2_for	At1g62180	5’-CAAGAAGGAAGATGACACCACC-3’
P744_BnAPR2_rev		5’-GCGAATCGACATCTCTATGCTC-3’
P745_Bn_APR3_for	At4g21990	5'-CATCAAGGAGAACAGCAACGCA-3'
P746_Bn_APR3_rev		5'-TCGGGAACACTAGTATCGTCGG-3'
P782_Bn_18S rRNA_for	X16077.1	5'-ATGAACGAATTCAGACTGTG-3'
P783_Bn_18S rRNA_rev		5'-ACTCATTCCAATTACCAGAC-3'
P968_Bn_PP2A_f	At1g69960	5’-ACGAGGACGGATTTGGTTCC-3’
P969_Bn_PP2A_r		5’-GCTCCGAGCTTGTCATCGAA-3’
P984_Bn_GDI1_f	At2g44100	5’-TGCACGTTTCCAAGGAGGTT-3’
P986_Bn_GDI1_r		5’-CGGTCTGAGGGTTGTCAGTC-3’
P954_Bn_PR2_f	At3g57260	5’-CTCCGCATTCGGCACACTTG-3’
P955_Bn_PR2_r		5’-CTTCCAGGCGATGCAGAACA-3’

2.3. Production of probes and Northern blot analysis

Total RNA was extracted according to Sokolowsky et al. (1990) from ground plant material and quantified spectrophotometrically. Fifteen µg of the RNA were separated on 1% denaturing agarose-formaldehyde gels. Equal loading was controlled by staining the gels with ethidium

bromide. After RNA transfer onto nylon membranes, they were probed with digoxigenin-labeled cDNA probes obtained by PCR (PCR DIG probe synthesis kit, Roche, Mannheim, Germany). To amplify the respective probes, the sequence-specific primers listed in Table 1 were used. The colorimetric detection method with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates for alkaline phosphatase was applied. Quantitative analysis of the Northern blot results was done by GelAnalyzer5 (http://www.gelanalyzer.com). Normalization of the genes of interests (GOI) was performed with a validated set of reference genes according to Rumlow et al. (2016).

2.4. Elemental analysis of plant material

For the analytical measurements, pooled samples were measured at least three times and up to six times. Dry plant material was ground to fine powder (MM 400, Retsch GmbH, Haan, Germany). About 38 mg of the ground powder was incinerated for a minimum of 8 h in a muffle furnace (M104, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA) for each sample. After cooling the samples to room temperature (RT) (between 21 and 23°C), 1.5 ml of 66% nitric acid was added. After 10 min, 13.5 ml of ultrapure water was pipetted to the samples. The solutions were filtered (0.45 µm pore size, Carl Roth, Karlsruhe, Germany) and stored in vials at −20°C before final analysis. The samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP- OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation).

2.5. Sulfate determination and extraction and analysis of soluble thiol compounds

Sulfate concentrations were analyzed by ion chromatography as described (Bloem et al., 2004; 2012). The determination of thiols was done according to Riemenschneider et al. (2005).

2.6. Analysis of GSLs

Samples were prepared as described by Gigolashvili et al. (2012) with some modifications. The content of GSLs was determined using 25 mg freeze-dried material. GSLs were extracted twice with 1 ml 80% (v/v) methanol and centrifuged at 13,000 *g* for 5 min. Before the centrifugation, samples were put on a shaker for 15 min after the first extraction and 30 min after the second extraction at RT. The supernatants were pooled and loaded onto a column containing 2 ml of a 5% (w/v) suspension of DEAE Sephadex A25 (Sigma-Aldrich, Taufkirchen, Germany) in 0.5 M acetic acid (pH 5). Columns were washed five times with 2 ml H_2O and two times with 2 ml

0.02 M acetic acid (pH 5). For desulfating the GSLs 50 µl of sulfatase (Sigma-Aldrich) solution was added to 450 µl 0.02 M acetic acid (pH 5) and loaded on to the columns as well (Thies, 1979). Desulfation took place over night at room temperature. Afterwards desulfated GSLs were eluted three times with 2 ml HPLC H_2O and dried overnight in a vacuum centrifuge, and then dissolved in a total amount of 300 µl HPLC H_2O. Analysis was performed with high-performance-liquid chromatography (HPLC) system (Knauer, Berlin, Germany) equipped with an Ultra AQ C-18 column (150 x 4.6 mm, 5 µm particle size) (Restek GmbH, Bad Homburg, Germany). A water (solvent A)-acetonitrile (solvent B) gradient at a flow rate of 0,5 ml min^{-1} at 45°C (injection volume 50 µl). The 52 min long run consisted of 100% A for 6 min, 100-70% A for 27 min, 70-40% A for 0.1 min, a 4.9 min hold of 40% A, 40-100 % A for 0.1 min and a 19.9 min hold of 100% A. The detection of the GSL was performed with DAD and FAD (Knauer, Berlin, Germany) at 229 nm. Quantification of the measured GSL was performed by using Sinigrin (Phytolab, Vestenbergsgreuth, Germany) and relative response factors.

2.7. Identification of GSLs in *B. napus*

For the identification of the GSL in *B. napus* samples were analyzed by LC-MS. A volume of 10 µl was injected in the HPLC system (Shimadzu, Darmstadt, Germany) and separated on a Knauer Vertex Plus column (250x 4mm, 5µm particle size, packing material ProntoSIL 120-5 C18-H) equipped with a pre-column (Knauer, Berlin, Germany). A water (solvent A)-methanol (solvent B), both containing 2 mM ammonium acetate and 0.01% acetic acid gradient was used with a flow rate of 0.8 ml min^{-1} at 30°C. For measuring the samples the following gradient was used: 10-90% B for 35 min, 90% for 2 min, 90-10% B for 1 min and 10% B for 2 min. Detection of the spectra in the range 190-800 nm was performed with a diode array detector (SPD-M20A, Shimadzu, Darmstadt, Germany). The HPLC system was connected to an AB Sciex Triple TOF mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA). At a temperature of 600°C and an ion spray voltage floating of -4500 V the negative electrospray ionization (ESI) was performed. For the ion source gas one and two 50 psi and for the curtain gas 35 psi were used. In the range of 100-1500 Da in the TOF range the mass spectra as well as the MS/MS spectra from 150-1500 Da at a collision energy of -10 eV were recorded. With the mass of characteristic fragments of every GSL peaks could be identified. Due to lack of a number of standards fractions of the measured samples were collected in a fraction collector (FRC-10A Shimadzu, Darmstadt, Germany). Afterwards the fractions were dried in a vacuum centrifuge and dissolved in 300 µl ultrapure water. The retention time for every GSL was then determined by measuring them in the HPLC system as described before.

2.8. Databases used for the expression analysis

The database AGRIS (www.arabidopsis.med.ohio-state.edu, Davuluri et al., 2003; Palaniswamy et al., 2006; Yilmaz et al., 2011) was used to search for circadian clock related binding site motifs. The tool from Mockler et al. (2007) (http://diurnal.mocklerlab.org/) was used to compare array-based transcriptome analysis in *A. thaliana* with the data obtained in this study.

2.9. Statistical analysis

Statistics were performed using a Two-Way and Three-Way ANOVA with the values of the expression data and metabolic content as dependent variable and sulfur concentration, time point of harvesting, and light condition (diurnal/circadian) as independent factors. Significance of factors and their interactions was assessed by means of F-tests. In the presence of interactions, the Tukey comparisons were carried out separately for each level of the interacting factor. Statistical computations were done in InfoStat (http://www.infostat.com.ar, InfoStat version 2016).

The evolution of GOIs and sulfur-containing metabolites over time were analyzed using statistical model selection with AICc, a small-sample version of the widely known Akaike Information Criterion (Hurvich and Tsai, 1989; Burnham and Anderson, 2002). A set of "candidate models" with a linear time response as well as trigonometric functions (sinus and cosine) were assembled. The model with the lowest AICc value was considered to be the one that gets most support from the experimental data. The "candidates" are linear models with the corresponding data for the expression and content of the sulfur-containing metabolites as endpoint; the independent variables include light, sulfur, time, and replication, as well as any reasonable interaction terms. Considering both linear and sine functions modeling the influence of time and allow for interactions so that slope and intercept or amplitude, average, and phase shift may or may not depend on light and sulfur. Statistical computations were done in R3.1.1 (RCoreTeam, 2014). All graphs were generated with SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

3. Results

3.1. Establishment of a highly controlled cultivation system and investigation of the role of sulfur in a circadian-regulated system by expression analysis

For investigating the interaction of the circadian clock and sulfur metabolism a growth system had to be established first. Confirming a suitable growth system for our purposes the expression of clock-controlled genes as well as genes involved in sulfate transport and assimilation were analyzed by Northern blot analysis (Fig. 2, Fig. S1). Plants grown under sufficient sulfur supply with 1 mM $MgSO_4$ and under sulfur-deficient conditions with 0.01 mM were harvested every 4 h, beginning 1 h before the light was switched on. For analyzing the expression data in a quantitative way, results of the Northern blot analysis were normalized according to Rumlow et al. (2016) with a validated set of reference genes (Fig. 2A). To check the stability and reproducibility of controlled manipulation of the cultivation system, a segment of the *B. napus CCA1* gene was isolated and used in Northern blot hybridization as a probe to follow the *CCA1* expression (Fig. 2A). Its expression pattern in light/dark (LD) conditions showed a maximum of expression antemeridian 3 h after the light was switched on and remained undetectable before midnight, 3 h after the light was switched off. This pattern is comparable to other plant species (Mizoguchi et al., 2002; Harmer, 2009). Regarding the sulfur fertilization, statistics revealed a significantly higher transcript amount of *CCA1* under sulfur-deficient conditions (Table S1). In samples from plants harvested under light/light (LL) conditions the expression pattern remained the same. However, a significant reduction in its amplitude was observed (Table S2). Taking the influence of all three factors on the expression of *CCA1* together there is a highly significant ($p<0.0001$) interaction between them (Table S2). To be able to follow the sulfur status in the plants the sulfate transporter *Sultr4;2*, known as a gene highly expressed under sulfur-deficient conditions in several species was included into the investigation. Plants grown in our experimental system showed a significant up-regulation of *Sultr4;2* indicating a successful application of sulfur limitation (Fig. 2A). Statistical analysis of the *Sultr4;2* expression in plants grown under sufficient sulfur supply resulted in relatively high standard deviations due to low signals. However, the transcript levels fluctuated significantly in the course of a day with higher transcript levels in the middle of the light phase (Table S1). In plants exposed to continuous light the expression of *Sultr4;2* was not significantly influenced (Table S2) except for 28 h where lower transcript amounts were detected. Furthermore, the influence of the sulfur status on the expression is independent from the light (Table S2). To analyze the influence of the LD conditions on the key genes of sulfur metabolism known from *A. thaliana* homologous genes

were identified in *B. napus*. Data known from literature (Kopriva et al., 1999) and motif research (http://arabidopsis.med.ohio-state.edu/AtcisDB/bindingsites.html) gave hints about genes which might be clock-controlled. Northern blot analysis of *Sultr3;1* in plants grown under sufficient sulfur supply (Fig. 2A) showed a significant up-regulation under LD conditions for approximately 8 h beginning 1 h before the light was switched on. Followed by a significant decrease in the transcript level transcript amounts were maintained at a lower level in the dark phase and started to increase again at 24 h (Table S1). At 28 h transcript amounts of *Sultr3;1* were 2 fold higher compared to the measured transcript amount at 4 h. At 32 h the expression was already down-regulated and transcript amounts of *Sultr3;1* decreased further in the light phase. Regarding the sulfur status there was no influence on the expression of *Sultr3;1* measureable. In accordance with statistical analysis a p-value of 0.6414 was calculated for the factor sulfur supporting the independence of the expression of *Sultr3;1* on the sulfur status (Table S1). In LL conditions analysis of the transcript levels resulted in a significantly decrease in the subjective night compared to LD conditions (Table S2). Furthermore, the transcript maximum of *Sultr3;1* was detected 8 h later than in plants grown under LD conditions. In accordance with statistics there is a highly significant interaction of the light and the harvesting time point independent from the sulfur status (Table S2).

Furthermore, the expression of members of the *APR* gene family was analyzed (Fig. 2A). The expression of the *APR2* gene oscillated for both sulfur regimes significantly in the course of a day in plants harvested under LD conditions with an up-regulated expression in the light phase. In plants grown under sufficient-sulfur supply and harvested at 0, 4, and 8 h the measured transcript level of *APR2* was relatively constant. In the beginning of the dark phase at 16 h a down-regulation of *APR2* in the plants was detected. At 24 h the expression was up-regulated again reaching 4 h later a maximum in the transcript level. Afterwards the expression of *APR2* was down-regulated again. In plants grown under sulfur-deficient conditions transcript amounts of *APR2* were significantly higher compared to plants grown under sufficient sulfur supply (Table S1). The highest transcript amounts were measured in the beginning and in the middle of the light phase respectively. In plants harvested under LL conditions the oscillations detected in the transcript levels of *APR2* were significantly affected by the light dependent on the time point the plants were harvested (Table S2). For the second isoform *APR3* expression analysis in the plant harvested under LD conditions resulted in nearly the same oscillations compared to *APR2*. In plants grown under sulfur-sufficient conditions a higher degree of up-regulation of *APR3* was measured in the beginning of the light phase and in the end of the dark phase. Sulfur-deficient conditions led to a significant increase in the transcript amount measured in the plants

(Table S1). The oscillations in the transcript level of *APR3* in these conditions were comparable to those in plants grown under sufficient sulfur supply. Analyzing the expression of *APR3* in plants harvested under LL conditions resulted in a shift in the expression pattern which was highly dependent on the sulfur status (Table S2). Only in plants grown under sulfur-deficient conditions the up-regulation began 4 h earlier in LL conditions compared to the expression in LD conditions. Comparing both isoforms the expression of *APR3* was more influenced by the LL conditions (Table S2).

Results obtained by Northern blot analysis reflect the successful establishment of a reliable growth system investigating circadian aspects with sufficient sulfur supply as well as under sulfur-deficient conditions. This experiment was performed twice verifying the reproducibility. All GOIs analyzed in the plants harvested under LD conditions showed diurnal oscillations with an up-regulation in the light phase and down-regulation in the dark phase. Analysis of the expression in plants harvested in LL conditions resulted in increased or decreased transcript amounts, and partly in alterations of the expression pattern.

As all transcripts oscillated diurnally sine functions were generated based on the candidate model with a certain period chosen by AICc (see 2.9 for more information) for each GOI, respectively (Fig. 2B). Oscillations for the isoform *APR2* showed a 23 h rhythm under LD as well as under LL conditions, whereas for the latter one a lowered amplitude and an advanced phase (shifts earlier in time) was shown. For *APR3* oscillations comprise only a 20 h period and the amplitude under sulfur-deficient conditions was increased. Under sulfur-deficient conditions an advanced phase was shown. For the clock gene *CCA1* periodic oscillations of 23 h were determined. The amplitudes in the oscillations were unaffected by the sulfur status. Under LL conditions a delayed phase (shifts later in time) was shown. For the sulfate transporter *Sultr3;1* oscillations in the transcript level follow a 23 h rhythm. The amplitude was unaffected by the sulfur status but lowered under LL conditions and showed a delayed phase of 4 h. Higher transcript levels of *Sultr3;1* on the second day under LD conditions resulted in a higher amplitude. For the second transporter *Sultr4;2* the model of a 20 h rhythm was determined. In plants under sufficient sulfur supply the amplitudes of the oscillations were very low compared to the amplitudes of the oscillations under sulfur-deficient conditions. For both sulfur regimes the second amplitude of the oscillations was higher under LD conditions. As the expression was unaffected by light no differences in the amplitude between LD and LL conditions were observed.

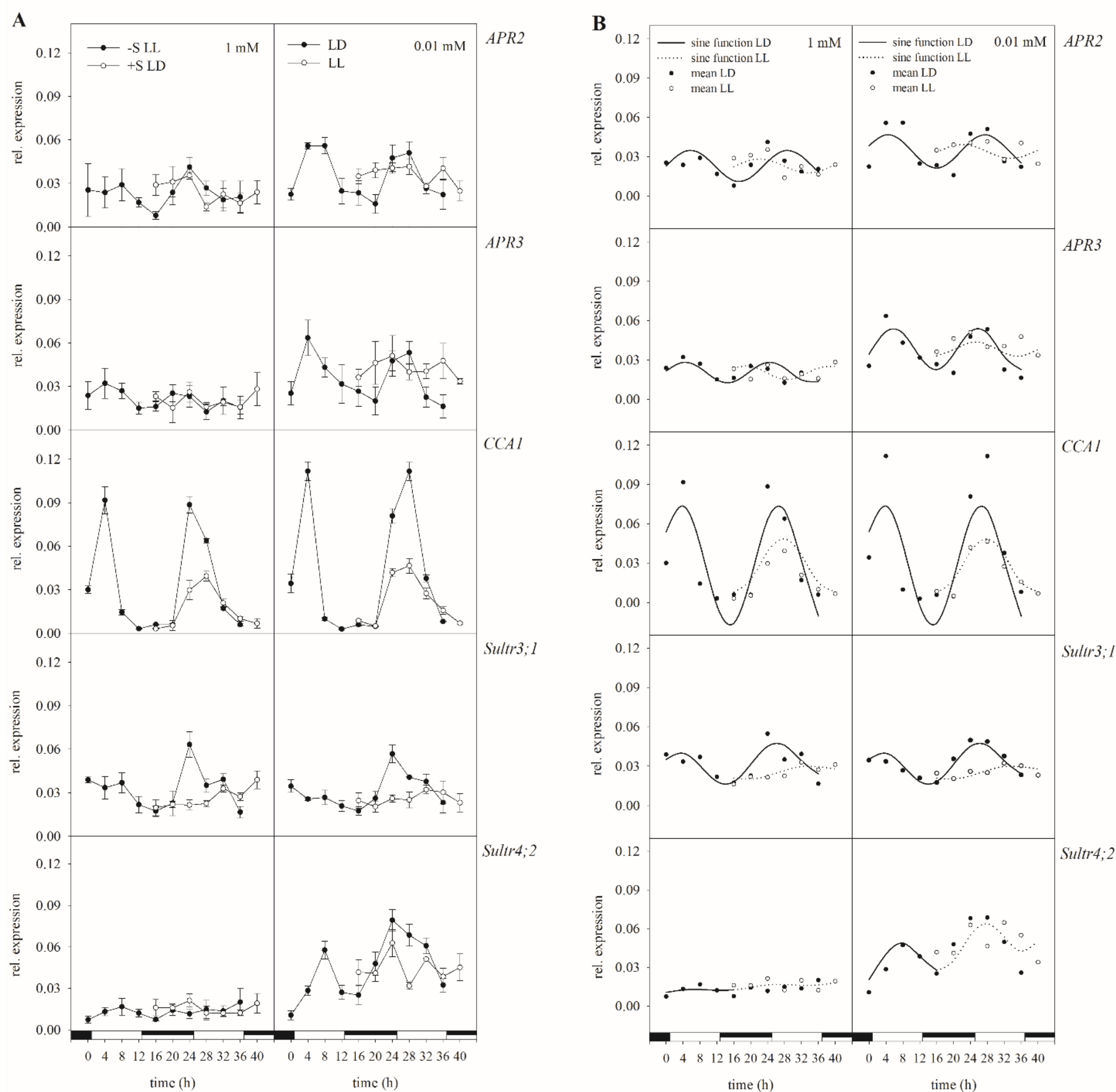


Figure 2. Expression of GOIs under circadian and sulfur-deficient conditions. Transcript amounts were determined in plants (with five fully expanded leaves) grown with 1 mM $MgSO_4$ as a control and 0.01 mM $MgSO_4$ for four days to obtain sulfur-deficient conditions. Plants grown under 12 h dark/ 12 h light (LD) were harvested over a period of 36 h every 4 h starting 1 h before the onset of light. In addition, plants grown in a chamber with 24 h light (LL) were parallel harvested beginning at 16 h and ending at 40 h fulfilling a 24 h rhythm. Total RNA was isolated, and for Northern blot analysis 15 µg RNA was electrophoretically separated and transferred onto membranes. For the detection DIG labeled probes were used. A) Normalization of the GOIs with a validated set of reference genes under LD and LL conditions. Data are shown as the mean of three technical replicates ± SD. Relative expression calculation was based on band intensity. B) Sine functions of the oscillations for the GOIs together with the according mean from the three technical replicates under LD and LL conditions. Abbreviations for probes see Table 1.

3.2. Metabolic analysis of sulfur-containing compounds in a circadian-regulated system

3.2.1. Measurements of total sulfur amounts in leaves

Previous results indicated changes of the total sulfur content during the day (Weese et al., 2015). To understand the influence of the light period on the total sulfur content in the leaves, dried material was analyzed by ICP-OES. In addition the effect of the sulfur treatments could be followed. The total sulfur content in plants grown under 1 mM sulfur supply showed significantly higher amounts in the light phase in plants grown under sufficient sulfur supply (Fig. 3, Table S1). Interestingly, in plants harvested under LD conditions the highest amount of sulfur with approximately 7 mg g^{-1} DM was measured. In plant material harvested from plants grown under sulfur-deficient conditions the sulfur content was significantly decreased reaching approximately 4 mg g^{-1} DM (Table S1). Furthermore, the measured content was maintained at relatively constant levels in the plants harvested under LD conditions. Under LL conditions the sulfur content measured in the plants decreased significantly independent from the sulfur status (Table S2). In the plant material from plants grown under sufficient sulfur supply and harvested under LL conditions the sulfur content decreased down to 4.4 mg g^{-1} DM in the course of the day. Under sulfur-deficient conditions the content reached a minimum of 2.7 mg g^{-1} DM at 36 h. For both sulfur regimes and light conditions oscillations in the content were rather random. Therefore, generating sine models with a certain period was not suitable.

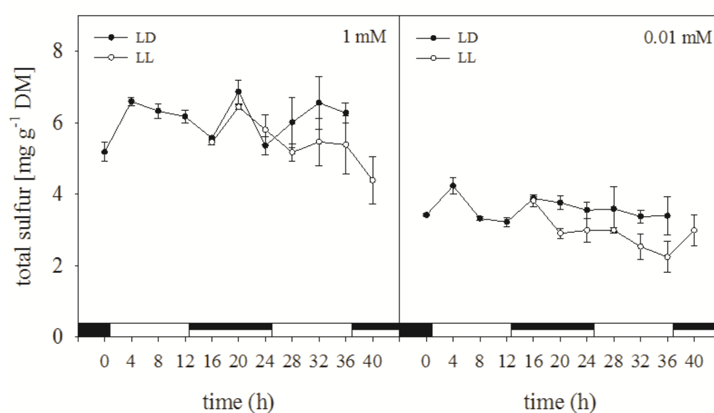


Figure 3. Total sulfur content under circadian and diurnal conditions. The elemental sulfur was measured in dried material (DM) of plants treated as described in Figure 2 with ICP-OES. Results calculated as mg g^{-1} DM represent the mean of two technical replicates ±SD.

3.2.2. Determination of sulfate levels in the leaves of *B. napus* plants

As the expression of the sulfate transporters *Sultr3;1* and *Sultr4;2* showed oscillations in the course of a day (Fig. 2) the sulfate contents measured in plants grown with sufficient sulfur supply oscillated significantly with maxima of approximately 5 mg g^{-1} DM 1 h before the onset of light and 1 h before the offset of light (Fig. S2, Table S1). Lowest amounts of sulfate were measured in plants harvested in the dark phase with 3.5 mg g^{-1} DM. In plants grown under sulfur-deficient conditions the measured sulfate content significantly decreased in the light phase from 3.6 to 2.1 mg g^{-1} DM (Table S1). After an increased content measured in the plants harvested in the dark phase the content of sulfate was further decreased down to 1.34 mg g^{-1}. For both sulfur regimes the measured sulfate amounts in the plants were significantly decreased under LL conditions which was highly dependent on the sulfate status and the time point of harvest (p-value <0.0001), respectively (Table S2). In plants grown under sufficient sulfur supply the decrease was significantly more drastic than under sulfur-deficient conditions; here the sulfate content was significantly decreased by 60% at 24 h and was then maintained at a constant level. In conclusion, the interaction of all three factors influencing the sulfate content in the plants was highly significant (p-value 0.0001, Table S2).

3.2.3. Cysteine and glutathione contents in leaves

As representatives of the primary sulfur assimilation pathway the cysteine content was analyzed via HPLC and the contents of the most important transport molecule of reduced sulfur, GSH (Fig. 4). The cysteine concentrations measured in plants grown with 1 mM $MgSO_4$ and harvested under LD conditions were higher during the day with a maximum of approximately 20.6 nmol g^{-1} FM than at night with a minimum of about 11 nmol g^{-1} FM (Fig 4A). Under sulfur-deficient conditions measurements of cysteine resulted in significant lower contents with a minimum of approximately 9.9 nmol g^{-1} FM without significant oscillations in the course of a day (Table S1). According to statistics with a p-value of 0.8352 the oscillations of the cysteine content in the course of the day were not significant independent from the sulfur status. Regarding the third factor light the measured cysteine contents in plants were not significantly influenced independent from the sulfur status (Table S2). The content of reduced GSH measured in plants grown with 1 mM $MgSO_4$ oscillated diurnally in the course of a day with high amounts up to 560 nmol g^{-1} FM at the end of the light phase and significant lower amounts of 240 nmol g^{-1} at the end of the dark phase (Fig. 4B, Table S1). The decrease of the GSH measured in plants grown under sulfur-deficient conditions was highly significant with a calculated p-value of 0.0002 (Table S1). With a maximum of approximately 403 nmol g^{-1} FM in the light phase and a minimum of 269 nmol g^{-1} FM the measured contents oscillated

significantly as well in the course of a day (Table S1). In agreement with statistics with a p-value of 0.1556 these oscillations were independent from the sulfur status (Table S2). Regarding the third factor light the GSH content measured in the plants was harvested under LL conditions was significantly influenced dependent on the time points the plants were harvested. Taking all three factors together the content of the measured GSH was not significantly influenced (Table S2). In agreement with the diurnal oscillations of the GSH content periodic oscillations with a period of 23 h was determined (Fig. 4C). The amplitude in LL conditions was lowered for both sulfur regimes. Under sulfur-deficient conditions an advanced phase could be observed. To summarize the results for the thiols the cysteine was only affected by the sulfur status and showed no oscillations in its content. The GSH content in contrast was influenced by all three factors. According to statistics the factor that influenced the oscillations of the GSH content the most was the time point the plants were harvested.

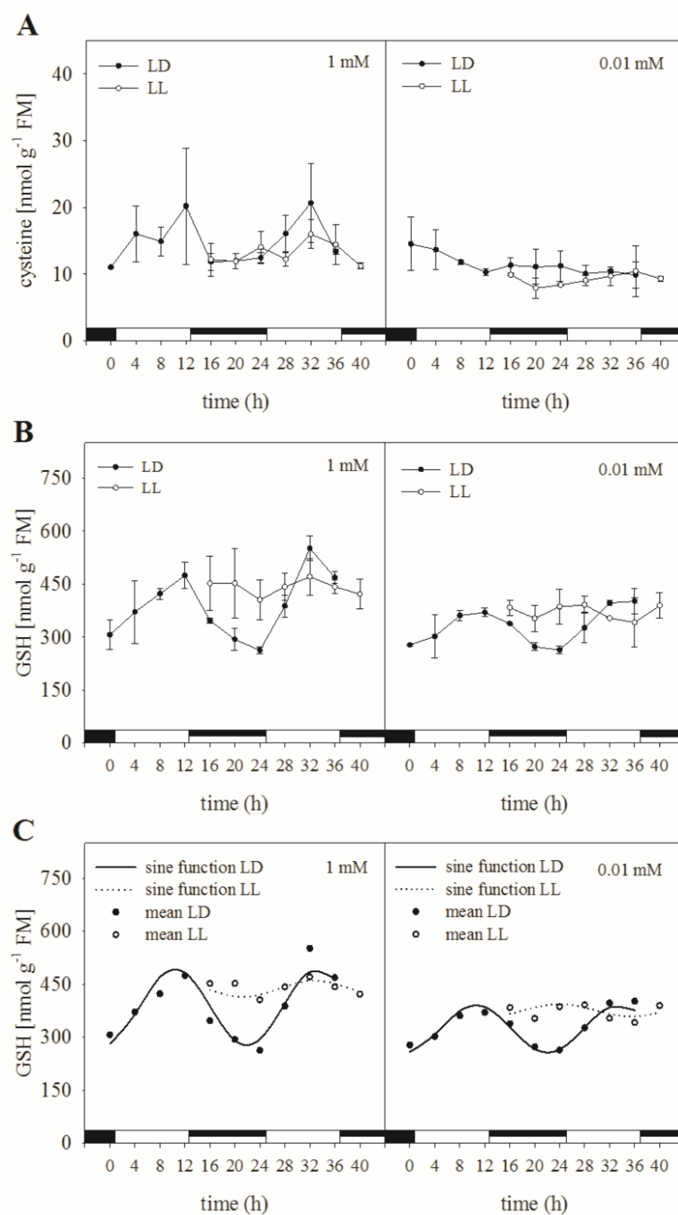


Figure 4. Thiol contents under circadian and diurnal conditions. Cysteine (A), GSH (B), the sine function of the periodic oscillations with the corresponding mean (C). The cysteine and GSH contents were determined in plants treated and collected as described in Figure 2 by HPLC. Data in nmol g^{-1} FM represent the mean of three technical replicates ±SD.

3.2.4. Quantification of GSLs in leaves

As representatives of the secondary sulfur metabolism intact GSLs were measured as desulfated GSLs in the leaves (Fig. 5, Fig. S3, S4). The identified GSLs showed different oscillations in the course of the day and were affected in different ways by the sulfur status and the light conditions. Taking all aliphatic GSLs together the measured content in plants grown with sufficient sulfur supply and harvested under LD conditions was maintained from 0 to 12 h at approximately 0.7 µmol g^{-1} DM (Fig. 5A). This was followed by a significant increase in the

content of aliphatic GSLs up to 1 µmol g^{-1} DM in the beginning of the dark phase (Table S1). After a slight decrease in the content in plants harvested in the dark phase the content reached 1 µmol g^{-1} DM again one hour after the onset of light. Afterwards the measured content of the aliphatic GSLs in the plants was decreased by 20% at 32 h and increased up to 0.9 µmol g^{-1} DM again at 36 h. The content of the aliphatic GSLs was significantly decreased in plants grown under sulfur-deficient conditions ($p<0.0001$) and the pattern in the oscillations in the content was altered (Table S1). Under sulfur-deficient conditions the measured concentration of the aliphatic GSLs was significantly decreased by 40% starting from one hour before the onset of light until the end of the light phase. In plants harvested in the dark phase the content was increased again reaching approximately 0.7 µmol g^{-1} DM. At the end of the dark phase the content of the aliphatic GSLs was decreased again by about 40%. In plants harvested at 32 h a maximum of 0.8 µmol g^{-1} DM was measured followed by a rapid decrease down to 0.5 µmol g^{-1} DM again. Considering the third factor light the content of the aliphatic GSLs was significantly influenced independent from the sulfur status (Table S2). Under sufficient sulfur supply the amount of aliphatic GSLs measured in plants harvested under LL conditions was lower in the subjective night at 16 and 20 h compared to plants harvested under LD conditions. Furthermore, from 24 to 36 h the content in the plants was decreased by about 30% followed by an increase in the subjective night at 40 h. In plants grown under sulfur-deficient supply and harvested under LL conditions the content of the aliphatic GSLs was significantly lower compared to plants harvested under LD conditions (Table S2). The content was maintained at approximately 0.45 µmol g^{-1} DM in the course of the day. Regarding the individual aliphatic GSLs nearly the same pattern in the oscillations could be observed (Figure S3). Except for glucoraphanin the oscillations in the content of the individual aliphatic GSLs were highly dependent on the sulfur status with p-values <0.0001 (Table S1). Interestingly gluconapin and glucoraphanin were not significantly influenced by the light independent from the sulfur status and the time point the plants were harvested.

In plants grown under sufficient sulfur supply and harvested in LD conditions, the highest concentration of the indolic GSLs with 0.15 µmol g^{-1} DM was measured at 0 h and 12 h respectively (Fig. 5B). Afterwards the content was decreased down to approximately 0.1 µmol g^{-1} DM and maintained at this level. Although the content of the indolic GSLs was not significantly decreased in plants grown under sulfur-deficient conditions an altered pattern in the oscillations were observed (Table S1). A maximum of approximately 0.25 µmol g^{-1} DM was measured in plants harvested three hours after the onset of light. Afterwards the content was decreased by about 70% in plants harvested at 8 h. In the beginning of the dark phase the

content of the indolic GSLs was increased again to 0.15 µmol g^{-1} DM. The content measured in the plants harvested at the end of the dark phase was decreased down to 0.1 µmol g^{-1} DM again. Afterwards the content of the indolic GSLs in the plants was slightly increased in the light phase and decreased again at the end of the light phase. In agreement with statistics the significant oscillations were highly dependent on the sulfur status with a p-value <0.0001 (Table S1). In plants harvested in LL conditions the content of the indolic GSLs was not influenced in a significant way independent from the sulfur status and the time point the plants were harvested (Table S2). Comparing the two GSLs glucobrassicn and neoglucobrassicin representing the indolic GSLs, for the former one oscillations were higher in the course of the day was (Fig. S4). The only aromatic GSL measured in *B. napus* was gluconasturtiin (Fig. 5C). In plants grown with sufficient sulfur supply and harvested under LD conditions a content of approximately 0.06 µmol g^{-1} DM was measured one hour before the onset of light. The decrease in the content down to 0.05 µmol g^{-1} DM measured in plants harvested at 4 and 8 h was followed by a significant increase up to 0.085 µmol g^{-1} DM four hours later (Table S1). In the middle of the night phase the content of gluconasturtiin was significantly decreased by 50%. Already 4 h later the measured content was increased again up to 0.065 µmol g^{-1} DM. At the end of the light phase the content in the plants was decreased by 30%. The sulfur-deficient conditions led to no significant decrease in the content of the aromatic GSL, whereas the pattern of the oscillations in the content in plants harvested under LD conditions was altered (Table S1). After the increase of the content in the beginning of the light phase up to 0.12 µmol g^{-1} DM a decrease by about 60 % occurred. At the end of the light phase the measured content of gluconasturtiin in the plants began to increase again reaching 0.09 µmol g^{-1} DM in plants harvested in the middle of the dark phase. This was followed by a decrease down to 0.05 µmol g^{-1} DM in plants harvested at 24 h. Maintained at this level the content of gluconasturtiin began to decrease again at 36 h. Regarding the third factor light the content in the plants of gluconasturtiin was affected in a significant way independent from the sulfur status (Table S2). In plants grown with sufficient sulfur supply and harvested under LL conditions the content was significantly decreased by 50% between 24 and 40 h. In plants grown under sulfur-deficient conditions the content of gluconasturtiin was significantly lower compared to plants harvested under LD dark conditions (Table S2). The same level in the content of gluconasturtiin as measured in plants harvested under LD conditions was reached at 32 h. To summarize the measurements of the GSLs in *B. napus* the oscillations in the content of the GSLs was dependent on the sulfur status. Only the aliphatic GSLs were reduced in their content under sulfur-deficient conditions. Furthermore, only for the indolic GSLs the content was unaffected by the circadian conditions.

For the individual GSLs oscillations in the content with a certain period could not be determined properly by statistical analysis.

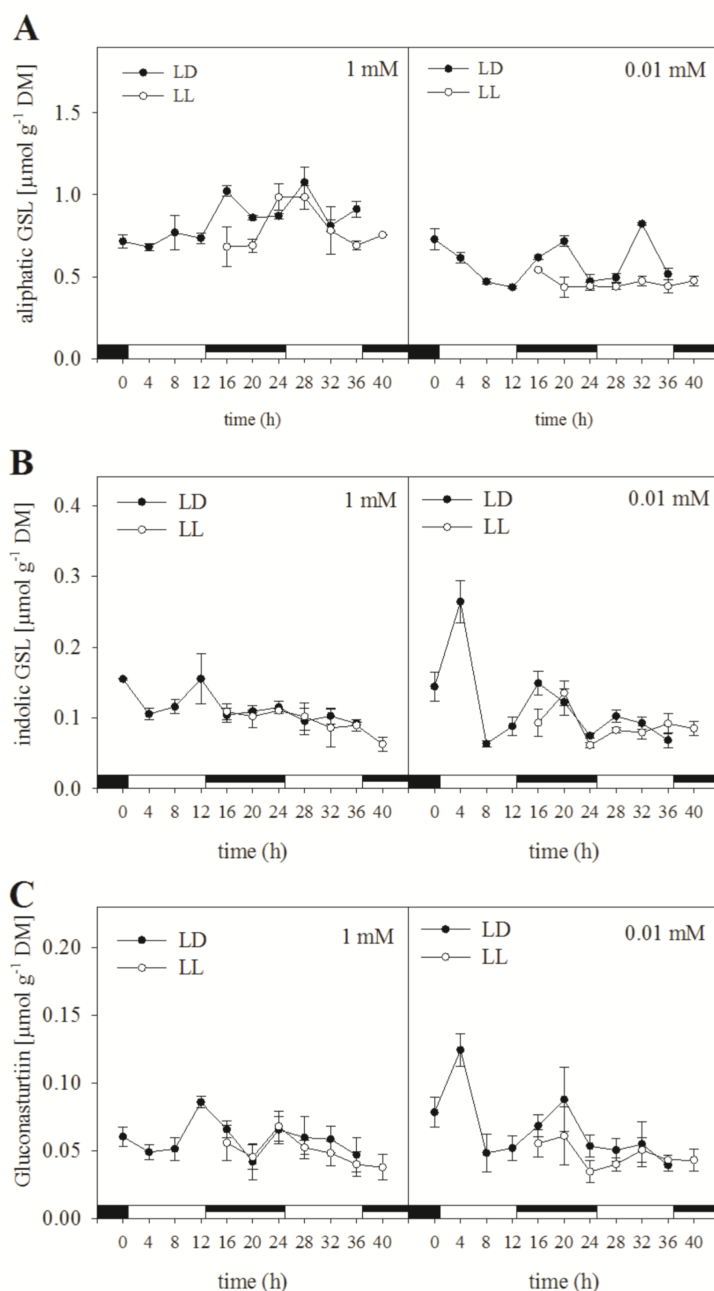


Figure 5. GSLs in leaves under diurnal/circadian conditions. Aliphatic (A) and indolic GSLs (B) as well as one aromatic GSL (C) were measured by HPLC in plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

3.3. Influence of different infection times on gene expression and sulfur-containing metabolites

As the resistance in plants is dependent on the time of the day *B. napus* plants were infected with the fungus *V. longisporum* at different time points in the morning and in the evening

respectively (Fig. 1). Plants were infected according to Weese et al. (2015). As here the fungus could not be directly detected at this early stage the increased total sulfur content and sulfur-containing metabolites as well as physiological measurements were taken as confirmation of the infection. However, results obtained in the study by Weese et al. (2015) for Genie could not be reproduced in this study. Either the infection was not successful or the infection rate was too low leading to underestimation of differences in comparison of infected to non-infected plants and differences between the different infection time points. On the other hand, a number of differences in the expression and metabolite analysis could be observed after 7 dpi in comparison to non-infected plants. For 14 and 21 dpi differences between infected and non-infected plants were indistinct. Therefore, only results for 7 dpi are shown.

3.3.1. Analysis of gene expression after infection at different time points

Transcript levels of *APR2* were higher under sufficient sulfur supply than under sulfur-deficient conditions. In infected plants the expression of *APR2* was down-regulated under sufficient sulfur supply compared to non-infected plants. At 0 h the highest expression was observed. In infected plants grown under sulfur-deficient conditions the expression was unaffected. In contrast the transcript amount of the second isoform *APR3* was down-regulated under sufficient sulfur supply and nearly reached undetectable levels. Under sulfur-deficient conditions higher transcript levels were detected. In infected plants grown under sufficient sulfur supply an increased expression was only determined at 0 h. In infected plants grown under sulfur-deficient conditions *APR3* was slightly down-regulated compared to non-infected plants. Regarding the time point of infection the lowest transcript level was measured at 16 h. As a gene involved in the pathogen response the expression of *PR2* was analyzed. In non-infected plants with sufficient sulfur supply at 0 h the highest transcript level was measured whereas under sulfur-deficient conditions at 0 h and 16 h the lowest levels were detected. In infected plants the expression of *PR2* was up-regulated. Under sufficient sulfur supply the lowest transcript amount was measured at 0 h and highest amount at 12 h. In plants grown under sulfur-deficient conditions at 0 h the lowest transcript amount was measured as well. Differences between the other three infection time points could not be determined. After 14 dpi transcript levels of *PR2* could not be detected any more in control plants. Only in infected plants under sulfur-deficient conditions *PR2* could be detected at 0 and 4 h (data not shown). The transcript amounts of *Sultr3;1* were very low and could not be properly detected. After 14 dpi transcript levels were increased (data not shown). Nevertheless, expression was neither affected by the infection nor

by the sulfur status the plants were grown under. The second sulfate transporter *Sultr4;2* was undetectable in non-infected plants under sufficient sulfur supply. The expression of this transporter was increased in non-infected plants under sulfur-deficient conditions. Interestingly, in infected plants at 0 h and grown under sufficient sulfur supply transcript amounts were detected. In infected plants and under sulfur-deficient conditions expression of *Sultr4;2* was drastically down-regulated in plants infected at 12 and 16 h, respectively. Analyzing the expression of *CCA1* a down-regulation of the expression in infected plants was determined which seemed to be independent from the time point of infection. In infected plants under sulfur-deficient conditions the degree of down-regulation appeared to be higher.

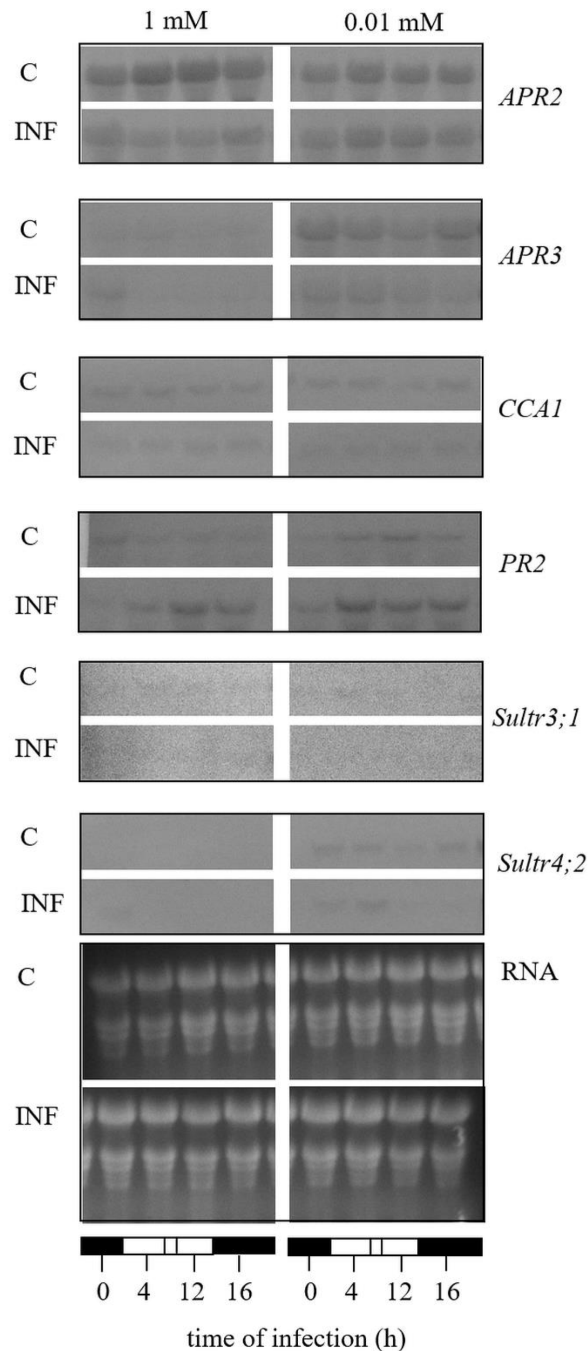


Figure 6. Expression of GOIs in leaves after infection at different time points. Seven days old seedlings were mock-inoculated or infected with *V. longisporum* spores at different time points. The time points were chosen according to Figure 1 at 0, 4, 12 and 16 h. Plants were grown under sufficient sulfur supply (1 mM $MgSO_4$) and under sulfur-deficient conditions (0.01 mM $MgSO_4$) in a climate chamber for 7 dpi. Four plants with leaves fully expanded per treatment were harvested. Northern blot analyses were done with specific digoxigenin-labeled probes. For abbreviations see Table 1.

3.3.2. Analyzing the effect of the infection time on total sulfur amounts in the leaves

The total sulfur amount measured in plants 7 dpi under sufficient sulfur supply was neither affected by the infection nor the time point of infection (Fig. 7). The content ranged from approximately 10 to 12.5 mg sulfur g^{-1} DM. Under sulfur-deficient conditions the content was decreased to a minimum of about 1 mg sulfur g^{-1} DM. In infected plants under these conditions the sulfur content was increased at 0, 12, and 16 h reaching a maximum of approximately 4.5 mg S g^{-1} DM at 16 h. The main factor influencing the total sulfur amount was the sulfur supply.

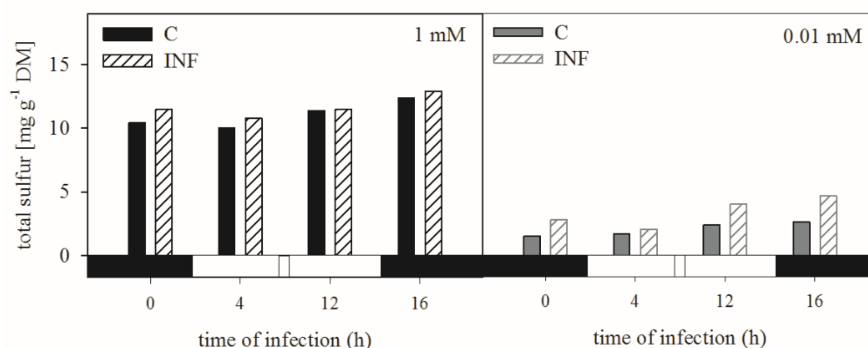


Figure 7. Total sulfur in leaves after infection at different time points. The elemental sulfur was measured in dried material (DM) of plants treated as described in Figure 3 with ICP-OES. Results calculated as mg g^{-1} DM represent one measurement. Filled bars represent the control plants (C) and striped bars infected plants (INF).

3.3.3. Thiol content in the leaves from plants infected at different time points

The cysteine content in the leaves harvested after 7 dpi reached under sufficient sulfur supply a maximum of approximately 30 nmol g^{-1} FM (Fig. 8A). In plants grown under sulfur-deficient conditions the cysteine content was decreased down to the half. Although in infected plants the cysteine content was increased, the infection and the time point of infection had no effect on the amount of cysteine. In plants 14 dpi the cysteine content was decreased further down to 7 to 15 nmol g^{-1} FW (data not shown). Again the content was not affected by the infection and the time point of infection.

The GSH content on the contrary was affected differently (Fig. 8B). After 7 dpi a maximum of approximately 350 nmol g^{-1} FM in the leaves of plants grown under sulfur-sufficient supply was measured. The content was not influenced due to the infection. In plants grown under sulfur-deficient conditions the content was decreased to a minimum of 100 nmol g^{-1} FM. In leaves of infected plants the GSH content was increased which was dependent on the time point of infection. At 4 h the GSH content was increased up to approximately 200 nmol g^{-1} FM. In plants infected 3 h after the light was switched off (16 h) the content was even doubled compared to the control plants. After 14 dpi highest amounts of GSH in the leaves of plants

grown under sufficient sulfur supply up to 400 nmol g^{-1} FM were measured (data not shown). In contrast to plants at 7 dpi the GSH was increased in infected plants compared to control plants independent from the time point of infection. In plants grown under sulfur-deficient conditions the amount of GSH was further decreased down to a minimum of 80 nmol g^{-1} FM. Interestingly, the GSH content was not increased due to infection with the fungus. Thus also the time point of infection did not affect the GSH content.

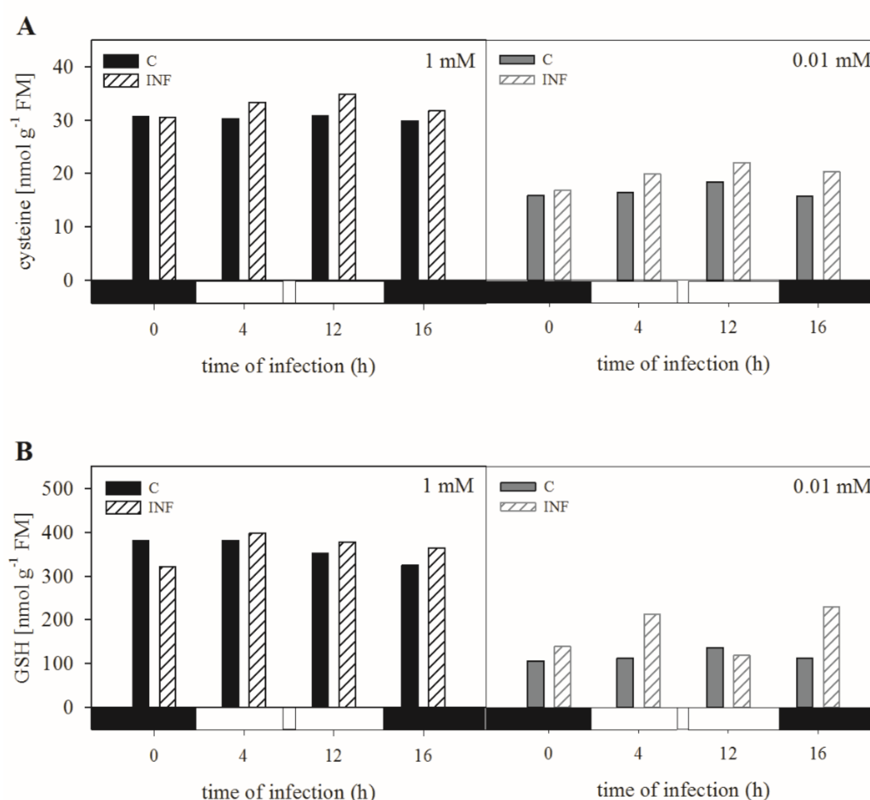


Figure 8. Thiol content in leaves after infection at different time points. cysteine (A) and GSH (B) were measured in plants treated as described in Figure 6 by HPLC analysis. Calculated data in nmol g^{-1} FM represent one measurement. Filled bars represent the control plants (C) and striped bars infected plants (INF).

3.3.3. Analysis of GSLs after infection at different time points

As it is hypothesized that breakdown products of GSL play a major role pathogen defense the GSL contents were analyzed as well. Measurement of the aliphatic GSL under sufficient sulfur supply showed no clear tendencies when comparing non-infected and infected plants (Fig. 9A). The highest amount was measured in plants infected at 12 h with approximately 0.6 µmol g^{-1} DM and the lowest amount with about 0.2 µmol g^{-1} DM at 0 h in non-infected plants. The latter one was doubled in putatively infected plants. As the content seemed to be increased in infected plants at 0 and 12 h the content at 4 h was decreased and at 16 h no differences at all were

shown. Under sulfur-deficient conditions, however, at 16 h the content was 2.5 fold higher in infected plants, whereas for the other infection time points no real differences were shown. Regarding the indolic GSLs the highest amount was measured under sufficient sulfur supply at 12 h with approximately 0.5 µmol g^{-1} DM in infected plants (Fig. 9B). Here the content was nearly doubled compared to the non-infected plant. For the other infection time points however, only small differences appeared between non-infected and infected plants. The content of indolic GSLs in control plants under sulfur-deficient conditions did not differ between the infection time points and were about 0.2 µmol g^{-1} DM. Except for the time point 0 h the content was increased in infected plants reaching the highest amount in plants infected at 16 h with approximately 0.35 µmol g^{-1} DM. For the one aromatic GSL gluconasturtiin an increase was measured in infected plants under sufficient sulfur supply for the infection time points 0, 12, and 16 h (Fig. 9C). Here the highest amount was reached in plants infected at 12 h with approximately 0.12 µmol g^{-1} DM. No differences for the infection time point at 4 h could be observed. Under sulfur-deficient conditions only in plants infected at 16 h an increase in the content up to 0.15 µmol g^{-1} DM could be determined. Here the content was doubled compared to non-infected plants. To summarize, the GSLs were differently affected due to the infection and the time point of infection. However, no clear tendencies were observed.

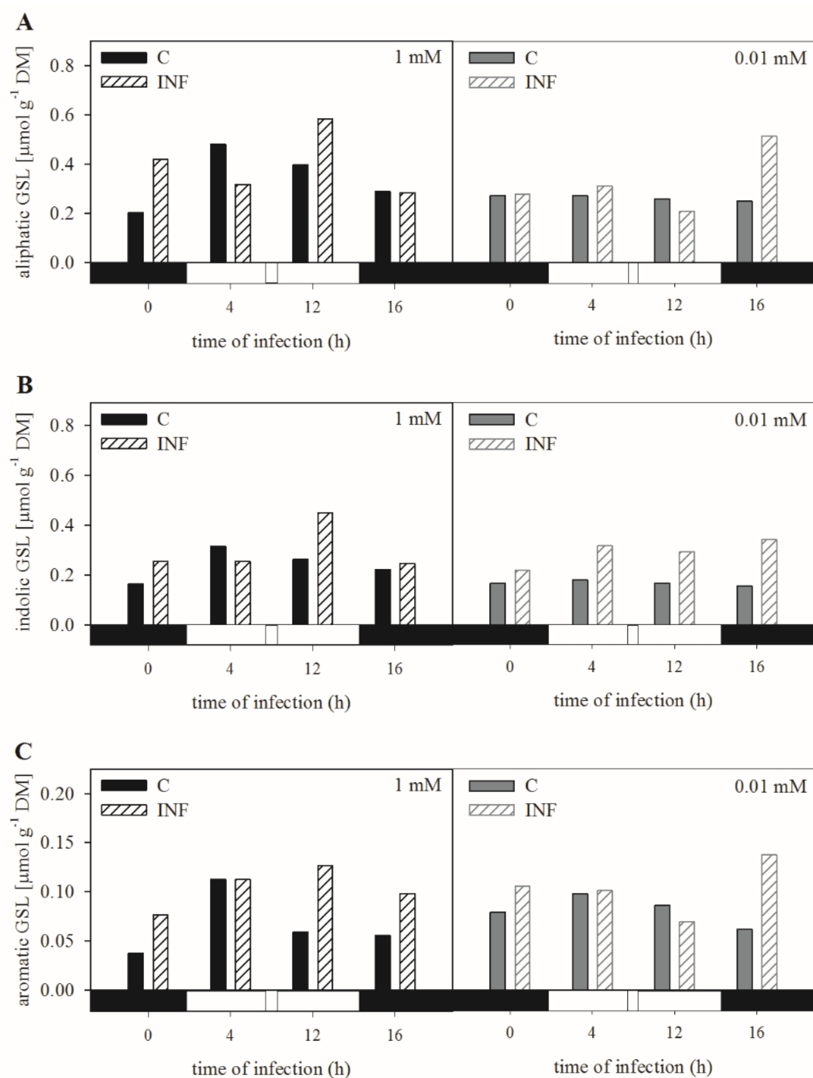


Figure 9. GSL content in leaves after infection at different time points. Aliphatic (A) and indolic (B) GSLs, as well as one aromatic (C) GSL were measured in plants treated as described in Figure 6 by HPLC analysis. Calculated data in µmol g^{-1} DM represent one measurement. Filled bars represent the control plants (C) and striped bars infected plants (INF).

4. Discussion

The aim of this work was to investigate a possible relationship between the circadian clock, the primary and secondary sulfur metabolism, and the infection with a fungus at different time points.

4.1. Investigating circadian aspects in *B. napus* in a reproducible way by establishing a suitable growth system is possible

For analyzing the influence of the circadian clock on the biosynthesis of sulfur-containing metabolites a highly controlled growth system had to be established. By analyzing the expression of the clock gene *CCA1* as a regulatory element of the core oscillator of the circadian clock stability of the growth system was verified. The expression was analyzed in plants collected every 4 h over a period of 40 h under diurnal conditions with 12 h light and 12 h dark as well as under free-running conditions with 24 h continuous light, respectively (Fig. 2, Fig S1). The expression pattern from Northern blot analysis of *CCA1* in *B. napus* is comparable to other plant species (Mizoguchi et al., 2002; reviewed in Harmer, 2009) indicating a suitable harvest pattern. However, the amplitude under constant light was significantly lowered. Transcriptome analysis of *CCA1* in *A. thaliana* where plants were grown in a light/dark cycle and exposed to constant light showed the same degree of down-regulation (Mockler et al., 2007). In the study of Kim et al. (2003) it was discussed that light may influence the activity of a positive effector of *CCA1* and *LHY* leading to an altered amplitude of the transcript level. As a possible candidate they named the TOC1 protein as it showed interactions with a phytochrome B-related transcription factor protein (Makino et al., 2002). On the basis of the expression analysis it was possible to verify a circadian period of 23 h for *CCA1* in *B. napus.* As in this study the zeitgeber was light, a circadian period of 24 h would have been assumed. It was already demonstrated in *A. thaliana* and *Brassica rapa* that the shortening in the period is dependent on the temperature (Lou et al., 2011; Kusakina et al., 2014). Plants grown under 17°C showed a circadian period around 24 h whereas under 27°C the period was shortened down to 20 h. It is postulated that a shorter period at higher temperatures may confer a performance advantage. Therefore, it can be suggested that the circadian period of the clock with 23 h in *B. napus* is more beneficial when grown at a temperature of 22°C. Investigation with other clock genes in *B. napus* would be helpful verifying this circadian period.

Furthermore, half of the plants were grown under sulfur-deficient conditions for 4 d. The plants were grown under sulfur-deficient conditions determining in the first place a possible influence of sulfur limitation on the circadian clock. There are indeed a number of nutrients which are

influenced by the circadian rhythm and vise versa (reviewed in Haydon et al., 2015). The nitrogen metabolism in *A. thaliana* as an example as it is regulated by *CCA1* which binds to the promoters of nitrogen-assimiliation genes (Gutiérrez et al., 2008). It was already shown that nitrate depletion affected the circadian clock by shortening the circadian period hours (Sweeney and Folli, 1984). In our study this was not the case as the period of *CCA1* was unaffected by sulfur-deficient conditions. Therefore, it can be concluded that in our study the given conditions have no influence on the expression of clock transcripts. This has to be further investigated by analyzing additional clock transcripts as well as prolonged growth under sulfur-deficient conditions. Therefore, to verify the sulfur deficiency in the plants the expression of the sulfate transporter *Sultr4;2* was analyzed (Fig. 2) which is predominantly detectable under sulfur-deficient conditions (Buchner et al., 2004). In plants grown under sulfur-sufficient conditions low amounts of transcripts were detected. One explanation would be that the supply of 1 mM $MgSO_4$ was not enough for a sufficient supply. Another explanation would be that a high degree of excessive sulfate triggered the up-regulation of the transporter. Thus the efflux of the sulfate out of the vacuole is triggered preventing an over-accumulation of sulfate in the vacuole (Kataoka et al., 2004; Reich et al., 2016). In plants grown with 0.01 mM $MgSO_4$ the expression of *Sultr 4;2* was up-regulated. This was also confirmed in a previous study with *B. napus* where this transporter was up-regulated under sulfur-deficient conditions (Buchner et al., 2004; Parmar et al., 2007; Weese et al., 2015). According to these results a suitable growth system was established for further detailed investigations.

4.2. The sulfate transport is differently affected by the diurnal and circadian conditions

In previous studies the expression of all members of the sulfate transporters ordered in the four groups in *Brassica oleracea* and all members of the five groups in *B. napus* under sulfur deprivation was analyzed in detail (Buchner et al., 2004; Parmar et al., 2007). In *B. oleracea* the sulfate transporter *Sutlr3;1* was expressed in the stem and roots independent from the sulfur supply of the plants. However, in leaves the expression of this transporter was only up-regulated under sulfur deprivation lasting at least 10 d (Buchner et al., 2004). Interestingly, in the study by Parmar et al. (2007) the expression of the transporter *Sultr3;1* could not be detected at all in the leaves from *B. napus* plants. This is in contrast to our results as the transporter was expressed at a relatively high level independent from the sulfur status (Fig. 2). Furthermore, transcript levels oscillated under diurnal and circadian conditions with a period of 23 h whereas under free-running conditions the amplitude was decreased and delayed in the phase. As the period matches the period of *CCA1* and remained the same in LL conditions a regulation by the

circadian clock can be assumed. This is even further supported as in *A. thaliana* the transporter *Sultr3;1* contains an evening element promoter motif (EE) (http://arabidopsis.med.ohio-state.edu/) where CCA1 can directly bind and regulate the expression (Wang et al., 1997; Hamer et al., 2000; Nagel et al., 2015). Genes with this motif are likely to be expressed in the evening as binding of CCA1 in this promoter region inhibits the expression. Therefore, one would expect a down-regulation in the expression of *Sultr3;1* in the morning and an up-regulation in the evening. This is contradictory to our results, as the expression was up-regulated in the morning and down-regulated in the evening (Fig. 2A, B). However, our results for the expression of *Sultr3;1* are in agreement with microarray analysis in *A. thaliana* under different light conditions (Mockler et al., 2007). In the study from Nagel et al. (2015) a number of target sequences for CCA1 next to the EE were genome-wide identified in *A. thaliana* revealing a morning-phased expression. Based on that it can be assumed that the sequence of the *Sultr3;1* in *B. napus* contains no EE and instead one of the other targets, thus leading to a peak of the expression in the beginning of the light phase. This is confirmed by our results as the transcript levels peak in the beginning of the light phase when *CCA1* was up-regulated as well. In the course of the day transcript amounts are decreasing such as the transcript amount of *CCA1*. Confirming a regulation by the circadian clock one would assume that under continuous light oscillations the transcript levels of *Sultr3;1* would be unaffected. However, in this study the amplitude was lowered and a delay in the phase occurred. A possible explanation would be the transcript amount of *CCA1* which was significantly decreased under free-running conditions. Thus the amplitude in the beginning of the light phase for the transcript level of *Sultr3;1* was decreased as well, underestimating differences in the course of a day. Nonetheless there is evidence that sulfur uptake in chloroplasts is regulated by the clock due to the circadian-regulated sulfate transporter 3;1 which is localized at the chloroplast membrane (Hayden et al., 2011; Cao et al., 2013). It is postulated that other members of group 3 transporters are also located at the chloroplast membrane (Cao et al., 2013). Therefore, expression analysis of the other members would be helpful confirming a circadian-regulated uptake into the chloroplast.

The expression of *Sultr4;2* was predominantly analyzed as detection for the sulfur limitation (Fig. 2). Interestingly, diurnal oscillations of the transcript levels under sulfur-deficient conditions were detected and were unaffected under free-running conditions in the subjective night. Based on that one would assume a regulation by the circadian clock. However, as the expression of the group 4 transporters is probably solely regulated by a sulfate gradient at the tonoplast a direct circadian regulation is likely to be excluded (Kataoka et al., 2004; Reich et al., 2016). This needs to be further investigated by analyzing the second member of the group

4 transporter as the transporter *Sultr4;2* plays only a minor role under sulfur-sufficient conditions (Buchner et al., 2004; Kataoka et al., 2004; Parmar et al., 2007).

4.3. The isoforms of the key enzyme in the sulfate reduction are affected differently in *B. napus* under diurnal and circadian conditions

As key enzymes of the sulfate assimilation pathway the expression of two isoforms of the adenosine-5'-phosphosulfate (APS) reductase (APR) was analyzed (Fig. 2). According to sequence analysis by using BLAST all three isoforms present in *A.thaliana* are also present in *B. napus.* As in the study from Kopriva et al. (1999) *APR1* and *APR3* showed a similar expression, for our study only the isoform *APR3* was included in the expression analysis next to *APR2*. For both isoforms transcript amounts oscillated diurnally with higher transcript amounts in the light phase under sulfur-sufficient as well as under sulfur-deficient conditions. This is in accordance with the expression of *APR* in *A. thaliana* and maize, oscillating diurnally with a maximum during the light period (Kocsy et al., 1997; Kopriva et al., 1999). Under sulfur-deficient conditions the amplitude of the oscillations increased as the expression of both isoforms was increased under sulfur-deficient conditions. The periodic oscillations in the transcript level of *APR2* comprise 23 h with a peak in the morning phase which is equal to the period of *CCA1*, thus supporting a regulation by the circadian clock. Under free-running conditions oscillations of 23 h were observed whereas the amplitude was lowered and showed an advanced phase with a peak in the subjective night. In the case of *APR3* oscillations comprise only 20 h. The amplitude was lowered by the continuous light and an advanced phase appeared under sufficient sulfur supply. In the study of Kopriva et al. (1999) the regulation of *APR* expression and APR activity by an endogenous rhythm was excluded as mRNA levels of all three isoforms decreased in continuous dark. However, it was already reported that the expression of *APR2* is under circadian control in *A. thaliana* (Harmer et al., 2000) which is in accordance with the presence of the EE in the sequence of *APR2* (http://arabidopsis.med.ohio-state.edu/). As already described for *Sultr3;1* genes with an EE are likely to peak in the night. This is in agreement with the results from the microarray analysis in *A. thaliana* as highest transcript amounts were measured in the night and lowest in the day, respectively, independent from the given light conditions (Mockler et al., 2007). As *APR2* transcripts contribute 75% of the APR activity in *A. thaliana* (Loudet et al., 2007) one would assume that the APR activity would show the same oscillations. However, in previous studies APR activity had only been shown to undergo diurnal rhythm in plants adapted to short days (Kopriva et al., 2009); when plants were grown in long days APR activity was again higher during the light period than in

the dark, but without the strong maximum observed under short days (Huseby et al., 2013). In summary, the results obtained in our study are contradictory for *APR2* regarding the circadian regulation thus making a proper conclusion difficult. That both isoforms differ in their period of the oscillations might be due to different regulation mechanisms which need to be further investigated.

4.4. Is there a circadian regulation in the transport and reduction of sulfate?

In this study the aim was to determine whether the transport and the reduction of sulfate might be under circadian control. For investigating an influence by the circadian clock the light was chosen as zeitgeber. Plants grown with sufficient sulfur supply as well as with sulfur-deficient supply were entrained to a 12 h light/dark rhythm. By exposing part of the plants to continuous light the external cue was absent. Under these conditions circadian regulated genes should show the same oscillations as under diurnal conditions. Except for *Sultr4;2* oscillations in the transcript levels of the analyzed GOIs showed lowered amplitudes and in some cases an advanced or delayed phase, whereas the period of the oscillations under these conditions remained the same. (Fig. 2B). Nevertheless, a circadian regulation could not be unambiguously determined. It can only be assumed that there might be a regulation by the circadian clock as the transporter *Sultr3;1* and *APR2* oscillate in the same period as *CCA1* with a peak in the morning under diurnal conditions. Therefore, a direct interaction of CCA1 with the target genes by binding to specific binding motives might be possible (Nagel et al., 2015). However, under LL conditions there was a shift in the phase and a lowered amplitude by up- or down-regulation even though the period of the oscillations remained the same. Potential targets of CCA1 were identified genome-wide in *A. thaliana* by ChipSeq analysis under LD and LL conditions (Nagel et al., 2015). A large portion of the putative target genes were non-cycling under LL conditions. It was discussed that this might be stress-related as plants were not expecting light in the night and consequently did not cycle in LL conditions (Velez-Ramilez et al., 2011). As a response to the LL induced stress the generation of reactive oxygen species (ROS) might be triggered. As GSH is involved in the detoxification of ROS (Foyer and Halliwell, 1976) it is likely that under LL conditions GSH accumulates in the plants thus leading to an altered activity of the enzymes involved in the sulfur assimilation as here the precursor for GSH cysteine is formed. It was shown in *A. thaliana* that the sulfate transport is negatively regulated by GSH thus leading to a decrease in the expression (Vauclare et al., 2002) which would be an explanation for the down-regulation of *Sultr3;1* under LL conditions. Based on that a possible regulation of the sulfur assimilation by the clock might be underestimated by the use of LL. As the sulfur assimilation

is dependent on the reducing equivalents produced in the photosynthesis, which is circadian regulated, a regulation by the circadian clock especially for the transport into the chloroplast is plausible (Harmer et al., 2000; Dodd et al., 2005; Haydon et al., 2011). Furthermore, is the nitrogen-metabolism known to be under circadian control and as these nutrients are dependent on each other a circadian regulation by the clock might be possible (reviewed in Haydon et al., 2015). Therefore, experiments with mutants of *B. napus* with a disrupted clock by the loss of, for example, *CCA1* are necessary.

4.5. Representatives of the primary and secondary metabolism differed in their oscillations under the given conditions

Measuring the total sulfur content in addition to the detection of the sulfate transporter *Sultr4;2* was a second way to detect sulfur deficiency in plants. *Brassia napus* plants with a sulfur content of 3.5 mg sulfur g^{-1} DM are considered to suffer deficiency (Scherer, 2001). This is in accordance with the data obtained in our study (Fig. 3). Following the total sulfur content in the course a day, can give first information on the regulation of the sulfur metabolism. The total sulfur content showed variations in the course of the day, however, statistics excluded diurnal oscillations and instead supported a more linear trend. As sulfate-sulfur comprises the biggest portion of the total sulfur in plants (Blake-Kalff et al., 1998; Zhao et al., 1999) it was not surprising that the content did not oscillate diurnally (Fig. S2). Interestingly, under constant light the sulfate-sulfur content and consequently the total sulfur showed a decrease. This might be due to a stress response induced by the LL as mentioned before resulting in an increased use of the sulfate for the generation of GSH. In the period of 40 h the plants were harvested, sulfate and consequently total sulfur levels decreased which might be due to decreasing sulfate amounts in the sand the plants were grown on. It would have been necessary to measure the sulfate in the substrate as well verifying the decrease which might have led to the decrease of the sulfur in the plants.

In previous experiments it was reported that the two major sulfur-containing metabolites, GSH and GSL, as representatives of primary and secondary sulfur metabolism showed only minor or no fluctuation during a light/dark period. GSH levels were higher during the light period than in the dark, without a clear maximum and GSL levels were relatively stable except for a peak 8 h after the onset of light (Huseby et al., 2013). On the other hand, no diurnal changes in cysteine or GSH contents were observed in poplar (Noctor et al., 1997). In our study, GSH showed diurnal oscillations with a period of 23 h with a maximum at the end of the light phase (Fig.4B, C). Same oscillations in the content of GSH could be observed in plants harvested in

LL conditions, whereas the amplitude of the oscillations was lowered and a delay in the phase under sulfur-deficient conditions occurred. As mentioned before, this incident might be based on the continuous light plants were exposed to. As a consequence, ROS might have been accumulated and thus GSH synthesis was up-regulated. This assumption needs to be clarified by determining an accumulation of ROS in the plants harvested under LL conditions, for example by a lipid peroxidation assay in the samples (Jambunathan et al., 2010). Nevertheless one could assume that the GSH synthesis is regulated by the circadian clock, as there is a CCA1 binding site motif in *GSH1* (http://arabidopsis.med.ohio-state.edu/) catalyzing the first step of the GSH synthesis. The precursor of GSH, cysteine, was measured as well and no diurnal oscillations with a certain period were determined (Fig. 4A). In the study by Huseby et al. (2013) it was demonstrated that the reduced sulfur is first incorporated in GSH and GSLs and at the end of the light phase in proteins thus it is not surprising that the content of cysteine as the source of the reduced sulfur was non-cycling. Furthermore, cysteine acts as a precursor for cofactors and iron-sulfur clusters (Hell, 1997; Beinert, 2000).

As representatives of the secondary metabolites the individual GSLs were measured in the leaves by HPLC (Fig. 5; Fig. S3, S4). As the sulfur-containing amino acid methionine acts as the precursor for the biosynthesis of aliphatic GSLs those are more sensitive to sulfur deficiency than the indolic and aromatic GSLs (Mailer, 1989). In agreement with our results after 4 d of sulfur-deficient conditions only the aliphatic GSL were significantly decreased in their content, whereas the indolic and the aromatic ones were unaffected in their content by the treatment. Regarding the oscillations of the content of the aliphatic, indolic and the one aromatic GSLs ultradian rhythms were shown which were altered under sulfur-deficient conditions. Ultradian rhythms show oscillations shorter than 20 h and were already reported to occur in plants for a number of processes such as glycolysis, sap flow, enzyme activity, root elongation, and leave movements (Salisbury and Ross, 1992; Solheim et al., 2009, Iijima and Matsushita, 2011)**.** Rosa et al. (1998) found a significant variation in the total and individual GSLs levels during a single day in *B. oleracea* as well, whereas these were most prominent when plants were grown at 30°C compared to the optimal growth temperature of 20°C. In this context the sensitivity to temperature of the ultradian rhythms is discussed. At an optimal temperature for the growth and development the photoperiod influences the synthesis of major GSLs, whereas under temperatures close to inducing stress variations are likely to derive from the temperature (Rosa et al., 1998). For vegetative growth of canola the optimum temperature is 25°C (Edwards and Hertel, 2011). The plants in our study were grown under 22°C which is in the ideal temperature range of canola plants. Thus, ultradian rhythms might not be derived from the temperature. As

the oscillations in the content of all GSLs measured in our study were dependent on the sulfur status the ultradian rhythms might derive from the sulfur supply the plants were grown under. In plants harvested in LL conditions the oscillations in the content of the indolic and aromatic GSLs were maintained under both sulfur regimes indicating that the biosynthesis might be regulated by an endogenous mechanism which can be altered by the sulfur supply. Furthermore, the rhythmic oscillations in the content of the aliphatic GSLs were absent under free-running conditions in plants grown under sulfur-deficient conditions. Regarding the total sulfur content and sulfate-sulfur measured in this study the content was further decreased in plants grown under sulfur-deficient conditions and harvested in LL conditions. It can be assumed that such low levels led to the loss of the rhythmic oscillations in the content of the aliphatic GSLs as the biosynthesis of the aliphatic GSL is more sensitive to sulfur-deficient conditions (Mailer, 1989). These results indicate that the sulfur status the plants are grown under, can indeed influence the rhythmic oscillations of the GSLs. This needs to be further investigated for example by reducing the sulfur supply even further or increasing the sulfur supply verifying the nature of the ultradian rhythms. Regarding the dependence of the oscillations in the content of the GSLs on the sulfur supply, the time of application of sulfur to the plants might also be a factor influencing the oscillations. This needs to be analyzed as well in further experiments. Nevertheless, so far only in the study by Rosa et al. (1998) ultradian oscillations in the content of GSLs in *B. oleracea* were reported. Diurnal oscillations in the content of the GSLs were already shown in *A. thaliana* (Huseby et al., 2013) where the total GSL content was increased during the day which is contradictory to measurement of the GLSs in *B. oleracea* as they accumulated in the night (Rosa et al., 1998). These different outcomes were reasoned due to different developmental stages of the plants. This is indeed a criterion when measuring the GSL content in plants. It was already shown in *B. napus* that the content of individual GSL can vary during the development (Clossais-Besnard and Larher, 1991; Fieldsend and Milford, 1994). Besides the temperature and the developmental stage the quality and intensity of light dependent on the genotype can lead to altered contents of GSLs as it was shown in *B. oleracea* (Pérez-Balibrea et al., 2008; Björkman et al., 2011). Interestingly, a circadian regulated accumulation of GLSs was already reported in *A. thaliana* and cabbage disks entrained to a 12 h light/ 12 h dark rhythm. Under continuous light elevated levels of GSLs were measured at the beginning of the subjective day and decreased amounts were measured in the subjective night (Goodspeed et al., 2013). Nevertheless, for the GSL content in our study oscillations in the content were comparable to ultradian oscillations, which were highly dependent on the sulfur status.

4.6. Regulation of the GSL biosynthesis seems to be more complex in *B. napus*

The expression analysis in this study did not include genes of the GSL biosynthesis. The biosynthesis of GSL in *A. thaliana* is well understood and a number of regulatory key genes have been identified so far (Sonderby et al., 2010). As genes in *B. napus* underwent duplications and subsequent functional divergence transferring the knowledge on *B. napus* might be problematic and regulation of the GSL biosynthesis might be more complex than in *A. thaliana* or another diploid *Brassica* spp. As a consequence multiple copies of homologues in *B. napus* are present and may exhibit a different expression pattern and functional divergence. As an example, for the transcript factor *MYB28* regulating the biosynthesis of aliphatic GSL there are actually six homologues in the genome of *B. napus* and four in *Brassica juncea* being highly similar in their sequence to the *AtMYB28* (Augustine et al., 2015; Long et al., 2016). However, these showed differences in their structural features exhibiting different expression patterns. When checking RNA-Seq derived data for GSL genes in *B. napus* a number of homologues could be found (data not shown). Comparing them resulted in most of the cases in different expression values regarding the sulfur status or were not even expressed at all. Thus, a brief analysis of all homologues have to be done first before appropriate expression analyses can be performed.

Therefore, microarray data from *A. thaliana* was used to compare the oscillations in the expression of key genes involved in the biosynthesis of the GSLs with the oscillations in the content of GSL measured in our study. Looking at the fluctuations in the microarray analysis in *A. thaliana* (Mockler et al., 2007) for key genes involved in the biosynthesis of aliphatic GSL most of the genes were mainly up-regulated in the light phase and down-regulated in the night. These changes were highly affected by different light/dark conditions as well as for long and short days. The expression of the transcription factor *MYB28* showed an up-regulation in the dark phase whereas this was only prominent in plants exposed to LL conditions. This was also the case when comparing the expression of genes involved in the biosynthesis of indolic and benzenic GSLs where the genes were highly affected also under light conditions. For example, *AtSOT16* and *SUR1* showed under light/dark conditions an up-regulation in the light phase whereas under LL conditions they were up-regulated in the dark phase instead. To summarize, the oscillations for the GSLs measured in our study are not comparable to the microarray of *A. thaliana* derived data for the corresponding genes.

4.7. The relationship of the time-dependent susceptibility in *B. napus* and the sulfur metabolism

That the defense response in plants is under circadian control was already shown in the model plant *A. thaliana* (Bhardwaj et al., 2011; Ingle et al., 2015). In the study from Bhardwaj et al. (2011) the susceptibility to the bacterial pathogen *Pseudomonas syringae* could be demonstrated as circadian controlled. In the morning, plants showed highest resistance and thus decreased bacterial growth compared to plants infected in the evening with an increase of bacterial growth under free-running conditions. This could also previously be demonstrated for infecting *A. thaliana* with the fungus *Botrytis cinerea* where the susceptibility towards the fungus was influenced by the circadian regulated jasmonate signaling pathway (Ingle et al., 2015). Therefore, a possible dependence of the susceptibility on the daytime in *B. napus* to the fungus *V. longisporum* was analyzed. Thus, plants were infected at different time points and additionally grown under sufficient sulfur supply as well as under sulfur-deficient conditions for enhancing the susceptibility (Fig. 1A). In our study this could not be determined properly as no significant differences among infected and non-infected plants and therefore, no differences between the infection time points could be observed. Preliminary qPCR data using primer for the ITS regions surrounding the 5.8S rDNA specific for *V. longisporum* were used based on previous studies (Eynck et al., 2007; Reusche et al., 2013). As the amount of fungal DNA might have been too low or not present at all in the plants harvested, detection by qPCR was not successful (data not shown). A promising candidate for the proof of successful infection was PR2 as this was only up-regulated in infected plants according to RNA-Seq derived data (data not shown). The *PR2* encodes a β-1,3-glucanase which is proposed to be involved in the degradation of the cell walls of invading fungal pathogens by catalyzing endo-type hydrolytic cleavage of the 1,3-ß-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger and Meins, 1999). It was also reported that the PR2 protein is involved in the generation of elicitors inducing defense responses (van Loon et al., 2006). Furthermore, *PR2* is suggested to function as a modulator of callose- and SA-dependent defense response (Oide et al., 2013). However, detection of PR2 by Northern blot analysis in plants 7 dpi (Fig. 6) could not determine the infection clearly as transcripts could also be determined in control plants. It can be postulated that *PR2* might be up-regulated at an early developmental stage as part of the defense system even in the absence of an infection. The samples used for the RNA-Seq analysis were from plants harvested at a later developmental stage (Weese et al., 2015). For the samples the *PR2* was only detectable in infected plants (data not shown).

Consequently, the infection itself might not have been successful or the infection rate was too low. Nevertheless, for plants harvested 7 dpi differences between non-infected and infected plants could be observed. Levels of cysteine and GSH have been suggested as markers for the elevated activity of primary sulfur metabolism after pathogen infection (Kruse et al., 2007). In experiments where *A. thaliana* plants grown under 50 or 500 µM sulfate supply were sprayed with *Alternaria brassicola* their contents increased upon infection until 7 dpi under both sulfate regimes. This was not the case for the results in our study, as in plants grown under sulfur-sufficient conditions supply no differences in the thiol content between the infected and non-infected plants were determined (Fig. 8). Under sulfur-deficient conditions differences were measured, but without a clear trend regarding the infection time point. If there is a higher susceptibility of *B. napus* against *V. longisporum* in the night, one would expect a higher degree of accumulation in the GSH content due to a higher infection rate. Thus expression of genes involved in the sulfur assimilation would be increased as well. This could not be determined in this study properly (Fig. 6). Furthermore, accumulating thiols would require a higher degree of uptake of sulfate compared to plants infected in the morning, fulfilling the demand for an efficient defense response. Expression analysis of *Sultr4;2* in this study would support this assumption as in plants infected in the evening and grown under sulfur-deficient conditions transcripts of the transporter could not be detected anymore. This can be correlated with the total sulfur amount measured in leaves 7 dpi where higher amounts compared to non-infected plants were measured (Fig. 7). Therefore, it can be postulated that the efflux of sulfate from the vacuole would not be necessary anymore leading consequently to a decrease of the transcript amount of the transporter *Sultr4;2*.

The plant secondary metabolites are also involved in the plant defense (reviewed in Bloem et al., 2014). Among them the GSLs, only present in the Brassicales, are involved in more than one way. The breakdown products exhibit anti-fungal effects (Brown and Morra, 1997). Thus one would assume that in infected plants the amount of GSLs would be decreased and might correlate with the rate of infection. This is contradictory to the measurements of the GSLs in our study (Fig. 9). Upon infection the GSLs were increased in the plants. It can be postulated that accumulation of the GSLs and therefore, an up-regulation of the genes involved in the biosynthesis might be involved in an early defense response of *B. napus* to the infection with the fungus *V. longisporum.* In an infection experiment with *A. thaliana* and *Sclerotinia sclerotiorum* genes involved in the GSL biosynthesis were up-regulated upon infection and elevated levels of aliphatic and indolic GSLs content could already be measured 48 h post-inoculation in infected plants (Stotz et al., 2011). Furthermore, in a study, where *Brassica rapa*

was treated with different signal molecules such as methyl jasmonate and jasmonic acid (JA) the content of indolic as well as well as of aliphatic GSLs was increased (Wiesner et al., 2013). As the JA is involved in the innate immunity in plants it is likely that upon infection the biosynthesis of the GSLs is up-regulated by the JA (Robert-Seilaniantz et al., 2011) An increase in the indolic GSLs after infection is likely to occur as these act as signal for the callose deposition (Clay et al., 2009). Regarding the differences in the measured contents for the GSLs for the different infection time points in plants grown under sufficient-sulfur supply most prominent differences were determined at 12 h as here the content of the GSLs was increased in a higher degree in infected plants compared to the other infection time points. Under sulfur-deficient conditions, however, infecting plants at 16 h resulted in a higher degree of accumulation of the GSLs compared to non-infected plants.

Results for this experiment gave only hints that there might be a dependence of the susceptibility on the time point of infection. One could suggest from the results that plants infected at 16 h were more susceptible or resistant as here the total sulfur and the GSH content as well as some GSLs accumulated in a higher degree compared to the other infection time points. Determining an increased or decreased susceptibility based on the data obtained in this study were not successful. Therefore, the experiment needs to be repeated as no final conclusions could be drawn yet. Before a reliable detection method need to be developed enabling the quantification of the fungus at an early infection stage, either by a PCR method or microscopically. Probably a more efficient infection system has to be applied as well. An option would be the use of a non-resistant variety to obtain larger differences between infected and non-infected plants. Although the variety Genie shows a *V. longisporum* resistance ranked from middle to good in a previous study this variety was next to Exocet more affected by the infection (Weese et al., 2015). Results clearly showed a reaction due to the infection with *V. longisporum* such as the decreased biomass and altered physiological parameters such as the leaf temperature as well as the accumulation of sulfur-containing metabolites. However, results obtained from the study described in Weese et al. (2015) could not be reproduced.

Conclusions

A growth system for *B. napus* was established to investigate circadian aspects in the sulfur metabolism. In this study the circadian period of the clock in *B. napus* plants entrained to a 12 light/ 12 dark rhythm was 23 h, probably as an adaption to the temperature the plants were grown under. We were able to show diurnal oscillations of genes involved in the transport and reduction of the sulfate with a period comparable to that of *CCA1*. As under free-running

conditions the amplitude was lowered and a shift in the phase was determined the circadian control could not be determined unambiguously. The same could be shown for the GSH content measured in the plants. The use of continuous light in this study might have underestimated circadian oscillations as it might have resulted in a stress response affecting the sulfur metabolism in the plants. The GSLs showed ultradian oscillations which were altered by the sulfur supply the plants were grown under. Probably the concentration of single GSLs is not regulated by the circadian clock but in an ultradian way. The infection experiment needs to be repeated as the infection rate was too low to be detected and to cause major significant transcriptomic and metabolic changes. The analysis of mutants or transgenes in key genes in cysteine and GSH biosynthesis could clarify whether contents of sulfur-containing metabolites are only regulated by the circadian clock because they need reducing equivalents produced in photosynthesis or whether they act as signal molecules.

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References

Angus, J. F., Gardner, P. A., Kirkegaard, J. A., and Desmarchelier, J. M. (1994). Biofumigation: isothiocyanates released from *Brassica* roots inhibit growth of the take-all fungus. *Plant Soil*, 162(1), 107–112. http://doi.org/10.1007/BF01416095

Anderson, D. R., and Burnham, K. P. (2002). Avoiding pitfalls when using information-theoretic methods. *JWM*, 912-918.

Augustine R., Majee M., Gershenzon J., and Bisht N. C. (2013). Four genes encoding MYB28, a major transcriptional regulator of the aliphatic glucosinolate pathway, are differentially expressed in the allopolyploid *Brassica juncea*. *J. Exp. Bot*. 2013; doi:10.1093/jxb/ert280

Beinert, H. (2000). A tribute to sulfur. *Eur. J. Biochem.* 267:5657–5664. doi:10.1046/j.1432-1327.2000.01637.x

Bhardwaj, V., Meier, S., Petersen, L. N., Ingle, R. A., and Roden, L. C. (2011). Defence responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. *PLoS ONE*, 1–8. http://doi.org/10.1371/journal.pone.0026968

Björkman, M., Klingen, I., Birch, A. N. E., Bones, A. M., Bruce, T. J. A., Johansen, T. J., Meadow R., Molmann, J., Seljasen, R., Smart, L. E., and Stewart, D. (2011). Phytochemicals of Brassicaceae in plant protection and human health-influences of climate, environment and agronomic practice. *Phytochemistry*, 72(7), 538–56. http://doi.org/10.1016/j.phytochem.2011.01.014

Blake-Kalff, M. M., Harrison, K. R., Hawkesford, M. J., Zhao, F. J., and McGrath, S. P. (1998). Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. *Plant Physiol.*, 118(4), 1337–1344. http://doi.org/10.1104/pp.118.4.1337

BLAST: Basic Local Alignment Search Tool. Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed 09 November 2016.

Bloem, E., Riemenschneider, A., Volker, J., Papenbrock, J., Schmidt, A., Salac, I., Haneklaus, S., and Schnug, E. (2004). Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus L. J. Exp. Bot.*, 55(406), 2305–12. http://doi.org/10.1093/jxb/erh236

Bloem, E., Haneklaus, S., Kesselmeier, J., and Schnug, E. (2012). Sulfur fertilization and fungal infections affect the exchange of H_2S and COS from agricultural crops. *J. Agr. Food Chem.*, 60(31), 7588–96. http://doi.org/10.1021/jf301912h

Bloem, E., Haneklaus, S., and Schnug, E. (2014). Milestones in plant sulfur research on sulfur-induced-resistance (SIR) in Europe. *Front. Plant Sci.*, 5, p.779. doi:10.3389/fpls.2014.00779

Bolouri Moghaddam, M. R., and den Ende, W. V. (2013). Sweet immunity in the plant circadian regulatory network. *J. Exp. Bot.*, pp.1439–1449. doi:10.1093/jxb/ert046

Brassica napus Genome Browser. Available: http://www.genoscope.cns.fr/brassicanapus/ Accessed 9 November 2016

Brown, P. D., and Morra, M. J. (1996). Hydrolysis products of glucosinolates in *Brassica napus* tissues as inhibitors of seed germination. *Plant Soil*, 181(2), 307–316. http://doi.org/10.1007/BF00012065

Buchner, P., Stuiver, C. E. E., Westerman, S., Wirtz, M., Hell, R., Hawkesford, M. J., and De Kok, L. J. (2004). Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulfate nutrition. *Plant Physiol.*, 136(2), 3396–408. http://doi.org/10.1104/pp.104.046441

Cao, M.-J., Wang, Z., Wirtz, M., Hell, R., Oliver, D. J., and Xiang, C.-B. (2013). SULTR3;1 is a chloroplast-localized sulfate transporter in *Arabidopsis thaliana*. *Plant J.*, 73(4), 607–16. http://doi.org/10.1111/tpj.12059

Chiasson, D. M., Loughlin, P. C., Mazurkiewicz, D., Mohammadidehcheshmeh, M., Fedorova, E. E., Okamoto, M., McLean E., Glass A. D. M., Smith, S. E., Bisseling, T., Tyerman, S. D., Day, D. A., and Kaiser, B. N. (2014). Soybean *SAT1* (*Symbiotic Ammonium Transporter 1*) encodes a bHLH transcription factor involved in nodule growth and NH_4^+ transport. *Proc. Natl. Acad. Sci. USA*, 111(13), 4814–9. http://doi.org/10.1073/pnas.1312801111

CLC Workbench. Available: http://www.clcbio.com/ Accessed 9 November 2016

Clay, N.K., Adio, A.M., Denoux, C., Jander, G., and Ausubel, F.M. (2009). Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science*, 323(5910), pp.95–101. doi:10.1126/science.1164627

Clossais-Besnard, N., and Larher, F. (1991). Physiological role of glucosinolates in *Brassica napus*. Concentration and distribution pattern of glucosinolates among plant organs during a complete life cycle. *J. Sci. Food Agric.*, 56: 25–38. doi:10.1002/jsfa.2740560104

Cooper, R. M., Resende, M. L. V., Flood, J., Rowan, M. G., Beale, M. H., and Potter, U. (1996). Detection and cellular localization of elemental sulfur in disease-resistant genotypes of *Theobroma cacao*. *Nature*, 379: 159-162. http://doi.org/10.1038/379159a0

Computional Biology and Functional Genomics Laboratory: Available http://compbio.dfci.harvard.edu/compbio Accessed 9 November 2016

Covington, M. F., Maloof, J. N., Straume, M., Kay, S. A., and Harmer, S. L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biol.*, 9, R130. http://doi.org/10.1186/gb-2008-9-8-r130

Davuluri, R. V., Sun, H., Palaniswamy, S. K., Matthews, N., Molina, C., Kurtz, M., and Grotewold, E. (2003). AGRIS: Arabidopsis gene regulatory information server, an information resource of Arabidopsis cis-regulatory elements and transcription factors. *BMC Bioinformatics*, 4(1), 25.

Dämmgen,U.,Walker, K., Grünhage, L., and Jäger, H.-J. (1998). "The atmospheric sulphur cycle," in *Sulphur in Agroecosystems*, eds E. Schnug, R. F. Hüttl, and H. Beringer (Dordrecht: Kluwer), 75–114.

Del Carmen M.-B., M., Moreno, D., and Carvajal, M. (2013). The physiological importance of glucosinolates on plant response to abiotic stress in *Brassica*. *Int. J. Mol. Sci.*, 14(6), 11607–11625. http://doi.org/10.3390/ijms140611607

Dodd, A. N., Kusakina, J., Hall, A., Gould, P. D., and Hanaoka, M. (2014). The circadian regulation of photosynthesis. *Photosynth. Res.*, 119(1-2), 181–90. http://doi.org/10.1007/s11120-013-9811-8

Dodd, A. N., Salathia, N., Hall, A., Kévei, E., Tóth, R., Nagy, F., Hibberd J. M., Millar A. J., and Webb, A. A. R. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science*, 309(July), 630–634.

DOSBox. Available: http://www.dosbox.com/ Accessed 9 November 2016

DSV: Deutsche Saatveredelung Available: http://www.dsv-saaten.de/raps/winterraps/sorten/genie.html Accessed: 9 November 2016

Dunker, S., Keunecke, H., Steinbach, P., and von Tiedemann, A. (2008). Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *J. Phytopathol.*, 156(11-12), 698–707. http://doi.org/10.1111/j.1439-0434.2008.01429.x

Edwards, J. and Hertel, K. (2011). Vegetative Growth. *Canola growth & development.* 2, pp.33-49. ISBN 978 1 74256 212 4

Eynck, C., Koopmann, B., Grunewaldt-Stoecker, G., Karlovsky, P., and von Tiedemann, A. (2007). Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. *Eur. J. Plant Pathol.*, 118(3), pp.259–274. doi:10.1007/s10658-007-9144-6

Eynck, C., Koopmann, B., Karlovsky, P., and von Tiedemann, A. (2009). Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology*, 99(43), pp.802–811. doi:10.1094/PHYTO-99-7-0802

Farré, E. M. (2012). The regulation of plant growth by the circadian clock. *Plant Biol.*, 14(3), 401–410. http://doi.org/10.1111/j.1438-8677.2011.00548.x

FastQC. Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ Accessed 9 November 2016

Fieldsend, J. and Milford, G. F. J. (1994). Changes in glucosinolates during crop development in single- and double-low genotypes of winter oilseed rape (*Brassica napus*): I. Production and distribution in vegetative tissues and developing pods during development and potential role in the recycling . *Ann. Appl. Biol.*, 124(3), 531–542. http://doi.org/10.1111/j.1744-7348.1994.tb04157.x

Filichkin, S. A., Breton, G., Priest, H. D., Dharmawardhana, P., Jaiswal, P., Fox, S. E., Michael T. P., Chory J., Kay, S. A., and Mockler, T. C. (2011). Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and cis-regulatory modules. *PloS ONE*, 6(6), e16907. http://doi.org/10.1371/journal.pone.0016907

Fowler, S. G., Cook, D., and Thomashow, M. F. (2005). Low temperature induction of *Arabidopsis CBF1*, *2*, and *3* is gated by the circadian clock. *Plant Physiol.*, 137(3), 961–968. http://doi.org/10.1104/pp.104.058354

Foyer, C.H. and Halliwell, B. (1976). The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta*, 133(1), pp.21–5. doi:10.1007/BF00386001

Galant, A., Preuss, M. L., Cameron, J. C., and Jez, J. M. (2011). Plant glutathione biosynthesis: diversity in biochemical regulation and reaction products. *Front. Plant Sci.*, 2, 45. http://doi.org/10.3389/fpls.2011.00045

Gelanalyzer: Available: http://gelanalyzer.com/ Accessed 9 November 2016

Gigolashvili, T., Geier, M., Ashykhmina, N., Frerigmann, H., Wulfert, S., Krueger, S., Mugford, S.G., Kopriva, S., Haferkamp, I., and Flügge, U.-I. (2012). The *Arabidopsis* thylakoid ADP/ATP carrier TAAC has an additional role in supplying plastidic phosphoadenosine 5'-phosphosulfate to the cytosol. *Plant Cell*, 24(10), pp.4187–204. doi:10.1105/tpc.112.101964 .

Goodspeed, D., Liu, J. D., Chehab, E. W., Sheng, Z., Francisco, M., Kliebenstein, D. J., and Braam, J. (2013). Postharvest circadian entrainment enhances crop pest resistance and

phytochemical cycling. *Curr. Biol.* 23: 1–7. http://dx.doi.org/10.1016/j.cub.2013.05.034

Gutiérrez, R. A., Stokes, T. L., Thum, K., Xu, X., Obertello, M., Katari, M. S., Tanurdzic, M., Dean, A., Nero, D. C., McClung, R., and Coruzzi, G. M. (2008). Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene *CCA1*. *Proc. Natl. Acad. Sci. USA* , 105(12), 4939–44. http://doi.org/10.1073/pnas.0800211105

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., and Kay, S. (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science*, 290(5499), 2110–2113. http://doi.org/10.1126/science.290.5499.2110

Harmer, S. L. (2009). The circadian system in higher plants. *Annu. Rev. Plant Biol.*, 60, 357–77. http://doi.org/10.1146/annurev.arplant.043008.092054

Harmon, F., Imaizumi, T., and Kay, S. (2005). The plant circadian clock: review of a clockwork *Arabidopsis*. *Annual plant reviews* 21, 1–23. http://doi.org/10.1002/9780470988527

Harris, P., Scullion, T., Sebesta, S. and Claras, G. (2003). Measuring sulfate in subgrade soil: difficulties and triumphs. *Transp. Res. Rec. J. Transp. Res. Board*, 1837, pp.3–11. doi:10.3141/1837-01 .

Hawkesford, M. J., and De Kok, L. J. (2006). Managing sulphur metabolism in plants. *Plant Cell Environ.*, 29(3), 382–395. http://doi.org/10.1111/j.1365-3040.2005.01470.x

Hawkesford, M. J., and Wray, J. L. (2000). Molecular genetics of sulphate assimilation. *Adv. Bot. Res.*, 33, 159–223. http://doi.org/10.1016/S0065-2296(00)33043-9

Haydon, M. J., Bell, L. J., and Webb, A. a R. (2011). Interactions between plant circadian clocks and solute transport. *J. Exp. Bot.*, 62(7), 2333–2348. http://doi.org/10.1093/jxb/err040

Haydon, M. J., Román, Á., and Arshad, W. (2015). Nutrient homeostasis within the plant circadian network. *Front. Plant Sci.*, 6, 1-6. http://doi.org/10.3389/fpls.2015.00299

Heale, J. B., and Karapapa, V. K. (1999). The verticillium threat to canada's major oilseed crop: canola. *Can. J. Plant Pathol.*, 21(1), 1–7. http://doi.org/10.1080/07060661.1999.10600114

Hell R. (1997). Molecular physiology of plant sulfur metabolism. *Planta*, 202: 138–148.

Hurvich, C. M., and Tsai, C. L. (1989). Regression and time series model selection in small samples. *Biometrika*, 76(2), 297-307.

Huseby, S., Koprivova, A., Lee, B.-R., Saha, S., Mithen, R., Wold, A.-B., Bengtsson, G. B.,

and Kopriva, S. (2013). Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in *Arabidopsis*. *J. Exp. Bot.*, 64(4), 1039–48. http://doi.org/10.1093/jxb/ers378

Iijima, M. and Matsushita, N. (2011). A circadian and an ultradian rhythm are both evident in root growth of rice. J. Plant Physiol., 168(17), pp.2072–80. doi:10.1016/j.jplph.2011.06.005 .

InfoStat. Available: http://www.infostat.com.ar/?lang=en Accessed 9 November 2016

Ingle, R. A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L. C., and Denby, K. J. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of *Arabidopsis* to the fungal pathogen *Botrytis cinerea*. *Plant J.* 84(5), 937-948. http://doi.org/10.1111/tpj.13050.

Jambunathan, N. (2010). Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. *Methods Mol. Biol.*, 639, pp.292–8. doi:10.1007/978-1-60761-702-0_18

Kataoka T., Watanabe-Takahashi A., Hayashi N., Ohnishi M., Mimura T., Buchner P., Hawkesford M. J., Yamaya T., and Takahashi, H. (2004).Vacuolar sulfate transporters are essen- tial determinants controlling internal distribution of sulfate in *Arabidopsis*. *Plant Cell* 16:2693–2704. doi:10. 1105/tpc. 104. 023960

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J. (2001). Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol.*, 126, 811–825.

Kerwin, R. E., Jimenez-Gomez, J. M., Fulop, D., Harmer, S. L., Maloof, J. N., and Kliebenstein, D. J. (2011). Network quantitative trait loci mapping of circadian clock outputs identifies metabolic pathway-to-clock linkages in *Arabidopsis*. *The Plant Cell*, 23(2), 471–85. http://doi.org/10.1105/tpc.110.082065

Kim, J.-Y., Song, H.-R., Taylor, B. L., and Carré, I. A. (2003). Light-regulated translation mediates gated induction of the *Arabidopsis* clock protein LHY. *EMBO J.*, 22(4), 935–44. http://doi.org/10.1093/emboj/cdg075

Kocsy, G., Owttrim, G., Brander, K., and Brunold, C. (1997). Effect of chilling on the diurnal rhythm of enzymes involved in protection against oxidative stress in a chilling-tolerant and a chilling-sensitive maize genotype. *Physiol. Plant.*, 99(2), 249–254. http://doi.org/10.1111/j.1399-3054.1997.tb05409.x

Kok, L. J. De, Westerman, S., Stuiver, C. E. E., and Stulen, I. (2000). Atmospheric H_2S as plant sulfur source: Interaction with pedospheric sulfur nutrition - A case study with

Brassica oleracea L. Sulfur Metabolism in Higher Plants 41–55.

Kopriva, S., Mugford, S. G., Matthewman, C., and Koprivova, A. (2009). Plant sulfate assimilation genes: redundancy versus specialization. *Plant Cell Rep.*, 28(12), 1769–80. http://doi.org/10.1007/s00299-009-0793-0

Kopriva, S., Muheim, R., Koprivova, A., Trachsel, N., Catalano, C., Suter, M., and Brunold, C. (1999). Light regulation of assimilatory sulphate reduction in *Arabidopsis thaliana*. *Plant J.*, 20(1), 37–44. http://doi.org/10.1046/j.1365-313X.1999.00573.x

Kruse, C., Jost, R., Lipschis, M., Kopp, B., Hartmann, M., and Hell, R. (2007). Sulfur-enhanced defence: effects of sulfur metabolism, nitrogen supply, and pathogen lifestyle. *Plant Biol.*, 9(5), 608–19. http://doi.org/10.1055/s-2007-965432

Kruse C., Haas F.H., Jost R., Reiser B., Reichelt M., Wirtz M., Gershenzon J., Schnug, E., and Hell, R. (2012). Improved sulfur nutrition provides the basis for enhanced production of sulfur-containing defense compounds in *Arabidopsis thaliana* upon inoculation with *Alternaria brassicicola*. *J. Plant Physiol*., 169: 740–3. doi:10.1016/j.jplph.2011.12.017

Kusakina, J., Gould, P. D., and Hall, A. (2014). A fast circadian clock at high temperatures is a conserved feature across *Arabidopsis* accessions and likely to be important for vegetative yield. *Plant Cell Environ.*, 37(2), 327-340

Lai, A. G., Doherty, C. J., Mueller-Roeber, B., Kay, S. A., Schippers, J. H. M., and Dijkwel, P. P. (2012). *CIRCADIAN CLOCK-ASSOCIATED 1* regulates ROS homeostasis and oxidative stress responses. *Proc. Natl. Acad. Sci. USA* , 109(42), 17129–34. http://doi.org/10.1073/pnas.1209148109

Legnaioli, T., Cuevas, J., and Mas, P. (2009). TOC1 functions as a molecular switch connecting the circadian clock with plant responses to drought. *EMBO J.*, 28(23), 3745–57. http://doi.org/10.1038/emboj.2009.297

Leubner-Metzger, G. and Meins Jr, F. (1999). Functions and Regulation of Plant β-(PR-2). in *Pathogenesis-related proteins in plants.* eds Datta, S. K. and Muthukrishnan S. (CRC Press) Chapter 3.

Leustek, T., Martin, M. N., Bick, J.-A., and Davies, J. P. (2000). Pathway and regulation of sulfur Metabolism revealed through molecular and genetic studies. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51, 141–165. http://doi.org/10.1146/annurev.arplant.51.1.141

Lewandowska, M., and Sirko, A. (2008). Recent advances in understanding plant response to sulfur-deficiency stress. *Acta Bioch. Pol.*, 55(3), 457–471. http://doi.org/20081696 [pii]

Long, Y., Wang, J., Wang, Y., Zhang, J., Wang, J., and Pei, X. (2016). Comparative analysis of *MYB28* homologs and development of a *MYB28*-specific marker in *Brassica napus L. Mol. Breed.*, 36(9), p.126. doi:10.1007/s11032-016-0551-2

Lou, P., Xie, Q., Xu, X., Edwards, C. E., Brock, M. T., Weinig, C., and McClung, C. R. (2011). Genetic architecture of the circadian clock and flowering time in *Brassica rapa*. *Theor. and Appl. Gen.*, 123(3), 397-409.

Loudet, O., Saliba-Colombani, V., Camilleri, C., Calenge, F., Gaudon, V., Koprivova, A., North, K.A., Kopriva, S., and Daniel-Vedele, F. (2007). Natural variation for sulfate content in *Arabidopsis thaliana* is highly controlled by APR2. *Nat. Genet.*, 39(7), pp.896–900. doi:10.1038/ng2050

Makino, S. (2002). The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: I. characterization with APRR1-overexpressing plants. *Plant Cell Physiol.*, 43(1), pp.58–69. doi:10.1093/pcp/pcf005

Mcclung, C. R. (2006). Plant circadian rhythms. *Plant Cell*, *18*, 792–803. http://doi.org/10.1017/CBO9781107415324.004

Michael, T. P. and McClung, C. R. (2003). Enhancer trapping reveals widespread circadian clock transcriptional control in *Arabidopsis*. *Plant Physiol.*, *132*(2), 629–39. http://doi.org/10.1104/pp.021006

Michael, T.P., Mockler, T.C., Breton, G., McEntee, C., Byer, A., Trout, J.D., Hazen, S.P., Shen, R., Priest, H.D., Sullivan, C.M., Givan, S.A., Yanovsky, M., Hong, F., Kay, S.A., and Chory, J. (2008). Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genet.*, 4(2), p.e14. doi:10.1371/journal.pgen.0040014

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.-R., Carré, I.A., and Coupland, G. (2002). *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev. Cell*, 2(5), pp.629–641. doi:10.1016/S1534-5807(02)00170-3

Mizuno, T. and Nakamichi, N. (2005). Pseudo-response regulators (PRRs) or true oscillator components (TOCs). *Plant Cell Physiol.*, 46(5), pp.677–685. doi:10.1093/pcp/pci087

Mockler, T.C., Michael, T.P., Priest, H.D., Shen, R., Sullivan, C.M., Givan, S.A., McEntee, C., Kay, S.A., and Chory, J. (2007). The diurnal project: diurnal and circadian expression profiling, model-based pattern matching, and promoter analysis. *Cold Spring Harb. Symp. Quant. Biol.*, 72(0), pp.353–63. doi:10.1101/sqb.2007.72.006

Murphy, R.L., Klein, R.R., Morishige, D.T., Brady, J.A., Rooney, W.L., Miller, F.R., Dugas, D. V, Klein, P.E., and Mullet, J.E. (2011). Coincident light and clock regulation of *pseudoresponse regulator protein 37* (*PRR37*) controls photoperiodic flowering in sorghum. *Proc. Natl. Acad. Sci. U. S. A.*, 108(39), pp.16469–74. doi:10.1073/pnas.1106212108

Nagel, D. H., Doherty, C. J., Pruneda-Paz, J. L., Schmitz, R. J., Ecker, J. R., and Kay, S. A. (2015). Genome-wide identification of CCA1 targets uncovers an expanded clock network in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, 112(34), E4802–10. http://doi.org/10.1073/pnas.1513609112

Noctor, G., Arisi, A.-C. M., Jouanin, L., Valadier, M.-H., Roux, Y., and Foyer, C. H. (1997). Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing γ-glutamylcysteine synthetase. *Planta*, 202(3), pp.357–369. http://doi.org/10.1007/s004250050138

Oide, S., Bejai, S., Staal, J., Guan, N., Kaliff, M., and Dixelius, C. (2013). A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *New Phytol.*, 200(4), pp.1187–99. doi:10.1111/nph.12436

Palaniswamy, S. K., James, S., Sun, H., Lamb, R. S., Davuluri, R. V., and Grotewold, E. (2006). AGRIS and AtRegNet. A platform to link cis-regulatory elements and transcription factors into regulatory networks. *Plant Physiol.*, 140(3), pp.818-829.

Parmar, S., Buchner, P., and Hawkesford, M. J. (2007). Leaf developmental stage affects sulfate depletion and specific sulfate transporter expression during sulfur deprivation in *Brassica napus* L. *Plant Biol.*, 9(5), pp.647–53. http://doi.org/10.1055/s-2007-965428

Pérez-Balibrea, S., Moreno, D.A., and García-Viguera, C. (2008). Influence of light on health-promoting phytochemicals of broccoli sprouts. *J. Sci. Food Agric.*, 88(5), pp.904–910. doi:10.1002/jsfa.3169

Quackenbush, J., Liang, F., Holt, I., Pertea, G., and Upton, J. (2000). The TIGR gene indices: reconstruction and representation of expressed gene sequences. *Nucleic Acids Res.*, 28(1), pp.141–5.

Rausch, T. and Wachter, A. (2005). Sulfur metabolism: a versatile platform for launching defence operations. *Trends Plant Sci.*, 10(10), pp.503–9. doi:10.1016/j.tplants.2005.08.006

Redovniković, I.R., Glivetić, T., Delonga, K., and Vorkapić-Furać, J. (2008). Glucosinolates and their potential role in plant. *Period. Biol.*, 110(4), pp.297–309.

Reich, M., Aghajanzadeh, T., Helm, J., Parmar, S., Hawkesford, M.J., and De Kok, L.J. (2016). Chloride and sulfate salinity differently affect biomass, mineral nutrient composition and expression of sulfate transport and assimilation genes in *Brassica rapa*. *Plant Soil*. doi:10.1007/s11104-016-3026-7

Riemenschneider, A., Nikiforova, V., Hoefgen, R., De Kok, L.J., Papenbrock, J. (2005). Impact of elevated H_2S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. *Plant Physiol. Biochem.* 43, 473-483.

Robert-Seilaniantz A., Grant, M. and Jones, J.D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343.

RobiNA. Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ Accessed 9 November 2016

Roden, L. C. and Ingle, R. A. (2009). Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant-pathogen interactions. *Plant Cell*, 21(9), pp.2546–52.http://doi.org/10.1105/tpc.109.069922

Rosa, A.S.; Rodrigues, P.M.F. (1998).The effect of light and temperature on glucosinolate concentration in the leaves and roots of cabbage seedlings. *J. Sci. Food Agric.* 78, 208–212.

Rumlow A., Keunen E., Klein J., Pallmann P., Riemenschneider A., Cuypers A., and Papenbrock, J. (2016). Quantitative expression analysis in *Brassica napus* by Northern blot analysis and reverse transcription-quantitative PCR in a complex experimental setting. *PLoS ONE* 11(9): e0163679.doi:10.1371/journal.pone.0163679

R Core Team (2014). *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing. Available online at: http:// www.R-project.org

Salisbury, F. B. and Ross, C. W. (1992). *Plant Physiol.* (4th edn). Wadsworth, Belmont, CA, USA.

Salomé, P.A. and McClung, C.R. (2004). The *Arabidopsis thaliana* Clock. *J. Biol. Rhythms*, pp.425–435. doi:10.1177/0748730404268112

Schnug, E. and Haneklaus, S. (2005). Sulphur deficiency symptoms in oilseed rape (*Brassica napus* L.)-the aesthetics of starvation. *Phyton*, 45(3), 79.

Scherer, H.W. (2001). Sulphur in crop production — invited paper. *Eur. J. Agron.*, 14(2), pp.81–111. doi:10.1016/S1161-0301(00)00082-4

Shaw, L.M., Turner, A.S., and Laurie, D.A. (2012). The impact of photoperiod insensitive *Ppd-1a* mutations on the photoperiod pathway across the three genomes of hexaploid

wheat (*Triticum aestivum*). *Plant J.*, 71(1), pp.71–84. doi:10.1111/j.1365-313X.2012.04971.x

Solheim, B.G.B., Johnsson, A., and Iversen, T.-H. (2009). Ultradian rhythms in *Arabidopsis thaliana* leaves in microgravity. *New Phytol.*, 183(4), pp.1043–52. doi:10.1111/j.1469-8137.2009.02896.x .

Sokolovsky, V., Kaldenhoff, R., Ricci, M., and Russo, V. (1990). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. *Fungal Genet Newslett*.

Stotz, H.U., Sawada, Y., Shimada, Y., Hirai, M.Y., Sasaki, E., Krischke, M., Brown, P.D., Saito, K. and Kamiya, Y. (2011). Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of *Arabidopsis* against *Sclerotinia sclerotiorum*. *Plant J.*, 67(1), pp.81–93. doi:10.1111/j.1365-313X.2011.04578.x .

Sweeney, B. M., and Folli, S. I. (1984). Nitrate Deficiency Shortens the Circadian Period in Gonyaulax. *Plant Physiol.*, 75(1), pp.242–245. http://doi.org/10.1104/pp.75.1.242

Takahashi, H., Buchner, P., Yoshimoto, N., Hawkesford, M. J., and Shiu, S.-H. (2012). Evolutionary relationships and functional diversity of plant sulfate transporters. *Front. Plant Sci.*, 2, 119. http://doi.org/10.3389/fpls.2011.00119

Thies, W. (1979). Detection and utilization of a glucosinolate sulfohydrolase in the edible snail, *Helix pomatia*." *Naturwissenschaften* 66.7 (1979): 364-365.

Turner, A., Beales, J., Faure, S., Dunford, R. P., and Laurie, D. A. (2005). The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. *Science*, 310(5750), 1031–4. http://doi.org/10.1126/science.1117619

Van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.*, 44, 135-162.

Vauclare, P., Kopriva, S., Fell, D., Suter, M., Sticher, L., von Ballmoos, P., Krähenbühl, U., den Camp, R.O., and Brunold, C. (2002). Flux control of sulphate assimilation in *Arabidopsis thaliana* : adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. *Plant J.*, 31(6), pp.729–740. doi:10.1046/j.1365-313X.2002.01391.x

Velez-Ramirez, A. I., van Ieperen, W., Vreugdenhil, D. and Millenaar, F. F. (2011). Plants under continuous light. *Trends Plant Sci.*, 16(6), pp.310–8. doi:10.1016/j.tplants.2011.02.003

Vollmann, J. and Rajcan, I. (2009). Oil crops. *Springer* http://doi.org/10.1007/978-0-387-77594-4. ISBN: 9780387775937

Wang, Z.Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S., and Tobin, E.M. (1997). A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis Lhcb* gene. *Plant Cell*, 9(4), pp.491–507. doi:10.1105/tpc.9.4.491

Wang, W., Barnaby, J.Y., Tada, Y., Li, H., Tör, M., Caldelari, D., Lee, D., Fu, X.-D., and Dong, X. (2011). Timing of plant immune responses by a central circadian regulator. *Nature*, 470(7332), pp.110–4. doi:10.1038/nature09766.

Weese, A., Pallmann, P., Papenbrock, J., and Riemenschneider, A. (2015). *Brassica napus* L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack. *Front. Plant Sci.*, 6. doi:10.3389/fpls.2015.00009 .

Wiesner, M., Hanschen, F., Schreiner, M., Glatt, H. and Zrenner, R., (2013) Induced Production of 1-Methoxy-indol-3-ylmethyl Glucosinolate by Jasmonic Acid and Methyl Jasmonate in Sprouts and Leaves of Pak Choi (Brassica rapa ssp. chinensis). *Int. J. Mol. Sci.*, 14(7), pp.14996–15016. doi:10.3390/ijms140714996 .

Williams, J. S., Hall, S. a, Hawkesford, M. J., Beale, M. H., and Cooper, R. M. (2002). Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiol.*, 128(1), pp. 150–159. http://doi.org/10.1104/pp.010687

Yamaguchi, Y. and Sano, H. (2001). The sulfate assimilation pathway in higher plants: Recent progress regarding multiple isoforms and regulatory mechanisms. *Plant Biotechnol.*, 18(1), pp.17–25. doi:10.5511/plantbiotechnology.18.17

Yilmaz, A., Mejia-Guerra, M. K., Kurz, K., Liang, X., Welch, L., and Grotewold, E. (2011). AGRIS: the *Arabidopsis* gene regulatory information server, an update. *Nucleic Acids Res.*, 39(1), D1118-D1122

Zhao, F. J., Wood, A. P., and McGrath, S. P. (1999). Effects of sulphur nutrition on growth and nitrogen fixation of pea (*Pisum sativum* L.). *Plant Soil*, 212(2), pp.207–217. doi:10.1023/A:1004618303445

Supporting information

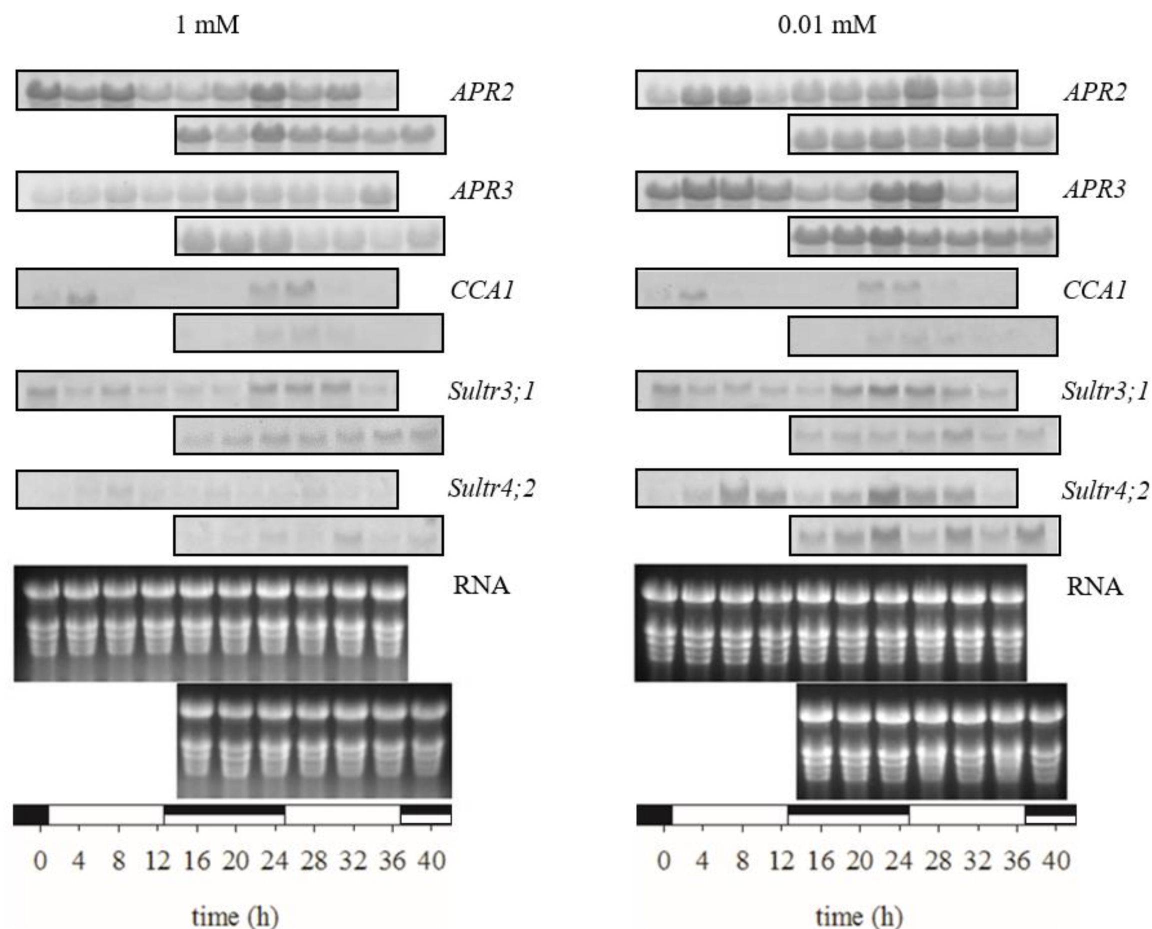

Figure S1. Northern blot analysis of the GOIs under diurnal/circadian conditions. Transcript amounts were determined in plants (with five fully expanded leaves) grown with 1 mM $MgSO_4$ as a control and 0.01 mM $MgSO_4$ for four days to obtain S-deficient conditions. Plants grown under 12 h dark/ 12 h light (LD) were harvested over a period of 36 h every 4 h starting 1 h before the onset of light (first row). In addition, plants grown in a chamber with 24 h light (LL) were parallel harvested beginning at 16 h (second row). Total RNA was isolated, and for Northern blot analysis 15 µg RNA was electrophoretically separated and transferred onto membranes. For the detection DIG labeled probes were used. Results for one technical replicate are shown. For abbreviations see Table 1.

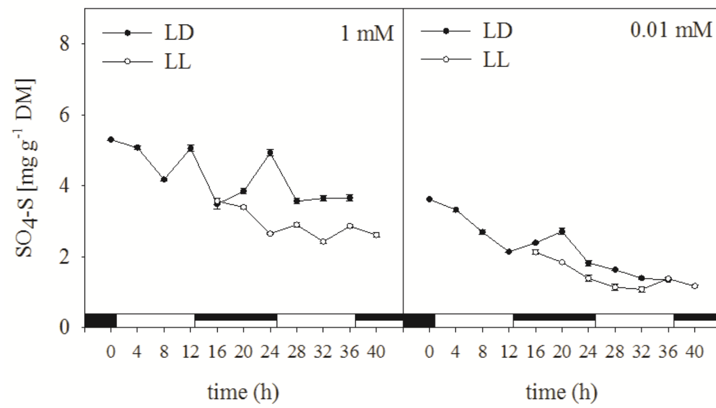

Figure S2. The content of sulfate-sulfur in the leaves under diurnal/circadian conditions. The sulfate content in the plant material treated and harvested as described for Figure 2 was determined in 500 mg freeze dried material by ion chromatography. Calculated data for the sulfate-sulfur in mg g^{-1} DW represent two technical replicates ±SD.

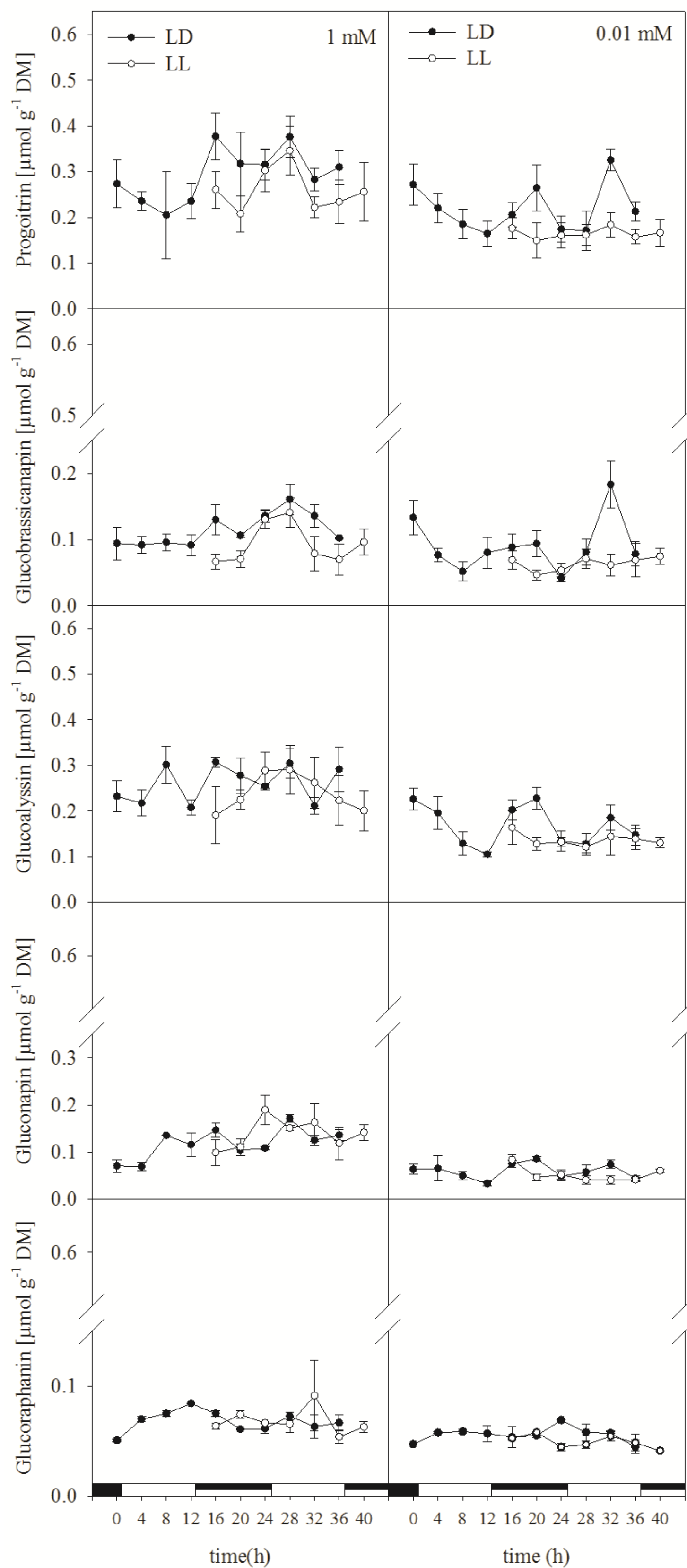

Figure S3. Individual aliphatic GSL in leaves under diurnal/circadian conditions. Individual aliphatic GSL were identified by LC-MS and quantified by HPLC measurements in plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

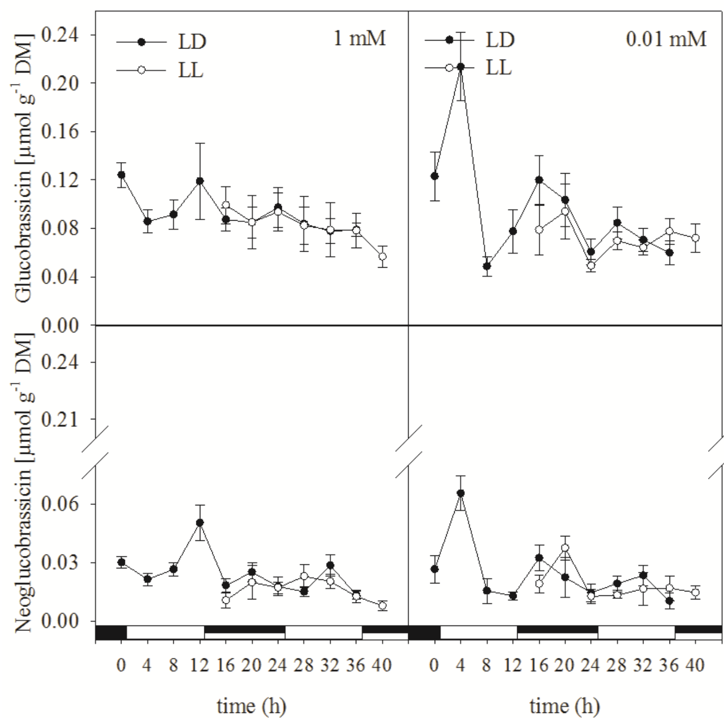

Figure S4. Individual indolic GSL in leaves under diurnal/circadian conditions. Individual indolic GSL were identified by LC-MS and quantified by HPLC measurements in plants treated and collected as described in Figure 2. The contents of the GSLs were calculated in µmol g^{-1} FM. Data represent the mean of three technical replicates ±SD.

Table S1: Two-way ANOVA analysis of the expression data and the measured sulfur-containing compounds under diurnal and circadian conditions.

	p-value		
	Sulfur status (S)	**Time point of harvest (T)**	**SxT**
APR2	0.0002	<0.0001	0.0245
APR3	<0.0001	0.0001	0.0048
CCA1	<0.0001	<0.0001	<0.0001
Sultr3;1	0.6414	<0.0001	0.4611
Sultr4;2	<0.0001	0.0001	0.0011
Total sulfur	<0.0001	0.0063	0.0281
Sulfate	<0.0001	<0.0001	<0.0001
GSH	0.0118	<0.0001	0.1556
Cysteine	0.0212	0.8352	0.4920
Aliphatic GSLs	<0.0001	0.0001	<0.0001
Indolic GSLs	0.7735	<0.0001	<0.0001
Progoitrin	<0.0001	0.0004	<0.0001
Glucoraphanin	<0.0001	0.0106	0.0535
Glucoalyssin	<0.0001	0.0002	<0.0001
Gluconapin	<0.0001	0.0057	0.0016
Glucobrassicanapin	0.0004	<0.0001	<0.0001
Glucobrassicin	0.7067	<0.0001	<0.0001
Gluconasturtiin	0.6170	<0.0001	<0.0001
Neoglucobrassicin	0.1013	<0.0001	<0.0001

Table S2: Three-way ANOVA analysis of the expression data and the measured sulfur-containing compounds under diurnal and circadian conditions.

	p-value						
	Sulfur status (S)	**Time point of harvest (T)**	**Light (L)**	**SxL**	**SxT**	**LxT**	**SxLxT**
APR2	<0.0001	<0.0001	0.0561	0.2427	0.0135	0.0007	0.2067
APR3	<0.0001	0.0217	0.0072	0.0119	0.0646	0.2059	0.0141
CCA1	<0.0001	<0.0001	<0.0001	0.0291	<0.0001	<0.0001	<0.0001
Sultr3;1	0.0723	<0.0001	<0.0001	0.6544	0.7347	<0.0001	0.3965
Sultr4;2	<0.0001	0.0183	0.2711	0.7722	0.0741	0.1772	0.1239
Total sulfur	<0.0001	0.0312	<0.0001	0.1850	0.0030	0.0938	0.6654
Sulfate	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
GSH	0.0002	0.0002	0.0018	0.1910	0.0396	0.0003	0.6312
Cysteine	0.0001	0.3206	0.1863	0.7663	0.3350	0.8574	0.7001
Aliphatic GSLs	<0.0001	0.1421	<0.0001	0.6490	0.0001	0.0212	0.0153
Indolic GSLs	0.3288	0.0066	0.1931	0.4712	0.0209	0.4621	0.2137
Progoitrin	<0.0001	0.2401	<0.0001	0.6282	0.0015	0.0321	0.0087
Glucoraphanin	0.0007	0.4794	0.5492	0.2016	0.9431	0.4455	0.4128
Glucoalyssin	<0.0001	0.6481	0.0015	0.4506	0.0196	0.0436	0.0065
Gluconapin	<0.0001	0.5670	0.5549	0.1429	0.1434	0.2888	0.0608
Glucobrassicanapin	<0.0001	0.0013	<0.0001	0.6470	<0.0001	0.0001	0.1741
Glucobrassicin	0.0757	0.0298	0.1026	0.1670	0.0004	0.2213	0.6741
Gluconasturtiin	0.4192	0.0082	0.0007	0.5925	0.0129	0.6681	0.7074
Neoglucobrassicin	0.3110	0.0001	0.4120	0.9839	0.0487	0.8406	0.1761

CHAPTER 5

General Discussion

Problems in detecting the infection in *B. napus* with *V. longisporum*

Infection of plants in general is accompanied by disease symptoms which can help detecting the infection by a certain pathogen. By using an assessment key disease symptoms as well as the progress of infection can be evaluated (Eynck et al., 2007). Typical symptoms upon infection with *V. longisporum* are wilting, stunting, chlorosis, vascular discoloration, and early senescence (Fradin and Thomma, 2006). These frequently observed symptoms cannot be found in infected *B. napus* plants under field conditions. Here only later in the growing season dark, unilateral striping on the stem appears (Heale and Karapapa, 1999). However, infecting *B. napus* by artificial root dip inoculation exhibited the typical symptoms mentioned above (Eynck et al., 2007, 2009; Zeise and Tiedemann, 2002). Therefore, by using an assessment key disease symptoms as well as the progress of infection can be evaluated (Eynck et al., 2007). Symptoms in this key were only focused on the yellowing and black veins which were absent in infected plants from the experiments described in Chapter 2, making the use of this key inappropriate. The only symptom detectable was the stunting growth of the infected plants by measuring the weight showing a decreased biomass as well as a lower shoot to root ratio. However, nowadays molecular methods have been developed for improving the accuracy and reliability detecting the infection. One of those is the PCR-based method with the internal transcribed spacer (ITS) as target DNA to amplify. This region enables the unambiguous molecular identification of fungi. The gold standard method for detecting the fungus in the plants is currently the quantitative real time PCR (qPCR). The high sensitivity and specificity of this technique enables the accurate quantification of the target pathogen. Therefore, the progress of the disease can be monitored (Garrido et al., 2009). This technique was successfully applied for detecting *V. longisporum* in *B. napus* plants (Eynck et al., 2007). After 7 dpi the fungus was already detectable in the hypocotyls of infected plants increasing steadily until 35 dpi. The detection of *V. longisporum* in the leaves was positive after 14 dpi. In this study, however, detecting the fungus by PCR-based detection of the ITS marker in the hypocotyl of infected plants from the experiments described in Chapter 2 and 4 at an early stage was not successful. Data of qPCR analysis revealed no differences between the control and infected plants. This might be explained by the use of resistant cultivars in this study. The systemic spread of *V. longisporum* in a resistant *B. napus* cultivar in comparison to a susceptible cultivar was already analyzed (Eynck et al., 2009). The authors observed that vascular occlusions and deposition of the cell wall-bound phenolics and lignin inhibited the systemic spread of the fungus. Thus detecting fungal DNA with qPCR in the hypocotyl tissues resulted in very low amounts of amplified DNA until 65 dpi. In a very recent experiment we were able to detect the fungus in the hypocotyl

of infected plants 28 dpi by qPCR analysis, whereas these plants were infected in a different way resulting probably in higher infection rates (data not shown). Based on these results the infection method should be modified and optimized for further experiments. For a distinct detection of the fungus infection experiments need to be prolonged as the fungus can only be detected in a later stage than 7 dpi. For analyzing the effect of the infection at an early stage it would be necessary to harvest only one part of the plants and let the other part grow longer. Then the infection could be detected at a later stage in the remaining plants. However, regarding the sulfur-deficient conditions the plants were grown in the experiments described in Chapter 2 and 4 it might be difficult to keep the plants alive. Another option would be to apply sulfur-deficient conditions at a later stage as it was performed for the experiments described in Chapter 3 and 4, respectively. Nevertheless, a distinct detection of the fungus may require weeks which could not be scheduled in this study for repeating the experiment described in Chapter 4. In addition, a more susceptible cultivar than the cultivars used in our studies with a resistance ranging from average to good, should be used for further experiments.

The sulfur-enhanced defense in *B. napus* against the fungus *V. longisporum* under different sulfur regimes

One aim of this study was to investigate the SED in *B. napus* in response to the infection with *V. longisporum* (Chapter 2 and 4). According to the SED plants are more susceptible under sulfur-limiting conditions. This was verified in the experiment described in Chapter 2 by chlorophyll fluorescence measurements where the photosynthesis efficiency was significantly decreased in infected plants grown under sulfur-deficient conditions. Furthermore, the reduction in biomass production was further increased by the infection under sulfur-limiting conditions. Regarding the contents of total sulfur and the sulfur containing compounds this relationship became less distinct. Therefore, it was calculated for both sulfur regimes how the content of the sulfur-containing compounds were changed with an infection and how much of the sulfur in these compounds is actually involved in the pathogen defense in relation to the total sulfur content (Table 1). In this calculation the GSLs content was included as well. The method for measuring the GSLs in *B. napus* had to be established first before the samples from the experiments described in Chapter 2 could be measured (Hornbacher, 2016). Only a small percentage of the sulfur in the measured compounds was involved in the pathogen defense (Table 1). In the cultivars Exocet and Compass the total of sulfur involved the pathogen defense accounted only for 0.51% and 0.60% under full sulfur supply. In Genie and King10 more sulfur

was involved in the pathogen defense under full supply accounting for 0.92% and 0.95% respectively. Under sulfur-limiting conditions in Compass nearly the same percentage was involved in the pathogen defense. For the other cultivars the sulfur involved in the pathogen defense was higher under sulfur-deficient conditions compared to sulfur-sufficient conditions. For Exocet the sulfur accounted for 0.68% and in King10 the percentage was doubled. In the cultivar Genie the highest percentage with 2.53% of sulfur was involved in the pathogen defense under sulfur-deficient conditions. Based on these data one would assume that the infection in Compass and Exocet is not necessarily dependent on the sulfur status the plants were grown under. Whereas for the cultivar Genie and King10 the infection is influenced by the sulfur status. The small fractions calculated are in accordance with field experiments where the infection of *B. napus* with *Pyrenopeziza brassicae* led to an increase in the cysteine-sulfur which only comprises 0.2% of the total sulfur in the leaves. The decrease of GSH-sulfur accounted for less than 1% after the infection (Salac, 2005, Haneklaus et al., 2007). Regarding the single sulfur-fractions the cultivars showed differences especially under sulfur-deficient conditions in their reaction to the fungus. For all cultivars under full sulfur supply the sulfur in the aliphatic GSLs was degraded. For the cultivar Genie and King10 the degradation comprised 0.8% and for the cultivar Exocet and Compass 0.45% of the total sulfur content respectively. Therefore, the aliphatic GSLs seems to play a major role in the pathogen defense in *B. napus* against *V. longisporum* when plants are grown with sufficient sulfur supply. The breakdown products of the aliphatic GSLs exhibit antifungal activity (Manici et al., 1997, Sarwar et al., 1998) and certain GSL breakdown products are able to inhibit the growth of *V. longisporum* (Witzel et al., 2013). This would be one explanation for the decrease in the aliphatic GSLs. Another possibility for the decrease would be the translocation of the aliphatic GSLs from the shoots to the roots as the primary infection site (Madsen et al., 2014, Witzel et al., 2015). The involvement of the breakdown products in the pathogen defense in *B. napus* in response to infection with *V. longisporum* might be clarified by including measurements of the breakdown products and GSL measurements in the roots. Under sulfur-deficient conditions the aliphatic GSLs were accumulating in infected plants except for the cultivar Compass. In the infection experiment with *A. thaliana* and *V. longisporum* from Witzel et al. (2015) the infection might have led to an inhibition of the production of breakdown products as these were reduced in their content. This could be a possible explanation for the accumulation of the aliphatic GSLs under sulfur-deficient conditions. The breakdown products from aromatic GSLs are also known to exhibit antifungal effects (Manici et al., 1997, Sarwar et al., 1998). However, gluconasturtiin was likely to be increased in infected plants under both sulfur regimes, whereas under sulfur-

deficient conditions a higher degree in the accumulation occurred. The indolic GLSs are known to be involved in the activation of the innate immunity protecting the plant against fungal penetration as possible precursor for the camalexin biosynthesis (Bednarek et al., 2009, Clay et al., 2009). Except for Compass the content of the indolic GLSs was increased after infection under full sulfur supply and accounted for less than 0.05% of the total sulfur. Due to sulfur-deficient conditions the accumulation in infected plants was further increased and accounted for a maximum in King10 of 0.46% of the total sulfur. Comparing the involvement of the sulfur in the GSLs in the pathogen defense the cultivars can be ranked in the following order starting with the cultivar with the highest percentage of GSL-sulfur: King10, Genie, Compass and Exocet. This can be correlated with the resistances in the cultivars as King10 is the least and Exocet the most susceptible cultivar (Chapter 2). As the double low cultivars are modified in their GSL content the contribution of the GSLs to plant defense was doubted. This was supported by experiments with Brassica lines differing in their GSL levels but the resistance to fungal attacks failed to correlate with high and low GSL levels (Mithen and Magrath, 1992; Mithen, 1992; Wretblad and Dixelius 2000). Results in our study clearly showed a change in the content of the GSLs in plants upon infection compared to non-infected plants and might therefore contribute to the plant defense in *B. napus* against *V. longisporum.* For all cultivars the sulfur incorporated in cysteine was likely to be increased after infection under both sulfur regimes and accounted for less than 0.04% of the total sulfur content. Regarding the involvement of cysteine in the HR an increase is likely to be occur upon infection as the metabolite (Álvarez et al., 2012). For the cultivars Genie and Exocet the GSH-sulfur was increased upon infection under full sulfur supply, whereas in Compass and King10 the sulfur in GSH was degraded. For the pathogen defense in the cultivars Genie, Exocet, and King10 the increase of the GSH-sulfur accounted for 1.4%, 0.40%, and 0.46% of the total sulfur content. In Compass the degradation of the GSH-sulfur comprised 0.02 % of the total sulfur content. The accumulation of GSH is induced by pathogen infection and rapidly accumulating after fungal attack and may act as a messenger for non-infected tissues by carrying information concerning the pathogen attack (Edwards et al., 1991; Foyer and Rennenberg, 2000). As the GSH for the cultivars was further increased in infected plants under sulfur-deficient conditions one can assume a higher susceptibility. As the cultivar Genie incorporated the highest percentage in GSH upon infection it can be postulated that this cultivar was more susceptible to the infection which is contradictory to the internal resistance in Genie. The relationship between the sulfur status and the infection is not always clear. The time of harvesting the plants seems to be one critical point as for example shown by Kuzniak and Sklodowska (2005).

Enzymes involved in the detoxification of ROS were significantly increased immediately after infection, whereas after two days the activity decreased again. Same could be observed for H_2S in the infection experiment with *B. napus* and *P. brassicae* as the H_2S increased relatively fast after the infection (Bloem et al., 2007). In the study from Kruse et al. in 2012 infected *A. thaliana* plants grown under optimal sulfur supply were able to react faster to the infection compared to plants under sulfur-limiting conditions. Already after 1 dpi only plants with optimal sulfur supply showed elevated levels of GSH and GSLs. The increase of GSH after infection was reported for a number of infection experiments whereas this was only the case over a period of time until the content decreased (Edwards et al., 1991, Foyer and Rennenberg, 2000). This indicates that measuring the sulfur containing compounds and corresponding genes involved in the pathogen defense is strongly dependent on the time the plants are harvested. There might have been greater differences for the sulfur-containing compounds between plants with full sulfur supply and plants grown under sulfur-deficient conditions in this study at an earlier stage of the infection. This should be investigated in further experiments. Therefore, conclusions on the SED cannot always be drawn properly. Nevertheless, results in this study clearly showed that the different resistances resulted in different reactions in the sulfur metabolism dependent on the sulfur supply. As the total sulfur content was likely to be increased upon the infection under both sulfur regimes it would be interesting to calculate the distribution of the sulfur fraction in infected plants. However, this was not possible as the sulfate measurement for all samples could not be performed. It is likely that the uptake of sulfate was increased upon infection as under sulfur-deficient conditions the expression of the sulfate transporter *Sultr4;2* was down-regulated in the experiments described in Chapter 2 and 4. This needs to be further investigated by the analysis of other sulfate transporters and sulfate measurements. As the measured sulfur-containing metabolites in the experiment described in Chapter 2 accounted for only a small percentage of the total sulfur (Table 1) measurements of other sulfur-containing compounds should be included in further studies as well. The sulfur-containing phytoalexin camalexin as an example is involved in the pathogen defense as well and accumulates during HR (Foyer and Rennenberg, 2000; Hammerschmidt and Nicholson, 2000).

Table 1: Change in the content of the sulfur-containing compounds in the of leaves of four *B. napus* cultivars after infection with *V. longisporum* grown under 0.01 and 1 mM $MgSO_4$ and the change in relation to the total sulfur content in the plants. The data for the GSLs were taken from Hornbacher, 2016. Values are based on the mean of the five harvesting time points.

cultivar	sulfur-fraction	after the infection [µg g^{-1}]		share in the total sulfur (%)	
		1 mM	0.01 mM	1 mM	0.01 mM
Compass	aliphatic GSLs-S	-19.64	-1.75	0.4530	0.2070
	indolic GSLs-S	-2.54	1.18	0.0587	0.1403
	aromatic GSLs-S	0.36	1.41	0.0082	0.1675
	Cysteine-S	1.08	0.34	0.0249	0.0399
	GSH-S	-2.46	-0.18	0.0568	0.0211
	total			**0.6016**	**0.5785**
Exocet	aliphatic GSLs-S	-17.46	0.69	0.4027	0.0817
	indolic GSLs-S	1.39	0.61	0.0321	0.0720
	aromatic GSLs-S	0.01	0.59	0.0002	0.0696
	Cysteine-S	1.12	0.20	0.0259	0.0242
	GSH-S	2.25	3.62	0.0519	0.4292
	total			**0.5128**	**0.6767**
Genie	aliphatic GSLs-S	-42.49	2.94	0.7901	0.3710
	indolic GSLs-S	2.16	2.95	0.0401	0.3724
	aromatic GSLs-S	-1.20	2.38	0.0224	0.3005
	Cysteine-S	0.19	0.60	0.0036	0.0754
	GSH-S	3.56	11.19	0.0663	1.4120
	total			**0.9225**	**2.5313**
King10	aliphatic GSLs-S	-33.69	5.10	0.7770	0.6035
	indolic GSLs-S	2.24	3.85	0.0517	0.4559
	aromatic GSLs-S	0.74	4.67	0.0172	0.5530
	Cysteine-S	-0.05	0.16	0.0011	0.0195
	GSH-S	-4.29	3.90	0.0990	0.4617
	total			**0.946**	**2.0936**

Influence of the circadian conditions on the sulfur distribution in the leaves of *B. napus* under different sulfur regimes

In the experiment described in Chapter 4 sulfate measurements were included. Therefore, it was possible to calculate the distribution of sulfur in the leaves of the cultivar Genie under conditions of sufficient sulfur supply and sulfur deficiency as well as under circadian conditions (Table 2). The two major pools of sulfur incorporated in proteins and sulfate account for approximately 95% (Table 1). Under sufficient sulfur supply and light/dark (LD) conditions 71% of the total sulfur was incorporated in sulfate. Under sulfur-limiting conditions only 53% of the total sulfur was incorporated in sulfate as it acts as the major sulfur source in the case of sulfur deficiency (Blake Kalff et al., 1998). The sulfur was incorporated in proteins accounting for 43%. Already in 1991 it was shown from Mengel that an increasing sulfur availability decreases the ratio of organic sulfur to inorganic sulfur. The fraction of sulfur incorporated into GSLs was unaffected by the sulfur status and accounted for approximately 1,4%. This is in agreement with literature where the GSLs in double low oilseed rape varieties comprise less than 5% in vegetative tissues (Blake Kalff et al., 1998). The sulfur incorporated in GSH account for 1.92% under sufficient sulfur supply and 2.40% under deficient sulfur supply respectively. The smallest fraction accounts for the cysteine with less than 0.07% under sufficient as well as under deficient sulfur supply. Therefore, neither the GSLs nor the thiols acts as a sulfur source under sulfur deficiency. The distribution was not only affected by the sulfur status. Under light/light (LL) conditions more sulfur was incorporated in proteins accounting compared to plants grown under LD conditions. The sulfur incorporated in sulfate accounted for 54% and 41% under sufficient sulfur supply and deficient sulfur supply of the total sulfur respectively. The incorporation of sulfur in the cysteine was unaffected by the LL conditions. In GSLs and GSH a slightly higher percentage of the sulfur was incorporated in these compounds under LL conditions.

Table 2: Distribution of the sulfur in the leaves of the *B. napus* cultivar Genie grown with 0.01 and 1 mM $MgSO_4$ under light/dark (LD) and light/light (LL). The values represent the mean of all harvesting time points ± SD in %.

	Percentage of sulfur in sulfur fractions %			
	1 mM		0.01 mM	
	LD	LL	LD	LL
SO_4-S	71.10 ± 15.58	53.84 ± 6.87	52.63 ± 16.31	40.54 ± 8.03
GSLs-S	1.30 ± 0.19	1.46 ± 0.35	1.38 ± 0.26	1.40 ± 0.19
GSH-S	1.92 ± 0.35	2.50 ± 0.47	2.40 ± 0.64	3.31 ± 0.33
Cysteine-S	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Protein-S	25.61 ± 15.57	42.14 ± 6.93	43.25 ± 16.15	54.68 ± 7.98

Sequence analysis among the canola cultivars

In the experiment described in Chapter 2 sequence analyses between the cultivars were performed determining possible differences in the sequence of key genes of the sulfur assimilation pathway. The sequence analysis of the transporters *Sultr3;1* and *Sultr4;2* was accompanied by difficulties in the amplification of the fragments using the cDNA as templates (Figure 1).

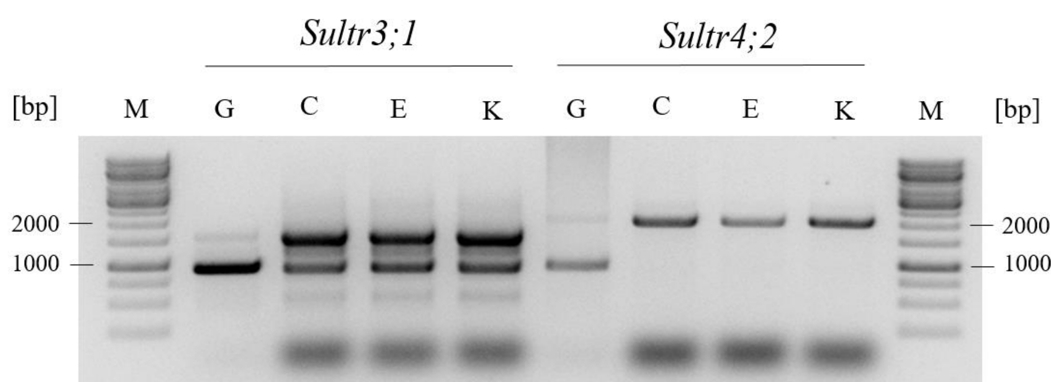

Figure 1: Amplification of the region of the transcripts used for expression analysis for the sulfate transporters *Sultr3;1* and *Sultr4;2* by PCR. The resulting amplicons were separated on a 1% agarose gel with a 1 kb DNA Ladder (Thermo Fisher scientific, Braunschweig). Abbreviations: bp, base pairs; M, marker; G, Genie; C, Compass; E, Exocet; K, King10; Sultr, sulfate transporters.

Except for the cultivar Genie amplification resulted in several products of different sizes for *Sultr3;1*. By changing the conditions of the PCR single bands could be amplified in the three cultivars Compass, Exocet, and King10 with the right size of the fragment (data not shown).

One possibility for the differences can be different splice variants caused by alternative splicing (AS). The amplificates for *Sultr4;2* of Compass, Exocet, and King10 seemed to be doubled as the fragments comprised ~2000 bp. Blasting the forward primer for the *Sultr4;2* resulted in two homologs of the transporter. Therefore, it might be possible that both homologs were amplified. This can only be clarified by sequencing the 2000 bp fragment in a further experiment. Changing the PCR conditions resulted in an amplificate with the right size. Sequencing results of the group 3 sulfate transporter revealed no differences among the cultivars. The identity of *Sultr3;1* fragments with the homologous partial sequence in *B. oleracea* was 99% and in *B. rapa* 98% respectively. When blasting the sequence of *Sultr4;2* from the cultivar Genie a GT-AG Intron was found which are the most common introns. Intron gain due to transcription is known. The most common intron gain event is the intron transposition where an intron transposes or "reverse splices" into a previously intron-less position in a transcript. The intron containing mRNA is then reverse transcribed and result in intron-containing cDNA. As this intron could not be found in the RNA Sequencing (RNA-Seq) derived data the intron might be the result of an error made by the reverse transcriptase in the cDNA synthesis. As this experiment was only performed once it should definitely be repeated with newly synthesized cDNA. However, one has to keep in mind that only a region of the sequence of the transporter was analyzed. Therefore, the whole sequence of the transporters should be analyzed. The best option in this case would be the use of RNA-Seq as here splice variants can be determined as well.

```
King10_Sultr4;2      TCTACCATTCTGGTGATCCTTCTAGTAATGAAGCATGTG---------------------
Genie_Sultr4;2       TCTACCATTCTGGTGATCCTTCTAGTAATGAAGCATGTGGTATGAGCTTTGTTCTTTCCT
                     ***************************************

King10_Sultr4;2      ------------------------------------------------------------
Genie_Sultr4;2       CTGTCAGTTTGTACTTTCCTTAATTACTTNTATTATGGTAAGACATGATGTGTCATGGCA

King10_Sultr4;2      -GGAAAAGCAAACAAGGAACTCCAGTTCATACGAGCAGCAGGGCCCCTCACAGGGCTTGC
Genie_Sultr4;2       GGGAAAAGCAAACAAGGAACTCCAGTTCATACGAGCAGCAGGGCCCCTCACAGGGCTTGC
                      ***********************************************************
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Figure 2: Part of the sequence alignment of the sulfate transporter 4;2 (Sultr4;2) between the cultivars King10 and Genie.

RNA-Seq analysis as a powerful tool for the expression analysis

In the infection experiment described in Chapter 4 RNA-Seq derived data were used determining genes involved in the pathogen defense. Nowadays high-throughput sequencing technology is the standard method for measuring RNA expression levels (Mortazavi et al., 2008). The mRNA fragmentation approach is used to gain sequence coverage of the whole transcript. The total number of reads for a given transcript is proportional to the expression level of the transcript multiplied by the length of the transcript. The longer the transcript the more reads are recorded compared to shorter transcripts which might be of similar expression (Oshlack and Wakefield, 2009). Therefore, it is necessary for the differential expression analysis to normalize the transcript length to the sequence which can be performed by a number of statistical algorithms. RNA-Seq analysis is indeed a powerful tool for expression analysis. However, even for this method the replicates of samples is the most significant factor for an accurate expression analysis (Robles et al., 2012). However, for a fast screening for possible candidates involved in the plant defense one replicate might be enough. Furthermore, with the help of the RNA-Seq derived data possible reference genes can be identified which are unaffected by the infection. Regarding the expression analysis for the circadian rhythm in the experiments described in Chapter 3 and 4 the use of only one replicate per harvesting time point would have been inappropriate. It would be interesting to perform the expression analysis for the circadian rhythm with RNA-Seq as here the whole transcriptome can be analyzed and not only one single gene. However, regarding the replicates and the sample amount the costs would be too high. The provided RNA-Seq analysis by different companies is still very expensive with prices ranging from 250-1200 € per sample, dependening on the type of RNA-Seq. Furthermore, as the genome of *B. napus* is very big (Chalhoub et al., 2014) the size of the data for one sample can comprise 10 gb. Handling the data for such a high sample amount would therefore, be very difficult.

Conclusions and further experiments

In this study it was determined that the sulfur metabolism of *B. napus* is involved in the pathogen defense against *V. longisporum* which differed among the cultivars according to their internal resistances. This was highly affected when plants were grown under sulfur-deficient conditions as here the susceptibility might have been enhanced. However, by using resistant cultivars typical symptoms upon the infection with the fungus were not exhibited and direct detection of the fungus in the plants was not possible as plants were analyzed at an early stage

of the infection. The involvement of the GSLs in the pathogen defense of *B. napus* could be determined. However, the way how they are actually functioning in the defense response needs to be further investigated. It was possible to investigate a circadian regulation in the expression of selected genes with a traditional method by optimizing the quantification resulting in reliable results comparable to modern techniques. Sulfur-containing metabolites which might be involved in the pathogen defense showed oscillations in their content in the course of a day as well. The dependence of the resistance in *B. napus* against the fungus *V. longisporum* on the time point could not be clearly validated in this study as the infection rate with the fungus might have been too low and therefore, needs to be repeated. Further experiments should be performed with a more susceptible cultivar enabling the quantification of the fungus for following the progress of the infection. The systemic spread in a more susceptible cultivar might be faster and therefore, detectable at an earlier stage. As the sulfur assimilation seems to be under circadian control experiments with mutants should be performed to clarify if the circadian control is only based on the need for the reduced equivalents of the photosynthesis or based on the role in the pathogen defense. It would be interesting to analyze in future experiments the oscillations in infected plants to see if the fungus is able to alter the clock period. As all the experiments were performed under a 12 h dark/ 12 h light cycle it would be interesting to perform these experiments under short and long day conditions as the oscillations might change.

References

Álvarez, C., Bermúdez, M.Á., Romero, L.C., Gotor, C. and García, I., (2012) Cysteine homeostasis plays an essential role in plant immunity. *New Phytol.*, 193(1), pp.165–77. doi:10.1111/j.1469-8137.2011.03889.x .

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A. and Schulze-Lefert, P., (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*, 323(5910), pp.101–6. doi:10.1126/science.1163732 .

Blake-Kalff, M.M. a., Harrison, K.R., Hawkesford, M.J., Zhao, F.J. and McGrath, S.P., (1998) Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. *Plant Physiol.*, 118(4), pp.1337–1344. doi:10.1104/pp.118.4.1337 .

Bloem, E., Haneklaus, S. and Schnug, E., (2007) Schwefel-induzierte Resistenz (SIR) – Schwefeldüngung als nachhaltige Strategie zur Gesunderhaltung von Pflanzen. *J. für Verbraucherschutz und Leb.*, 2(1), pp.7–12. doi:10.1007/s00003-006-0107-1 .

Bloem, E., Haneklaus, S., Salac, I., Wickenhäuser, P. and Schnug, E., (2007) Facts and fiction about sulfur metabolism in relation to plant-pathogen interactions. *Plant Biol. (Stuttg).*, 9(5), pp.596–607. doi:10.1055/s-2007-965420 .

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I.A.P., Tang, H., Wincker, P. et al., (2014) Plant genetics. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science*, 345(6199), pp.950–3. doi:10.1126/science.1253435 .

Clay, N.K., Adio, A.M., Denoux, C., Jander, G. and Ausubel, F.M., (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science*, 323(5910), pp.95–101. doi:10.1126/science.1164627 .

Edwards, R., Blount, J.W. and Dixon, R.A., (1991) Glutathione and elicitation of the phytoalexin response in legume cell cultures. *Planta*, 184(3), pp.403–9. doi:10.1007/BF00195343 .

Eynck, C., Koopmann, B., Grunewaldt-Stoecker, G., Karlovsky, P. and von Tiedemann, A. (2007) Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. *Eur. J. Plant Pathol.*, 118(3), pp.259–274. doi:10.1007/s10658-007-9144-6 .

Eynck, C., Koopmann, B., Karlovsky, P. and von Tiedemann, A., (2009) Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum. Phytopathology*, 99(43), pp.802–811. doi:10.1094/PHYTO-99-7-0802 .

Foyer, C. H. and Rennenberg, H., (2000) Regulation of glutathione synthesis and its role in abiotic and biotic stress defence. *Sulfur nutrition and sulfur assimilation in higher plants: Molecular, Biochemical and Physiological Aspects*, 127-153.

Fradin, E.F. and Thomma, B.P.H.J., (2006) Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. alboatrum*. *Mol. Plant Pathol.*, 7(2), pp.71–86. doi:10.1111/j.1364-3703.2006.00323.x .

Garrido, C., Carbú, M., Fernández-Acero, F.J., Boonham, N., Colyer, A., Cantoral, J.M. and Budge, G., (2009) Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. *Plant Pathol.*, 58(1), pp.43–51. doi:10.1111/j.1365-3059.2008.01933.x .

Hammerschmidt R. and Nicholson R.L., (2000) A survey of plant defense responses to pathogens. *Induced Plant Defenses Against Pathogens and Herbivores,* pp. 55–71 APS Press, Minneapolis, USA eds Agrawal, A. A., Tuzun, S., Bent, E.

Heale, J.B. and Karapapa, V.K., (1999) The verticillium threat to canada's major oilseed crop: canola. *Can. J. Plant Pathol.*, 21(1), pp.1–7. doi:10.1080/07060661.1999.10600114 .

Hornbacher, J., (2016) Determination of glucosinolates of species in the Brassicales order in response to abiotic and biotic stress factors. Master thesis. Gottfried Wilhelm Leibniz university.

Kuzniak, E. and Skłodowska, M., (2005) Fungal pathogen-induced changes in the antioxidant systems of leaf peroxisomes from infected tomato plants. *Planta*, 222(1), pp.192–200. doi:10.1007/s00425-005-1514-8 .

Kruse, C., Haas, F.H., Jost, R., Reiser, B., Reichelt, M., Wirtz, M., Gershenzon, J., Schnug, E. and Hell, R., (2012) Improved sulfur nutrition provides the basis for enhanced production of sulfur-containing defense compounds in *Arabidopsis thaliana* upon inoculation with *Alternaria brassicicola*. *J. Plant Physiol.*, 169(7), pp.740–3. doi:10.1016/j.jplph.2011.12.017 .

Madsen, S.R., Olsen, C.E., Nour-Eldin, H.H. and Halkier, B.A., (2014) Elucidating the role of transport processes in leaf glucosinolate distribution. *Plant Physiol.*, 166(3), pp.1450–62. doi:10.1104/pp.114.246249 .

Manici, L.M., Lazzeri, L. and Palmieri, S., (1997) In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. *J. Agric. Food Chem.*, 45(7), pp.2768–2773. doi:10.1021/jf9608635 .

Mengel K (1991) *Ernährung und Stoffwechsel der Pflanze*. Fischer Verlag, Stuttgart, ISBN: 3-334003-10-8

Mithen, R., (1992) Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. *Euphytica*, 63(1–2), pp.71–83. doi:10.1007/BF00023913 .

Mithen, R.F. and Magrath, R., (1992) Glucosinolates and Resistance to *Leptosphaeria maculans* in Wild and Cultivated Brassica Species. *Plant Breed.*, 108(1), pp.60–68. doi:10.1111/j.1439-0523.1992.tb00100.x .

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B., (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods*, 5(7), pp.621–8. doi:10.1038/nmeth.1226 .

Oshlack, A. and Wakefield, M.J., (2009) Transcript length bias in RNA-seq data confounds systems biology. *Biol. Direct*, 4(1), p.14. doi:10.1186/1745-6150-4-14 .

Robles, J.A., Qureshi, S.E., Stephen, S.J., Wilson, S.R., Burden, C.J. and Taylor, J.M., (2012) Efficient experimental design and analysis strategies for the detection of differential expression using RNA-Sequencing. *BMC Genomics*, 13(1), p.484. doi:10.1186/1471-2164-13-484 .

Salac, I., Haneklaus S. H., Bloem E., Booth, E. J., Sutherland, K. G., Walker, K. C. and Schnug, E., (2005) Sulfur nutrition and its significance for crop resistance – a case study from Scotland. *FAL Agricultural research* (special issue 283), 111–119 .

Sarwar, M., Kirkegaard, J.A., Wong, P.T.W. and Desmarchelier, J.M., (1998) Biofumigation potential of brassicas. *Plant Soil*, 201(1), pp.103–112. doi:10.1023/A:1004381129991 .

Witzel, K., Hanschen, F.S., Schreiner, M., Krumbein, A., Ruppel, S. and Grosch, R., (2013) Verticillium suppression is associated with the glucosinolate composition of *Arabidopsis thaliana* leaves. *PLoS ONE*, 8(9), p.e71877. doi:10.1371/journal.pone.0071877 .

Witzel, K., Hanschen, F.S., Klopsch, R., Ruppel, S., Schreiner, M. and Grosch, R., (2015) *Verticillium longisporum* infection induces organ-specific glucosinolate degradation in *Arabidopsis thaliana*. *Front. Plant Sci.*, 6, p.508. doi:10.3389/fpls.2015.00508 .

Wretblad, S. and Dixelius, C., (2000) B-genome derived resistance to *Leptosphaeria maculans* in near isogenic *Brassica napus* lines is independent of glucosinolate profile. *Physiol. Plant.*, 110(4), pp.461–468. doi:10.1111/j.1399-3054.2000.1100406.x .

Zeise, K. and von Tiedemann, A., (2002) Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *J. Phytopathol.*, 150(3), pp.112–119. doi:10.1046/j.1439-0434.2002.00730.x .

Abbreviations

ANOVA	analysis of variance
APK	adenosine phosphosulfate kinase
APR	adenosine phosphosulfate reductase
APS	adenosine phosphosulfate
ATP	adenosine triphosphate
BLAST	Basic local alignment searching tool
Bn	*Brassica napus*
C	non-infected plants
CCA1	Clock circadian associated 1
CE	Capillary electrophoresis
cDNA	complementary Deoxyribonucleic acid
DFCI	Dana Farber Cancer Institute Gene Index
DM	dry mass
Dpi	Day post inoculation
EE	Evening Element
EST	expressed sequence tags
Et al.	Et alii
FM	Fresh mass
F_v/F_m	maximal PS II quantum yield
GOI	gene of interest
GSL	Glucosinolate
GSH	reduced Glutathione
H_2S	hydrogen sulfide
HPLC	High pressure liquid chromatography
HR	hypersensistive response
ICP-OES	inductively coupled plasma optical emission spectroscopy
ITS	Internal transcribed spacer
INF	infected plants
LD	light/dark
LL	light/light

$MgSO_4$	Magnesiumsulfate
mRNA	messenger ribonucleic acid
NPQ	non-photochemical quenching
PAM	pulse-amplitude-modulation
PAPS	3‘-phosphoadenosine 5‘-phosphoslufate
PCR	Polymerase chain reaction
PDB	potato dextrose broth
PR	pathogen related
PS II	photosystem II
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SD	Standard deviation
Sultr	sulfate transporter
Y (II)	effective PS II quantum yield

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Cejas, I., Rumlow, A., Turcios, A.E., Engelmann, F., Martínez, M.E., Yabor, L., Papenbrock, J., Lorenzo, J.C. (2016) Exposure of common bean seeds to liquid nitrogen modifies mineral composition of young plantlet leaves. American Journal of Plant Sciences, 7: 1612-1617. DOI: 10.4236/ajps.2016.712152

Rumlow, A., Keunen, E., Klein, J., Pallmann, P., Riemenschneider, A., Cuypers, A., Papenbrock, J. (2016) Quantitative expression analysis in *Brassica napus* by Northern blot analysis, and reverse transcription-quantitative PCR in a complex experimental setting. PLoS ONE 11(9): e0163679. doi:10.1371/journal.pone.0163679

Weese, A., Pallmann, P., Papenbrock, J., Riemenschneider, A. (2015) *Brassica napus* L. cultivars show a broad variability in their morphology, physiology and metabolite levels in response to sulfur limitations and to pathogen attack. Front Plant Sci. Frontiers; 2015;6. doi:10.3389/fpls.2015.00009

Konferenzen und Seminare

ICP-OES Anwenderseminar 05.-06.11.2015 in Hannover, Präsentation mit dem Titel: Elementanalyse von salzhaltigem Material mittels ICP-OES als Substrat in einem Fermentationsprozess

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ICP-OES Anwenderseminar 06.-07.11.2014 in Göttingen, Präsentation mit dem Titel: Einfluss von Pathogenbefall auf die Elementgehalte in Raps-Pflanzen

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