Response of starch potato (*Solanum tuberosum* L.) genotypes to osmotic stress in vitro and drought stress in vivo

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Abstract

Potato (*Solanum tuberosum* L.) is one of the most important crops in the world. In addition to food and fodder, potato is also used for industrial purposes like production of adhesives, paper, and cosmetics. The vegetative growth phase of potato correlates with dry periods in spring and early summer, which are increased by climate change. Drought stress leads to morphological, physiological, and biochemical changes in the plant that have an extensive negative impact on the size and quality of the tubers. Since potato is a drought-sensitive species with its shallow root system, the interest in drought-tolerant cultivars is immense. Because ex vitro test systems are expensive and labor-intense and because additional parameters like other abiotic and biotic stressors influence the stress response, investigations in vitro are of great interest. Advantages of in vitro systems are the controlled light intensity, temperature, and supply of nutrients. Furthermore, pathogens can be excluded from the culture, and experiments require less space. Osmotic stress in vitro can be induced by adding an osmoticum, which lowers the osmotic potential in the culture medium.

In the context of this work, an existing in vitro test system was optimised. For this purpose, the solid medium was replaced by liquid medium to enable that sorbitol can be added stepwise with increasing concentration. This resulted in two advantages: 1. the stress induction was gradual, and thus no osmotic shock was induced; 2. the plants were able to establish roots prior to the addition of the osmoticum, which allowed the stress that occurred by cutting the explants to be mitigated. This experiment showed that sorbitol was probably taken up by the roots and transported into the shoots, where it was detected by GC-MS. Furthermore, selected potato genotypes were investigated for their early drought stress response in open greenhouse and shelter experiments and their early osmotic stress response in vitro. For this purpose, candidate proteins for drought stress were selected after identification by LC-MS in material from rainout shelter trials. Identified proteins were further selected based on differential abundance in the genotypes ‘Eurostarch’ and ‘Tomba', which were postulated to be rather tolerant. From the identified candidate proteins, eight genes were selected, and their expression was investigated by RT-qPCR in leaves after seven days of water withdrawal in two trials in an open greenhouse, where differences between treatments but no genotypic effects were detected. Expression of *peroxidase 51-like (POD)*, *subtilase family protein (SBT1.7)*, and *cell wall/vacuolar inhibitor of fructosidase* (*INH1)* responded strongly to drought stress in all genotypes. Dry masses of the shoots also demonstrated stress induction ex vitro without reaching the permanent wilting point in the open greenhouse. The analysis under osmotic stress in two experiments in vitro also showed altered shoot dry mass and differential gene expression under osmotic stress. *SBT1.7* was regulated in vitro in all genotypes under osmotic stress. *POD* showed similar regulation to the open greenhouse experiments in three of the four genotypes analysed. Furthermore, *INH1* was only regulated in ‘Eurostrach’ and 'Tomba'. Additionally, *13-LOX*, a gene of the family of lipoxygenases linked to osmotic adjustment, was upregulated in all genotypes under osmotic stress. Finally, differentially abundant proteins were identified in leaves of two shelter experiments under drought stress, nitrogen deficiency, and combined stress in two genotypes that differ in tolerance towards those stresses. Results showed differences in proteomic responses under combined as well as single stresses. The sensitive genotype ‘Kiebitz’ showed a higher abundance of proteases, whereas the rather tolerant genotype ‘Tomba’ showed a lower abundance of such proteins.

In summary, important insights into the stress response of potato to drought stress and osmotic stress were gained. Further studies with earlier sampling could help to better understand genotypic differences and develop biomarkers for early drought stress. An alternative osmoticum for the in vitro system should be considered.

Keywords: *Solanum tuberosum*, drought, osmotic stress, proteomics, gene expression, sorbitol

Zusammenfassung

Die Kartoffel (*Solanum tuberosum* L.) ist eine der wichtigsten Kulturpflanzen der Welt. Neben der Nutzung zur Ernährung und als Viehfutter wird Kartoffelstärke auch für industrielle Zwecke wie zur Bindemittel- und Papierherstellung, sowie für die Kosmetikindustrie verwendet. Der Klimawandel verursacht Trockenperioden im Frühling und Frühsommer, wenn sich die Kartoffelpflanzen in der vegetativen Wachstumsphase befinden. Trockenstress führt zu morphologischen, physiologischen und biochemischen Veränderungen in der Pflanze, die sich negativ auf die Knollengröße und -qualität auswirken. Da die Kartoffel mit ihrem flachen Wurzelsystem eine trockensensitive Kultur ist, ist das Interesse an trockentoleranten Sorten immens. Da Ex-vitro-Testsysteme kosten- und arbeitsintensiv sind und zusätzliche Parameter wie abiotische und biotische Stressoren die Stressreaktion beeinflussen, sind In-vitro-Untersuchungen von großem Interesse. Vorteile von In-vitro-Systemen sind die kontrollierte Lichtintensität, Temperatur und Nährstoffzufuhr. Außerdem können Krankheitserreger aus der Kultur ausgeschlossen werden und die Versuche benötigen weniger Platz. Das osmotische Potenzial im Kulturmedium kann durch Zugabe eines Osmotikums reduziert werden, was zu osmotischem Stress führt.

Im Rahmen dieser Arbeit wurde ein bestehendes In-vitro-Testsystem optimiert. Dazu wurde das Festmedium, in das das Osmotikum Sorbitol nur einmalig eingebracht werden konnte, durch ein Flüssigmedium ersetzt, dem Sorbitol in zunehmender Konzentration zugesetzt wurde. Daraus ergaben sich zwei Vorteile: 1. die Stressinduktion erfolgte schrittweise, so dass kein osmotischer Schock ausgelöst wurde; 2. die Pflanzen konnten vor der Zugabe des Osmotikums Wurzeln bilden, so dass der Stress, der durch das Schneiden der Explantate entstand, gemildert werden konnte. Dieser Versuch zeigte, dass Sorbitol von den Wurzeln aufgenommen und in die Sprosse transportiert wurde, wo es mittels GC-MS nachgewiesen werden konnte. Darüber hinaus wurden ausgewählte Kartoffelgenotypen in Experimenten im offenen Gewächshaus und im Shelter auf ihre Reaktion auf Trockenstress und in vitro auf ihre Reaktion auf osmotischen Stress untersucht. Zu diesem Zweck wurden Kandidatenproteine für Trockenstress nach Identifizierung durch LC-MS in Material aus Rain-out Shelterversuchen ausgewählt. Die identifizierten Proteine wurden aufgrund der differentiellen Abundanz in den Genotypen ‚Eurostarch' und ‚Tomba', die als eher tolerant gelten, ausgewählt. Aus den identifizierten Kandidatenproteinen wurden acht Gene ausgewählt, deren Expression mittels RT-qPCR in Blättern nach sieben Tagen ohne Bewässerung in zwei Versuchen im offenen Gewächshaus untersucht wurde. Es wurden Unterschiede zwischen den Behandlungen, aber keine genotypischen Effekte festgestellt. Die Expression der *peroxidase 51-like (POD)*, *subtilase family protein (SBT1.7*) und *cell wall / vacuolar inhibitor of fructosidase* (*INH1)* reagierte bei allen Genotypen stark auf Trockenstress. Auch die Wachstumsdaten (Spross- und Wurzeltrockenmasse) zeigten, dass ex vitro Stress induziert wurde, ohne dass im offenen Gewächshaus der permanente Welkepunkt erreicht wurde. Die Analyse unter osmotischem Stress in zwei Experimenten in vitro zeigte ebenfalls eine verringerte Sprosstrockenmasse und eine regulierte Genexpression unter osmotischem Stress. *SBT1.7* wurde auch in vitro in allen Genotypen unter osmotischem Stress reguliert. *POD* zeigte bei drei der vier untersuchten Genotypen eine ähnliche Änderung der Genexpression wie in den Experimenten im offenen Gewächshaus. *INH1* wurde nur bei ‚Eurostrach' und ‚Tomba' reguliert. Zusätzlich wurde *13-LOX*, ein Gen aus der Familie der Lipoxygenasen, das mit der osmotischen Anpassung zusammenhängt, in allen untersuchten Genotypen unter osmotischem Stress hochreguliert. Schließlich wurden in den Blättern von zwei Shelter-Experimenten unter Trockenstress, Stickstoffmangel und kombiniertem Stress bei zwei Genotypen, die sich in ihrer Toleranz gegenüber diesen Stressfaktoren unterscheiden, differentiell abundante Proteine identifiziert. Die Ergebnisse zeigten sowohl bei kombiniertem als auch bei Einzelstress Unterschiede in den Proteinabundanz. Der sensitive Genotyp ‚Kiebitz' wies eine höhere Abundanz von Proteasen auf, während der eher tolerante Genotyp ‚Tomba' eine geringere Abundanz solcher Proteine zeigte.

Zusammenfassend ließen sich wichtige Erkenntnisse über die Stressreaktion der Kartoffel auf Trockenstress und osmotischen Stress gewinnen. Weitere Studien mit früheren Probenahmen könnten dazu beitragen, die genotypischen Unterschiede besser zu verstehen und Biomarker für frühen Trockenstress zu entwickeln. Ein alternatives Osmotikum für das In-vitro-System sollte in Betracht gezogen werden.

Schlagwörter: Genexpression, osmotischer Stress, Proteomik, *Solanum tuberosum*, Sorbitol, Trockenheit

Abbreviations

•OH	hydroxyl radical
13-LOX	lipoxygenase
^{14}C	radioactive isotope of carbon with an atomic nucleus containing 6 protons and 8 neutrons
ABA	abscisic acid
ATP	adenosine triphosphate
BC	Before Christ
BMEL	Bundesministerium für Ernährung und Landwirtschaft
C	carbon
CO_2	carbon dioxide
CRISPR/Cas	clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein
DM	dry mass
DRYM	deviation of relative starch yield from the experimental median
FNR	Fachagentur Nachwachsende Rohstoffe e. V.
GC-MS	gas chromatography-mass spectrometry
GenTG	Gentechnikgesetz
Glyx	lactoylglutathione lyase/glyoxalase I family protein
GMO	genetically modified organism
GOI(s)	gene(s) of interest
H_2O_2	hydrogen peroxide
ha	hectar(s)
IEF/SDS-PAGE	isoelectric focussing/sodium dodecyl sulfate–polyacrylamide gel electrophoresis
INH1	cell wall / vacuolar inhibitor of fructosidase
LC-MS	liquid chromatography-mass spectrometry
LEA	late embryogenesis abundant protein
MG	methylglyoxal
N	nitrogen

NAD-SDH	nicotinamide adenine dinucleotide dependent sorbitol dehydrogenase
NCBI	National Center for Biotechnology Information
NO_3^-	nitrate
O_2	oxygen
O_2^-	superoxide
OSML	osmotin-like
P5CS	pyrroline-5-carboxylate synthase
PEG	polyethylene glycol
PGSC	Potato Genome Sequencing Consortium
POD	peroxidase
PROKAR	Charakterisierung des Proteoms unter Stickstoff- und Wassermangelstress als Grundlage für die züchterische Entwicklung stickstoffeffizienter und trockentoleranter Stärkekartoffeln
RFS	raffinose synthase
ROS	reactive oxygen species
RPT5a	regulatory particle triple-A ATPase 5A
RT-qPCR	reverse transcription quantitative real-time PCR
RuBP	ribulose-1,5-bisphosphat
SBT1.7	subtilase family protein
SHMT	serine transhydroxymethyltransferase
SNF1	sucrose nonfermenting 1
SNP	single nucleotide polymorphism
SnRK2	sucrose non-fermenting-1-related protein kinase 2
SSI	stress susceptibility index
TF	transcription factor
VALPROKAR	Validierung identifizierter Markerproteine als Grundlage für die züchterische Entwicklung stickstoffeffizienter und trockentoleranter Stärkekartoffeln
ZBD	zinc-binding dehydrogenase family protein

Content

1. General Introduction

1.1 *Solanum tuberosum* L. – economic importance and breeding

Solanum tuberosum L. (common: potato) is a heterozygous, autotetraploid species with a total of 48 chromosomes (2n=4x=48) (Bonierbale et al. 2020). Potato belongs to the family Solanaceae and the genus Solanum (Dolničar 2021). The plant is characterized by its herbaceous growth with white or purple flowers and tubers formed on belowground organs (botanically shoots), the stolons. The maturation time varies from 75-90 days for early varieties, 90-100 days for mid-season varieties, and 100-110 days for late varieties (Nasir and Toth 2022).

Potato originated from Peru and Bolivia, where it was cultivated as early as 8000 to 5000 BC (National Research Council (U.S.) 1989). At the end of the 16th century, potato also came to Europe with spanish conquistadors, and displaced the turnip from the plates of the people until the 19th century due to their nutritious nature and affordability (Salaman 1985).

Today, potato is one of the most important food crops in the world and is grown worldwide in over 100 countries (Dahal et al. 2019). In 2021, 369 million tons of potato were produced in an area of 18,132,649 ha with an increasing tendency (FAO 2022). The largest production was recorded for China (94 million tons), followed by India (54 million tons), Ukraine (21 million tons), and Russia (18 million tons). Germany ranked 6th in the global comparison, where 11.3 million tons of potato tubers on 258,300 ha were produced in 2021 for food purposes, seed, or fodder, and for industrial use. 44.6 % of this area were in Lower Saxony (BLE 2022; BMEL 2022) making it the biggest region with potato cultivation in Germany and north-west Europe (Goffart et al. 2022). 18% of all the potatoes cultivated in Germany were industrial potatoes used for starch production. Their starch is used in the paper industry, production of adhesives and cosmetics (Kraak 1992; Röper 2002).

Solanum tuberosum is well adapted to the northern hemisphere. The most common method of breeding for improved potato lines is based on phenotypic recurrent selection and clonal reproduction. Any seedling has the potential to become a new variety via clonal breeding (Bonierbale et al. 2020). Therefore, two heterozygous parental plants are crossed, leading to a high genetic variation in the offspring due to dominance and epistatic effects (Grüneberg et al. 2009). Seedlings of the crossing are grown in the first year and A-clones grow from these seedling tubers in the second year. The selection of B-, C-, and D-clones, which are tested for the desired traits, over the next years, results in less and less genotypes which are tested in increasing numbers

(clonally propagated) and can then serve as a new variety (Grüneberg et al. 2009). This breeding technique is time consuming, and the selection of suitable parents is not easy due to various desirable traits. An optimal duration for developing a new cultivar would be 13-14 years, though it can take longer for breeding optimised lines due to the most important traits like tuber yield being quantitative in a tetraploid species, rapid inbreeding depression and slow propagation through tubers (Ghislain and Douches 2020; Bradshaw 2021). Molecular markers can speed up the process of breeding and may help to find the best parents to start with. Also *A. tumefaciens*- mediated transformation or genome editing via e.g. CRISPR/Cas9 are methods to decrease breeding time for optimised varieties (Nadakuduti et al. 2018; Bonierbale et al. 2020; Nahirñak et al. 2021).

1.2 Challenges of potato production

Potato as a crop that is cultivated throughout the world, faces many challenges in production reaching from biotic stress (viruses, bacteria, fungi, and oomycetes in the microbiome, and insects) to abiotic stress. Challenging pathogens and pests that Solanaceae like potato are still struggling with are late blight, caused by *Phytophthora infestans* which can lead to 70 % of yield loss when plants are infected (Mekonen and Tadesse 2018), as well as potato cyst nematodes (*Globodera spp.*), which are quarantine restricted pests in Germany, and lead to massive yield loss (Mburu et al. 2020; Judelson and Blanco 2005).

Furthermore, quality of potato yield highly depends on the soil features like organic matter, nutrient status, and soil structure. In intensive potato production tillage and subsoiling can be used for weed control, soil preparation, and harvest, which may cause structural damage to the site, e.g. soil erosion, compaction, and loss of organic matter (Ghosh and Daigh 2020). The soil must be preserved and maintained in terms of organic matter synthesis, as well as fertility and soil biome. The microbiome plays an important role in potato production by influencing nutrient availability, plant growth, and development (Song et al. 2021). Organic matter in the soil influences the need for nitrogen (N) fertilisation. As potato cultures need N for maximum yield, it needs a good fertiliser management so that this is not a threat for ground water. The N uptake efficiency varies between varieties due to root morphology and differences in N uptake kinetcs (Sharifi and Zebarth 2006). Drought limits the N efficiency of the plants and N can influence the drought sensitivity (Da Silva et al. 2011). Because of predicted and already happening drought periods leading to artificial irrigation, and heavy rainfalls due to climate change during this period, there is the risk of N to be leached into the groundwater as NO_3^- (Zebarth and Rosen 2007).

Climate change and associated rising global surface air temperatures will further increase potato growing problems (Haverkort and Verhagen 2008). Currently, cumulative industrial CO_2 emissions since 1850 have reached 2400 +- 240 Gt CO_2. Various scenarios of CO_2 emission development allow predictions of 0.5 to over 4 °C rising global surface air temperature (Intergovernmental Panel on Climate Change 2022). For potato cultivation, this means increased weather extremes such as storms and heavy rainfall, as well as hot and dry periods during the growing season. Especially in spring and early summer, when the plant is in the vegetative growth phase, stress periods will occur more frequently (Fig. 1).

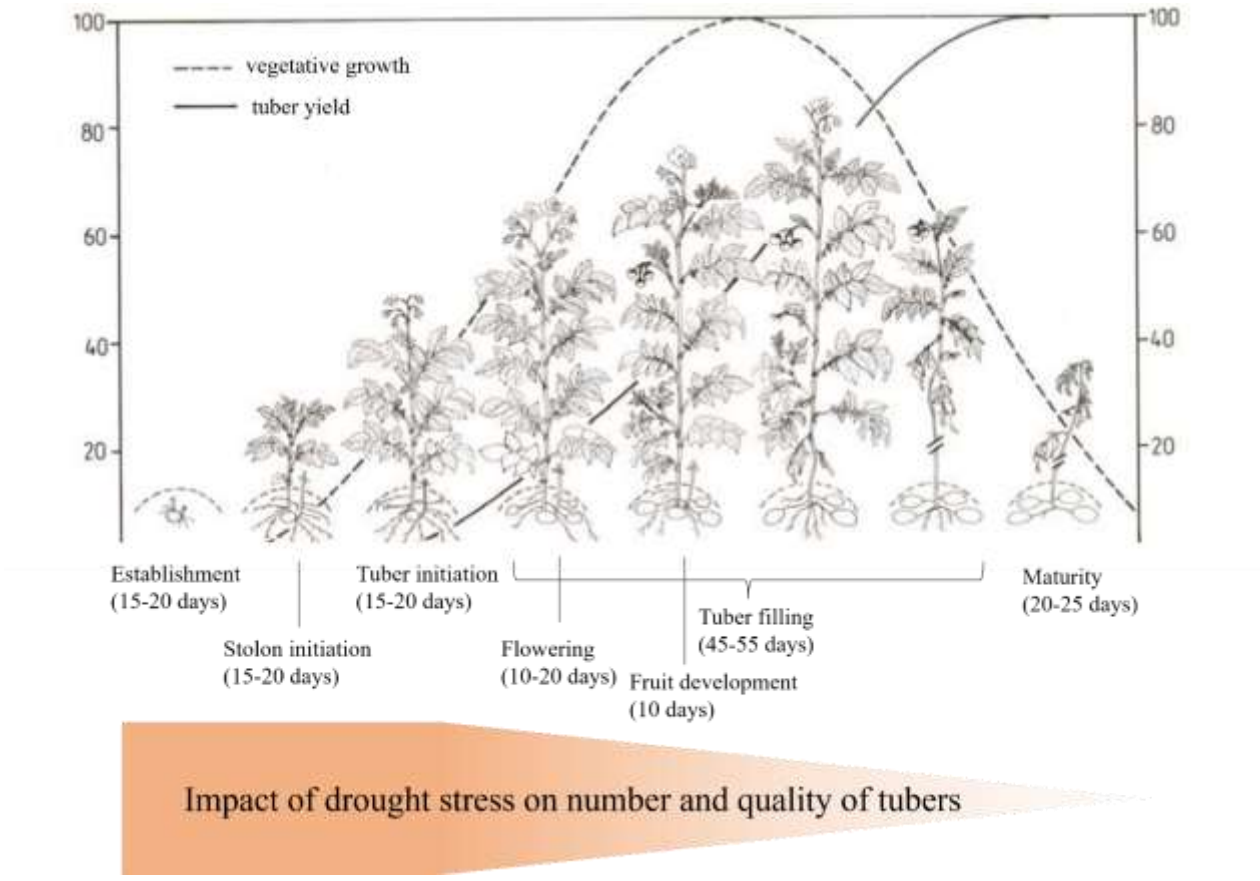

Figure 1 Growth stages of potato and impact of occurring drought stress on tuber yield adjusted after Landwirtschaftskammer Nordrhein-Westfalen (2015) and Obidiegwu et al. (2015). Vegetative growth starting at establishment phase, tuber growth starting after stolon initiation phase. Impact of drought stress on tuber yield decreases by progressing in the life cycle.

Abiotic stresses like heat, drought, and salt stress have a severe impact on potato yield and are key issues of potato production (Devaux et al. 2020). Heat and drought have the greatest impact on tuber quality and quantity when they occur in the vegetative growth phase (MacKerron and Jefferies 1986; Walworth and Carling 2002) whereas after tuber formation the impact on tuber yield decreases (Haverkort et al. 1990). Because of their shallow root system, which is concentrated

in the upper 30 cm of the soil, potato is considered a drought-susceptible crop (Iwama and Yamaguchi 2006; Iwama 2008). As a result, farmers have to irrigate their fields artificially, which is very cost-intensive and water can become scarce in places. Since up to 92 % of freshwater are used for agriculture (varying within the year) and the world faces water shortages, breeding new varieties with increased drought tolerance is crucial (Hoekstra et al. 2012).

1.3 Responses of potato to drought stress

Drought stress can induce morphological, as well as physiological and biochemical alterations in potato (Hanász et al. 2022), which are depending on the developmental status of the plant and the intensity of the stress. Reactions to drought stress lead to acclimatisation of the plant. This is due to epigenetic modifications like methylation. Epigenetic modifications can be induced by plant hormones, microbial interactions, or abiotic factors like drought and play a key role in acclimatisation by regulating gene expression (Akhter et al. 2021).

Drought, compared to other abiotic stresses, has a very pronounced effect on morphology of potato plants (Mańkowska et al. 2022). Drought decreases canopy development (e.g. plant height, number of leaves, leaf area), tuber number and tuber size (Luitel et al. 2015; Mańkowska et al. 2022). The root is not affected in the same way as the above-ground plant material. Some studies show that the root/shoot ratio was increased after drought stress (Jefferies 1993; Mańkowska et al. 2022). This is a reaction of the plant to minimise transpiration and therefore water loss through the leaves on the one hand, while reaching into more distant soil for increased water uptake through longer and more branched roots on the other hand.

Despite morphological alterations being the first visible response of potato to drought stress, changes in physiological and biochemical responses start before visible reactions. An important response to drought stress is stomatal closure (Jia and Zhang 2008). It helps to maintain the leaf water potential by reducing water loss through evaporation. Stomatal closure is maintained by ABA regulation in the plant. ABA signaling is caused by stress sensed by roots and ABA is transported to shoots under drought stress (Obidiegwu et al. 2015). This was also reported for osmotic stress, which is part of drought stress, by Yang et al. (2019), who found ABA-related genes like SnRK2 and several transcription factors (TFs) upregulated under osmotic stress in potato. SnRK2, also known as SNF1-related protein kinase 2, is a group of protein kinases of the Sucrose Non-fermenting 1-related protein kinase family. SnRK2 proteins are involved in responses to drought,

salt, and cold as well as in growth and development of the plant (Mazur et al. 2021; Hasan et al. 2022; Li et al. 2022). The trade-off of stomatal closure is the reduction of CO_2 uptake and thus photosynthesis rate (Chaves et al. 2003; Obidiegwu et al. 2015). In addition, photosynthesis is directly affected by shortage of water and by multiple effects on canopy growth, too (Obidiegwu et al. 2015; Chaves et al. 2002). Reduced water content in the plants leads to decreased turgor pressure and reduced cell volume. To counteract this, the plant accumulates high concentrations of ions and osmolytes, like proline, soluble sugars or sugar alcohols to lower the osmotic potential of the cells and maintain water uptake (Girma and Krieg 1992, Mane et al. 2008). This process is called osmotic adjustment and leads to inhibited ATP synthesis and thus D-ribulose-1,5-bisphosphate (RuBP) production, the principal CO_2 acceptor in photosynthesis (Nasir and Toth 2022). Tourneux and Peltier (1995), among others, found photosynthesis to be lower in potato under drought stress. Chen et al. (2019) showed osmotin-like (OSML), delta-1-pyrroline-5-carboxylate synthase (P5CS), late embryogenesis abundant protein (LEA) and galactinol-sucrose galactosyltransferase (raffinose synthase, RFS) gene expression upregulated under drought stress in potato.

Another consequence of drought stress is oxidative stress due to ROS (reactive oxygen species) accumulation. ROS are molecules, that occur in plants in form of hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals (•OH). As byproducts of normal metabolism, plants have regulators for ROS content like superoxide dismutases, catalases, and peroxidases (Huang et al. 2019). As stress responses, ROS can accumulate up to levels, that lead to cells being deprived of oxygen and eventually leading to cell death. ROS production is known to be a signal for defense responses by specific signal transduction pathways that involve H_2O_2 (Cruz de Carvalho 2008).

Responses to combined abiotic stressors may differ from drought stress responses. This is due to counteraction of metabolic pathways (Mittler 2006). Heat, and alteration in nutrients are able to impede the stress level and additional stresses like osmotic stress or oxidative stress may occur (Wang et al. 2003). Regarding the various artificial experiments used to analyse drought stress, it is important to consider combined stress and the changes that occur in the stress response.

1.4 In vitro versus ex vitro systems to identify stress-tolerant genotypes

Drought or related stresses such as osmotic stress can be applied artificially by several test systems to determine response mechanisms on a morphological, biochemical, and molecular level and to eventually identify drought tolerant potato genotypes. The advantages and disadvantages of field experiments, experiments in rainout shelters and open greenhouses, greenhouses and climate chambers, and in vitro experiments are discussed in this chapter.

1.4.1 Field experiments

In the field, potato genotypes can be tested under the most genuine, production-like conditions. There is enough space for the roots to develop in field trials. But since soil conditions may differ on a small regional scale already, multiple locations are needed to make a robust statement about the genotypes' stress response (Zaki and Radwan 2022). It is also difficult to distinguish drought stress reactions in the field from other stresses occurring next to drought in an open environment. Temperature, light, mechanical stress from storm or heavy rain, or biotic stressors cannot be easily excluded and may change the plant's morphological and biochemical response. As drought stress can be reached by water withdrawal, it is obvious that this is difficult to achieve in the field, where rain can occur in the period of the experiment. Therefore, field trials should take place several times over multiple years to cover and help determine a broad mass of possible co-variables. Moreover, field experiments are cost- and time-consuming, and big areas have to be available for the scientists.

1.4.2 Rainout shelter and open greenhouse

Rainout shelters can be designed in two ways. Either, they have a mobile roof on top of the trial area that can be rolled over the crop as needed. This type allows to create normal field conditions in clear weather, but keeps out any precipitation. Or they have a static roof on top of the experimental area, which cannot be moved, but still allows parameters such as wind and temperature to occur close to field conditions. This type will be referred to as open greenhouse in the following. The exclusion of precipitation and the simultaneous possibility to cultivate the plants in the soil is a great advantage of this system. However, like in field trials, it is not possible to influence the soil properties or other factors such as temperature, or light intensity.

In an open greenhouse, in addition to the roof there can be one to three walls that prevent water or wind to disturb the experimental setting. Plants are cultivated in pots either on the ground or on tables, where substrate composition and soil water content can be better controlled. Sampling and

visual checks for pests and other problems are easy to implement. However, the plant has little space to develop its root system, which can lead to altered morphology of the root system. The walls of the open greenhouse can also create problems by reflecting heat on warm days and, in the worst case, trapping heat. This may lead to a temperature gradient and higher temperature than in the field within the experimental area and thus an unwanted influence on plant growth.

1.4.3 Greenhouse and climate chamber

Stress experiments in a greenhouse or climate chamber are common to analyse stress responses of plants. In the controlled environment, many parameters can be directly influenced, and experiments can be started throughout the whole year, largely independent of the season. Water can be added exactly as needed by growing the plants in the pots or containers. Damage to the plant by wind and pests are minimised and the temperature can be controlled manually. However, as in the open greenhouse, heat build-up can occur in the greenhouse if it is not cooled properly. In addition, heating/cooling is costly, space is limited, and light intensity may differ from field trials. Furthermore, the root system is limited by pots and the substrate used is artificial. All parameters like stress duration, stress development, and stress intensity must be considered carefully.

1.4.4 In vitro experiments

In vitro propagation is implemented during the breeding process, as well as in biotechnological approaches in potato. The antiseptic environment can be used to produce virus-free material and cultivate endangered species. For the pharmacological and food-related industries, it is a cost-effective way to produce large quantities of plants and their active ingredients. Stress responses can also be analysed under in vitro culture conditions. The high number of rather uniform plants that can be used is a great advantage. As in the climate chamber, many parameters in vitro can be regulated and adjusted as desired. Factors include the composition and solidification of the culture medium, the carbon (C) source, light intensity, and temperature. Furthermore, it is a space-efficient method and pathogens and microorganisms can be mostly excluded (Schum et al. 2016). However, there are drawbacks to in vitro culture of plants that complicate the interpretation of the stress response. First, in vitro cultivation is stress for plants, which can influence the response to other desired stresses. The morphology of plants in in vitro culture differs from that of plants ex vitro. Due to the high humidity in the vessels, which cannot be regulated, the stomata of the plants are open throughout, because they don’t need them to regulate the water balance. In line with this, the cuticle is only rudimentary developed in plants in vitro (George et al. 2008). Genetic modifications

also occur in vitro and are due to propagation via e.g. nodal cuttings. Especially after the use of a callus culture somaclonal variation occurs, which can change properties of the plant, and thus also the stress response. Application of drought stress in vitro cannot be realised due to the high relative humidiy in the culture vessels. Instead, an osmoticum can be added to the medium, through which the osmotic potential is reduced, thus limiting the water availability.

1.5 Responses of potato to osmotic stress in vitro

Osmotic stress arises ex vitro as a part of drought and salt stress and can be achieved in vitro by adding an osmoticum to the culture medium resulting in a lower osmotic potential. Morphological responses like reduction of shoot length, fresh and dry weight, and a reduced number and length of roots occur (Hanász et al. 2022). Molecular responses to osmotic stress are complex and reach from osmolyte biosynthesis to membrane transport of ions, signal transduction and cellular protection (Zhu et al. 1997).

An indicator for stress is the increase of the concentration of proline and soluble sugars as osmoregulators (osmolyte synthesis). Both can act as osmoprotectants (Dorneles et al. 2021). Osmoprotectants are synthesised by plants in response to stress and help stabilising the cell by maintaining the cell turgor, and regulating water movement (Singh et al. 2015). Bündig et al. (2016a) showed that proline is an indicator for stress, but the proline content is not a suitable parameter for distinguishing between genotypes regarding their stress tolerance. For the purpose of determining their tolerance, the root/shoot ratio based on dry mass was more reliable in the in vitro study by Bündig et al. (2016a) on a solid MS-medium (Murashige and Skoog 1962). Dobránszki et al. (2003) also suggested to use traits like survival rate, number and length of roots or the rate of rooted explants of potato to analyse the potato genotypes tolerance towards osmotic stress in an in vitro approach with mannitol as an osmoticum.

Osmotic stress responses, like drought stress responses, are postulated to be genotype-dependent in potato (Gopal and Iwama 2007). However, the stress response of plants can be induced in vitro. If there might be the chance to draw conclusions about drought stress ex vitro must be investigated.

Bündig et al. (2016a) have used an in vitro test system that allows to osmotically stress potato plants and detect differences in stress response between genotypes. For this purpose, the plants were grown on solid medium to which sorbitol was added directly. However, since sorbitol was assumed to be taken up through the cut surfaces of the shoots as sorbitol was found by metabolite

analysis in the shoots of treated plants (Bündig et al. 2016b), the system was proposed to be improved in a way, that plants can regenerate roots, before stress is applied. Furthermore, as solid medium was used, an osmotic shock for the plants occurred. As soil also partially dries over time, it was proposed, that a gradual increase through the addition of the osmoticum to a liquid test system might mimic more closely the natural occuring drought spells for potatoes. The osmoticum should be considered carefully, as it should have a high osmotic potential to induce osmotic stress, should not be taken up and metabolised by the plants. Further, it must be non-toxic to the plants and chemically stable to minimise effects on the results. It is difficult to compare osmotic stress in vitro, and drought stress ex vitro. However, the attempt to find a test system that approximates field conditions is promising for identifying biomarkers for a pre-screening of cultivars.

1.6 The project VALPROKAR

This thesis and experimental work were conducted as part of the cooperation project “Validation of identified marker proteins as a basis for the breeding development of nitrogen-efficient and drought-tolerant starch potatoes (VALPROKAR)”, which was funded by the German Federal Ministry for Food and Agriculture (Bundesministerium für Ernährung und Landwirtschaft, BMEL) and the Federal Agency for Renewable Resources (Fachagentur Nachwachsende Rohstoffe, FNR; funding number Hannover: 22001917).

In the previous project (PROKAR), different starch potato genotypes were characterised with respect to their nitrogen efficiency and their drought and osmotic stress tolerance. For that purpose, divergently responding genotypes to osmotic stress were analysed by IEF/SDS-PAGE, resulting in differentially abundant proteins assigned to proteolysis, and ROS-detoxification. Differences between potato genotypes under drought stress, N deficiency, and combined stress regarding their growth and tuber yield were analysed in rainout shelter experiments. The aim of VALPROKAR was the validation of proteins found in PROKAR in leaf material of those rainout shelter experiments, and identification of new proteins connected to drought stress tolerance and nitrogen efficiency.

One part of the project was implemented by researchers of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben (funding number: 22007018). Candidate proteins from the nitrogen deficiency response were validated in the plasma membrane proteome of the roots. Also, candidate proteins should be detected by directed mass spectrometry.

The other part of the project, which is also the base for this thesis, was conducted at the Leibniz University of Hannover. The in vitro test system, which was set up in PROKAR, was optimised. Furthermore, the proteomic responses and alterations in candidate gene expression level of potato leaves to drought stress ex vitro and shoots to osmotic stress in vitro were analysed. The candidate genes were identified in an proteomic LC-MS analysis in leaf material from two rainout shelter stress experiments in PROKAR. The set was chosen based on the proteomic response to drought stress of two potato genotypes, that were considered rather tolerant to drought stress ('Tomba') and combined drought stress and N deficiency ('Eurostarch'). Combined stress of N deficiency and drought stress was analysed in leaf material of the same two experiments from PROKAR regarding their proteomic response.

1.7 Thesis objectives

The vegetative growth of potato mainly takes place in spring and early summer, which correlates with the time of predicted and already occuring drought periods. This results in cost- and freshwater-intensive artificial irrigation. The need for identification of drought tolerant potato genotypes for breeding by early selection is immense. For this purpose, the aim of this work was to validate known and identify new proteins, which indicate drought stress and may be assigned to drought stress tolerance for subsequent development of biomarkers. These will enable breeders to develop improved varieties with respect to future climate events.

Objectives:

1. Identification of candidate proteins altered in leaves of rather tolerant genotypes under drought stress from two rainout shelter experiments of the predecessor project PROKAR (adressed in chapter 2.1).
2. Identification of genes of interest (GOI) for biomarker development for drought tolerance based on results of objective 1. Gene expression analysis in leaf samples of two open greenhouse experiments (adressed in chapter 2.2).
3. Identification of GOI for osmotic stress tolerance from results of objective 1. Gene expression analysis in shoot samples of in vitro experiments with sorbitol as osmoticum (adressed in chapter 2.3).
4. Optimisation of an existing in vitro test system for osmotic stress. The system should allow a gradual application of the osmoticum to better depict the increasing stress intensity of

drought in the field as soil dries over time and to prevail osmotic shock. Also, the system should allow the plants to form roots prior to the stress treatment to prevail the uptake of sorbitol through the cut surface of the in vitro explants and exclude the overlaying stress of the wounding (adressed in chapter 2.3).

5. Analysis of proteomic alteration under drought stress, nitrogen deficiency, and combined stress in leaf samples of two rainout shelter experiments of PROKAR (adressed in chapter 2.4).

2. Manuscripts

2.1 Identification of candidate proteins in drought stress tolerant and sensitive starch potato genotypes (*Solanum tuberosum* L.) for biomarker development

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Abstract

Due to the foretold climate change, droughts will become more frequent in spring and early summer. This time represents the period of highest vegetative growth in potato development, which highly correlates with later starch yield. Since potato plants are depending on a high supply of nitrogen and water during this period, breeding starch potato varieties with high nitrogen efficiency and increased drought tolerance is of great interest especially for the production of starch for industrial use. Drought tolerance is based on the activation and regulation of specific pathways that lead to physiological, morphological and biochemical stress responses of the plant. In a previous proteomic study, we found 138 differentially abundant proteins by comparing a drought stress tolerant and a sensitive starch potato genotype after application of osmotic stress in an *in vitro* test system on solified medium.

In the present study, the drought-tolerant starch potato genotypes 'Eurostarch' and 'Tomba' as well as the more sensitive genotypes 'Kiebitz' and 'Kolibri' had been submitted to drought stress in rain-out shelter experiments in 2013 and 2015. Proteins were extracted from leaf material of both experiments to identify divergently responding pathways and potential marker proteins for drought tolerance. By means of liquid chromatography-mass spectrometry (LC-MS) and subsequent bioinformatic analysis, 1535 proteins were identified. Out of these, 233 showed a significantly different abundance between control and stressed plants in at least one genotype. Proteins with increased abundances that were exclusively detected in the tolerant genotypes in the drought stress treatment were for example involved in ROS detoxification. These proteins will be of great interest for gene expression studies and will be validated in further experiments involving drought stress *in vivo* as well as osmotic stress treatments *in vitro*.

1. Introduction, Knowledge, Objectives

Solanum tuberosum L. is the world's fourth most important crop after rice, maize and wheat. In 2020, the planted acreage for starch potatoes in Germany was about 58,000 ha (BLE 2021). More than 20 % were used for the production of starch potatoes for industrial purposes, e. g. to produce biogas, bioethanol or adhesives (Röper 2002).

Despite increasing acreage, starch yields decreased dramatically from 2017/18 to 2020. The reason was found in the increasing drought spells during the early growth phases of potato plants. Strong winds and warm or even hot days in early summer months have led to high evaporation and increased land aridity. Precipitation of previous years could not compensate for this aridity (BLE 2021). In many cases the soil-borne water was not enough to supply the shallow-rooted potato plants and irrigation became obligatory. However, irrigation is both labor- and cost-intensive (Iwama and Yamaguchi 2006).

If the vegetative growth phase of *Solanum tuberosum* coincides with the expected dry periods, this will have severely negative consequences for the potato yield and quality at harvest time. Besides inhibition of shoot growth, shift of root/shoot ratio towards the roots and reduced leaf size, drought stress also inhibits photosynthesis, reduces tuber size and –quality, and enhances ROS production (George et al. 2017; Jozefowicz et al. 2017). Thus, selecting and breeding of drought stress tolerant potato genotypes is of high economic and ecological interest.

In previous works, the proteome of potato under nitrogen deficiency and drought stress was characterized in the project PROKAR to increase the knowledge for breeding nitrogen efficient and drought stress tolerant starch potatoes. Therein, differences in the proteome were observed in differently responding genotypes. For example, proteins of proteolysis, specific stress proteins, and proteins of ROS-detoxification were found to play a role. In addition, differences in metabolite concentrations (proline, glycine, sucrose) were found and morphological differences between genotypes were detected in rain-out shelter experiments (Bündig et al. 2016a; 2016b; 2016c). The objectives of our current collaborative project VALPROKAR result from findings in the project PROKAR (FNR 2016; 2022). In VALPROKAR, we are working on the validation of candidate proteins for drought stress tolerance using leaf material of drought-stressed and non-stressed potato plants and plants from a rain-out-shelter experiment. Proteins of interest will be evaluated for their usability as biomarkers in drought stress tolerance breeding for tolerance of starch potato. In this study, we present the identification of new proteins of interest showing differences in abundance between different potato genotypes under drought stress.

2. Data, Methods and Approach

Based on results of Meise et al. (2019), the drought stress tolerant genotypes 'Eurostarch' and 'Tomba' and the drought stress sensitive genotypes 'Kiebitz' and 'Kolibri' were selected for the proteomic analyses. These genotypes were identified in two rain-out shelter experiments based on tuber and starch yields at the end of the growth period (Meise et al. 2019). In our study, we analyzed plant material from these two experiments. These experiments were performed in 2013 and 2015 (Meise et al. 2019). After tuber induction, plants were exposed for five days to drought stress at < 20 % water holding capacity (WHC), whereas control plants were cultivated at 60 % WHC. On the 5th day of stress treatment, the third leaflet of the youngest fully developed pinnate leaf was harvested from stressed and control plants (Fig. 1).

Per genotype, four leaf samples per experiment were analyzed. About 100 mg frozen leaf sample was grinded using 3 mm steel beads and a mixing mill (Retsch, Haan, Germany). Protein extraction was performed using the TCA method (Tsugita and Kamo 1999). TCA solutions A and B consisted of 20 mM DTT instead of 0.07 % (v/v) 2-mercaptoethanol. Next steps were the quantification of the proteins using the 2D-Quant Kit (GE Healthcare,

Munich, Germany) and the filter-based digestion with trypsin (Jozefowicz et al. 2020). Peptides were taken up in 2 % acetonitrile (ACN), 0.1 % formic acid (FA) (v/v), separated by LC (Dionex UltiMateTM 3000 RSLCnano System from Thermo Fisher Scientific, Dreieich, Germany) using analytical column Acclaim PepMap RSLC C18 (50 cm x 75 µm, Thermo Fisher Scientific) and measured by ESI-QTOF-MS/MS (Impact II from Bruker Daltonics, Bremen, Germany). LC-MS data were analyzed using Progenesis QI (Version 3.0, Nonlinear Dynamics, Newcastle upon Tyne, U.K.) for mass correction, alignment, normalization, peak picking, quantification, and statistics. All samples were normalized to one sample automatically selected as normalization reference, using 'normalise to all proteins' default method. MS/MS spectra were used for peptide identification with Mascot v2.5.1.

The database analysis was based on the potato genome Phureja DM1-3 (PGSC_DM_v3.4_pep_representative, 39,031 entries) (Potato Genome Sequencing Consortium et al. 2011) matching against NCBI entries via Blast2GO. The following search parameters were applied: 15 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation as fixed modification, and methionine oxidation as variable modification. Proteins identified with at least two unique peptides and a maximum fold change > 1.5 were quantified. Only proteins that passed the significance limits were considered as differentially abundant proteins (students t-test p value < 0.05 and fold change stress/control <0.66 or >1.5).

Figure 1: Experimental setup of rain-out-shelter experiments in 2013 and 2015. WHC: water holding capacity. Analyzed genotypes were 'Eurostarch', 'Tomba', 'Kiebitz' and 'Kolibri'. Plants were grown until tuber induction. Leaves were harvested on the 5th day of drought stress. Measurement of tuber yield and starch content took place after maturation.

3. Results

In total, 1.535 proteins were found in all samples. Out of these, 444 passed the maximum fold change > 1.5 and 233 proteins of these showed different abundance in at least one of the four genotypes when comparing stressed and control plants passing the student's t-Test (p-value < 0.05) and a fold change stress/control < 0.66 or > 1.5 (Fig. 2). Most of the differentially abundant proteins were identified in the tolerant genotypes 'Eurostarch' and 'Tomba'. For example, 93 proteins showed lower abundance in stressed plants compared to control plants of cultivar 'Eurostarch' in 2013, whereas 54 proteins showed higher abundance. In cultivar 'Tomba', there were 85 proteins lless abundant in stressed plant samples, whereas in 'Kolibri' and in 'Kiebitz'

69 and 39 proteins showed lower abundance in stressed plants, respectively. Comparing proteins with different abundance in drought tolerant and sensitive genotypes, we found that 14 proteins were specifically downregulated in stressed plants of the tolerant genotypes 'Eurostarch' and 'Tomba' in 2013 (13 proteins in 2015), whereas eight proteins were of higher abundance in both experimental years. These proteins are, among others, involved in plant hormone regulation and ROS detoxification and could be a reason for higher drought stress tolerance of 'Eurostarch' and 'Tomba'.

From these proteins, a total of 15 were selected for future studies according to following criteria: All of these proteins were identified in both experimental years in all genotypes. The selected proteins were significantly differentially abundant in both tolerant genotypes in at least one experimental year. Selected proteins are listed in Table 1. Two proteins were differentially abundant in both years and both genotypes (PGSC0003DMT400011762 Protein invertase inhibitor and PGSC0003DMT400055410 Protein subtilisin-like protease-like).

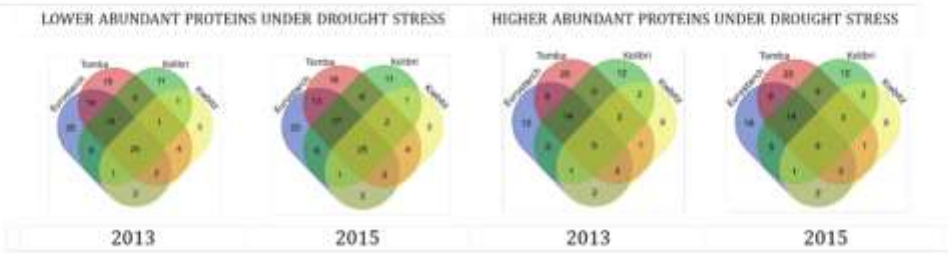

Figure 2: Venn diagrams presenting proteins with differential abundance in drought stressed and control plants of four starch potato genotypes in 2013 and 2015. n= 4 (one leaf sample from the third leaflet of the youngest fully developed leaf from four plants)

Nine proteins were higher abundant under drought stress. Besides some proteins involved in plant hormone regulation, abiotic stress response and proteins of secondary metabolism, some differentially abundant proteins were identified which support the plant in coping with oxidative stress such as 26S protease regulatory subunit 6b homolog, cbs domain-containing protein mitochondrial-like and 2-alkenal reductase (NADP(+)-dependent)-like. There is also one protein of lower abundance under drought stress which is connected to ROS detoxification (catalase isozyme 1-like protein).

Table 1: Proteins with differential abundance in drought stressed and control plants. Higher abundance in stress treatment (S) than in controls (C) in the rather tolerant genotypes 'Eurostarch' and 'Tomba': 1-9. Lower abundance: 10-15.

	Protein	FC 'Eurostarch' [S/C]	FC 'Tomba' [S/C]	year	Pathway
1	phosphoribosylformylglycinamidine cyclo- chloroplastic mitochondrial-like	5.62	5.95	2013	secondary metabolism
2	probable carotenoid cleavage dioxygenase chloroplastic-like	3.65	2.34	2013	hormone regulation, abiotic and biotic stress response
3	26s protease regulatory subunit 6b homolog	1.62	2.53	2013	tolerance to oxidative stress, heat shock protection
4	linoleate 13s-lipoxygenase 2- chloroplastic-like	1.54	1.50	2013	oxylipin biosynthesis
5	cysteine protease inhibitor 1-like	1.52	2.74	2013	inhibition of programmed cell death
6	invertase inhibitor	2.21	1.56	2015	stress response to drought, temperature, salt, ABA
7	cbs domain-containing protein mitochondrial-like	1.87	1.89	2015	ROS detoxification
8	2-alkenal reductase (nadp(+)-dependent)-like	1.72	1.85	2015	ROS detoxification
9	serine mitochondrial-like	1.52	1.94	2015	photorespiratoric enzyme (glycine → serine)
10	catalase isozyme 1-like protein	0.62	0.60	2015	ROS detoxification
11	subtilisin-like protease-like	0.43	0.36	2015	inhibition of leaf senescence
12	peroxidase 51-like	0.64	0.63	2013	NADPH oxidase signaling pathway
13	early tobacco anther 1	0.63	0.63	2013	catalysis of structural changes of molecules
14	dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex-like	0.63	0.64	2013	transferase activity
15	high mobility group family	0.48	0.60	2013	mitochondrial electron transport

4. Conclusions

Drought stress in potato leads to many different stress responses. One of them, oxidative stress, can be triggered by several factors, including the imbalance of ROS synthesis and degradation/scavenging (antioxidation) mechanisms and the *de novo* synthesis of ROS as a defense and adaptation mechanism (Demidchik 2015). Hence, tolerant potato plants might reduce these radicals by synthesizing new antioxidants or ROS scavenging elements. Pieczynski et al. (2018) also found genes encoding oxidative stress-related proteins to be connected to drought stress in potato, *A. thaliana* and rice. Furthermore, Liu et al. (2018) reported a role of ROS scavenging proteins during drought stress in potato.
The tolerant genotypes 'Eurostarch' and 'Tomba' withstood short periods of drought stress better than the sensitive genotypes 'Kiebitz' and 'Kolibri'. One reason for this could be the handling of reactive oxygen species. Specific proteins involved in the formation of antioxidants might allow plants to maintain a protective mechanism against oxidative stress, resulting in less growth deprivation.
As a next step, the proteins of interest will be investigated at gene transcript level in order to draw conclusions about the level of regulation. Further metabolite analyses will be performed to characterize the observed pathways and to identify important pathways. Furthermore, the stress response of the plants will be investigated proteomically and metabolomically in further rain-out shelter experiments to study younger plant stages.

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2.2 Expression analysis of candidate genes as indicators for commencing drought stress in starch potatoes

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Author contribution statement

Material preparation and data collection were performed by KW and CB with help of staff of the section of woody plant and propagation physiology. KW and JS contributed to data analysis. CB and TW conceived and coordinated the project. The first draft of the manuscript was written by KW and CB. The manuscript was revised by JS and TW. All authors have read and approved the final document.

Data availability statement

The data that support the findings of this study are openly available in Research Data Repository of the Leibniz University Hannover at https://doi.org/10.25835/td4w2pg9.

Conflict of interest disclosure

The authors have declared no conflict of interest.

Key Points

- Drought stress was applied in all analysed genotypes without passing the permanent wilting point as indicated by growth reduction
- Setup of stress experiments under open greenhouse conditions is of major importance regarding classification of tolerance levels
- *Invertase inhibitor 1* represents a promising candidate for the detection of early drought stress in young potato plants

Abstract

Drought stress is a major problem for potato production and will be of grave importance due to climate change and the resulting temperature peaks along with drought periods in the vegetative growth phase of potato. Plants, as sessile organisms, adapt to their environment morphologically as well as biochemically. To cope better with abiotic stresses like drought, plants developed strategies like reactive oxygen species (ROS) detoxification and fast reacting stomatal closure, as well as signaling cascades leading to a quick response to stress. This study aimed at analysing eight genes of interest, derived from a former proteomic study, and determining their suitability for detection of commencing drought stress in early growth stages of potato. For this aim, six starch potato genotypes, which differed in stress response in previous studies, were examined for plant growth and physiological parameters in two experiments in an open greenhouse after seven and 14 days of stress. Besides lower shoot biomass after drought stress, which was already visible after seven days and became stronger after 14 days, weaker root growth was also detected after 14 days. The observed differences between the experiments can presumably be explained by temperature peaks and high radiation prior and during the first experiment, which took place earlier in the year. The expression of the eight genes was studied in young leaves of four genotypes after seven days of water withdrawal. Gene expression patterns were dependent on the studied genes. Three genes, *cell wall / vacuolar inhibitor of fructosidase (INH1)*, *peroxidase 51-like (POD)*, and *subtilase family protein (SBT1.7)* showed consistent changes in gene expression after seven days of stress between all genotypes. The *INH1* gene was found to be upregulated in all genotypes in two independent experiments after drought stress. This correlates with the results at the protein level, where INH1 was also found to be higher abundant in two genotypes of potato (Wellpott et al., 2021). Therefore, this gene might be an appropriate candidate for the detection of commencing drought stress in potato.

Introduction:

Potato is one of the most important food crops together with rice, wheat, and maize comprising around 5000 cultivars worldwide. Based on the high adaptability of the plant, potatoes are cultivated in many parts of the world (Bundesanstalt für Landwirtschaft und Ernährung [BLE], 2022). In addition to direct consumption of table potatoes and its use as fodder for animals, starch potatoes are of importance due to their high starch content for industrial purposes such as the production of paper, adhesives and thermoplastics (Röper, 2002; Vreugdenhil et al., 2014).

There are considerable differences in potato yields between the individual continents. In addition to technical and economic development in individual regions, this is due to climatic differences (BLE, 2022). Because of the foretold climate change, potato production worldwide is under severe pressure. Although being adaptable, the plant is rather sensitive to drought stress due to their shallow root system (van Loon, 1981). Drought influences plant growth in form of overall poor growth, reduced photosynthesis rate, reduced leaf area, smaller tubers, and lower starch content (Gervais et al., 2021; Sprenger et al., 2015). Especially prolonged drought and heat periods are known to negatively affect the appearance and physiological properties of the tuber, which drastically reduces the overall quality and market value.

Drought stress is a major problem in potato production and recent years have displayed more severe weather extremes, leading to an obligation in alteration in culture management e.g. irrigation of cultures (Haverkort & Verhagen, 2008). More intense heavy rains occur, followed by dry periods, during which there is not enough water available for the plants in the soil (Intergovernmental panel on climate change [IPCC], 2022). The forecast of a higher frequency and severity of drought periods in spring and early summer, which correlates with the time of highest vegetative growth, will increase the need for more tolerant potato varieties to this abiotic stress.

One of the first reactions of plants to drought stress is a reduction in growth (Dahal et al., 2019). Reduced stem elongation can provide hydration of the plants due to shorter transport distance (Aliche et al., 2020) and a reduction in canopy area decreases the overall transpiration area to avoid further water loss. Plants also react to drought on a molecular level. Abscisic acid (ABA) is shown to be increased after drought stress and induces processes such as the regulation of stomatal closure and primary metabolism (Mustilli et al., 2002; Ruan et al., 2010; Yang et al., 2020). Further, plants respond to drought stress by activating signaling processes (Schaller et al., 2018) and generating ROS (Demidchik, 2015).

Previous transcriptomic studies investigating reactions to drought stress in potato either analysed long-term drought stress (Evers et al., 2010; Aliche et al., 2022) or short-term drought stress under greenhouse conditions or in cell cultures (van Muijen et al., 2016). Complementing these previous reports, this study examined candidate genes after short-term drought stress in an open greenhouse and in an early vegetative growth phase.

The candidate genes were selected based on a previous proteomic study and were encoding proteins of differential abundance in more tolerant potato genotypes after drought stress in a rain-out-shelter trial (Wellpott et al., 2021). Based on this study, we selected eight genes of interest (GOIs) which might play a role and represent potential marker genes for drought stress or drought stress tolerance in potato. From these eight GOIs, Wellpott et al. (2021) found five associated proteins to be higher abundant in two rather tolerant to drought stress genotypes ‘Eurostarch’ and ‘Tomba’: ZBD (Zinc-binding dehydrogenase family protein; enzymes), RPT5a (regulatory particle triple-A ATPase 5A; folding, sorting and degradation), 13-LOX (lipoxygenase; lipid metabolism), SHMT (serine

transhydroxymethyltransferase; carbohydrate metabolism/amino acid metabolism), and INH1 (cell wall / vacuolar inhibitor of fructosidase; enzymes). Three of the eight proteins were found to be lower abundant on protein level after drought stress: Glyx (lactoylglutathione lyase/glyoxalase I family protein; signal transduction), POD (peroxidase 51-like; biosynthesis of other secondary metabolites), and SBT1.7 (subtilase family protein; folding, sorting and degradation) (Table 1).

The aim of this study was to analyse whether the regulation of these differentially abundant proteins also occurred at the transcriptional level. Therefore, we determined plant growth and physiological responses to drought stress of six starch potato genotypes in an open greenhouse after seven and 14 days of commencing drought stress. Because yield loss was reported to be greatest when drought occurred in the vegetative and tuber initiation phase (van Loon, 1981), drought was presented to the plants in this study four weeks after acclimatization. The responses of the eight GOIs were analysed by quantitative reverse transcription (qRT)-PCR in four contrasting genotypes after seven days.

Material and methods

Plant material and experimental setup

Six starch potato genotypes (‘Eurobravo’, ‘Eurostarch’, ‘Kiebitz’, ‘Maxi’, ‘Ramses’, and ‘Tomba’), kindly provided by the respective breeders, were used in the drought stress experiments of this study. These genotypes were selected based on their stress susceptibility index (SSI) according to Fischer & Maurer (1978) calculated for the tuber yield (Meise et al. 2019). ‘Tomba’ and ‘Maxi’ responded rather tolerant under drought stress based on tuber yield. ‘Eurostarch’ was between tolerant and sensitive, whereas ‘Kiebitz’ and ‘Eurobravo’ responded rather sensitive in the test set under drought stress (Meise et al., 2019). ‘Ramses’ was not tested in the study by Meise et al. 2019, however was described as more tolerant compared to a test set (Schumacher et al., 2021).

Nodal cuttings were propagated *in vitro* on solid MS medium (Murashige & Skoog, 1962) containing 3 % sucrose and 7.5 g L^{-1} Plant Agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). Cultivation took place at 18° C in a 16 h photoperiod with a PPFD-PAR of 35 µmol m^{-2} s^{-1}. Three-week-old plants were transferred to pot substrate (70 % peat, 30 % clay, limed to pH 5.5 to 6.5) and were acclimatized for three days by reducing air humidity to regular greenhouse conditions. Cuttings were taken for greater stem stability and after a rooting period of twelve days, they were planted in 2 l containers (⌀ 14 cm, height 18 cm) with 1700 g of a growing medium consisting of pot substrate:sand (1:1 [v/v]; substrate: Einheitserde T, Einheitserdewerke Werkverband e.V., Sinntal-Altengronau; and sand: size 0-2 mm, washed, declared as sand, Lehmann, Burgdorf). All pots were fertilized three times over two weeks with a 1 ‰ solution of Ferty 3 Mega fertilizer (N-P-K: 18-12-18 + 1.2 MgO, total volume per plant: ~ 300 ml). The experiments took place in an open greenhouse (glass roof, open sides) in Hanover, Germany (52°23'36.4"N 9°42'14.3"E) from June 23 to July 16 (experiment 1) and from July 20 to August 12 (experiment 2). The total of 672 experimental plants and 96 boundary plants per experiment were arranged in 24 blocks in a randomized complete block design (RCBD). Drought stress was applied for seven or 14 days. Stressed plants were not irrigated until a water holding capacity (WHC) of 15 % was reached (~ day 7). Control plants were irrigated to a WHC of 60 % by daily weighing. These levels were maintained until evaluation (Fig. 1). Six

additional plants per variant served as recovery plants after seven and 14 days of water withdrawal, respectively. After stress application for seven or 14 days, they were rewatered for nine days to a WHC of 60 %.

Throughout the whole experiment, the shoot length (from the soil surface to the shoot tip) was recorded and SPAD values were measured with a chlorophyll meter SPAD-502 (Konica Minolta Sensing Europe B.V., Nieuwegein, the Netherlands) on the first fully developed leaf of each plant (Table S1). At each evaluation, eight (start of experiment, day 7, day 14) or six (recovery day 7 and recovery day 14) plants (=biological replicates) were harvested and the roots were thoroughly washed to remove the substrate to record the fresh mass. Shoots were separated from roots carefully and weighed. After 48 h at 70 °C, the dry mass of shoots and roots was determined. For gene expression analysis, the third leaflets of the first fully grown leaf of five biological replicates were harvested from extra plants, immediately frozen in liquid N, and stored at -80 °C until further use. Additionally, the relative water content (RWC) in percent in leaves was calculated from the weight of the youngest fully developed leaf of a plant after harvest, after 24 h (100 %) in water, and after 48 h of drying (0 %).

RNA isolation and cDNA synthesis

Frozen leaf samples of five biological replicates of the four genotypes ‘Eurobravo’, ‘Eurostarch’, ‘Maxi’, and ‘Tomba’ from control conditions and after seven days of drought stress (commencing-drought) were separately homogenized in a mixer mill at 27 Hz for 2.5 min (MM400, Retsch, Haan, DE). RNA was extracted from 100 mg of homogenized plant material by using the InviTrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, Germany). Instructions of the manufacturer were followed and the DCT lysis buffer was used. Genomic DNA was removed with DNase I according to the manual (Thermo Scientific, Waltham, MA, USA), and the integrity of RNA was determined in a 1 % agarose gel. For cDNA synthesis, the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used following the instructions of the manufacturer using the oligo-dT primer and 1 µg RNA as a template. The cDNA was diluted 1:10 and stored at -20 °C until further use.

Primer selection

Eight candidate genes were selected based on identified differentially abundant proteins in starch potato leaves under drought stress (Wellpott et al., 2021). For their selection, a focus was set on proteins that were differentially abundant in rather tolerant genotypes ‘Eurostarch’ and ‘Tomba’. Primers were designed meeting the criteria of 18-24 bp length, GC content 40-60 %, amplification product 80-250 bp, and a melting temperature T_M 60 °C (Table S2). Primers were tested for specificity with Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) aligning it to the *Solanum tuberosum* subsp. *tuberosum* genome (NCBI: txid4113). Sequence information for all GOIs was provided by Spud DB (http:// spuddb.uga.edu) using the genomic sequence of *Solanum tuberosum* group Phureja DM1-3 v6.1. All primers were tested in a standard PCR with cDNA of genotype ‘Eurostarch’ as a template and an annealing temperature T_A = 60 °C and checked on a 1.5 % agarose gel. The PCR products were sequenced by Sanger sequencing (Sanger et al., 1977). A list of all used primers is provided in Table S2. Sequencing results can be found in the LUH data repository under the following link:

https://doi.org/10.25835/td4w2pg9. Alignments were performed via MAFFT v7 (Katoh & Standley, 2013) using Benchling (benchling.com).

RT-qPCR

The real-time quantitative RT-PCR was performed using the Applied Biosystems QuantStudio 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). All primers were tested with a pool of all cDNA samples for their efficiency. Primer efficiencies calculated in the software QuantStudio™ Real-Time PCR Software v1.3 are listed in Table S2. Only primers with single peaks in the melt curve analysis were selected for further analysis. This resulted in eight genes of interest (GOI) that were analysed. Genes *EF1a* (elongation factor α), *APRT* (adeninphosphoribosyltranferase), and *Cyclo* (cyclophilin) were used as reference genes (Nicot et al., 2005). They were tested for stability in RStudio (2022.07.1 Build 554) based on R version 4.1.3 using the NormFinder algorithm (Andersen et al., 2004). Because of a stability value > 0.25, *EF1a* was excluded from calculations of the normalized gene expression. Each sample was measured in three technical replicates. Five biological replicates were analysed for each genotype ('Eurobravo', 'Eurostarch', 'Maxi', 'Tomba') at the start of the experiment (T0) and after seven days under control conditions (T7C) and drought stress (T7S). In total, diluted cDNA of 120 samples was mixed with Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) diluted 1:4 (v/v) for analysis with every primer pair (final concentration in reaction: 0.2 µM). Following PCR conditions were used: one cycle at 95 °C for 60 s, 40 cycles at 95 °C for 15 s, one cycle at 60 °C for 60 s. Subsequently, melting curve analysis (60 °C to 95 °C with an increment of 0.5 °C/15 s) was conducted to determine specificity of amplification. Data was further processed with QuantStudio™ Real-Time PCR Software v1.3. Data are shown as normalized gene expression (Pfaffl, 2001).

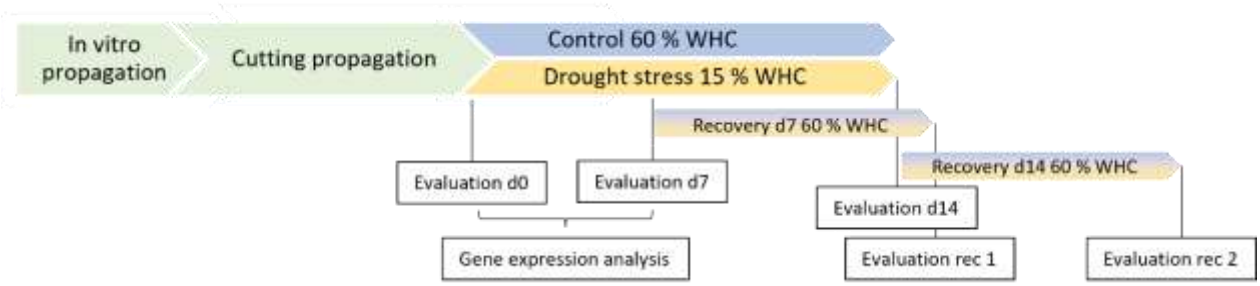


Fig 1 Timeline of drought stress experiments in an open greenhouse. Six starch potato genotypes were propagated in vitro, acclimatized, and once propagated via cuttings. Drought stress variants were watered daily to a WHC of 15 %, control plants received water to 60 % WHC. Evaluations took place on d0: start of the experiment, d7: seven days under drought stress, d14: 14 days under drought stress, rec 1: nine days of recovery (60 % WHC) after seven days of drought stress, rec 2: nine days of recovery (60 % WHC) after 14 days of drought stress. Samples for gene expression analysis were taken at d0 and d7.

Statistical analysis

Graphics and statistical analysis for growth data as well as for gene expression data were performed in R version 4.1.3 (R Core Team, 2022) using RStudio v. 2022.07.1 Build 554 (RStudio Team, 2022). Figures were produced

using the packages ‘ggplot2’ (Wickham, 2016), ‘cowplot’ (Wilke, 2020), ‘ggpubr’ (Kassambara, 2020), ‘ggsci’ (Xiao, 2018), and ‘RcolorBrewer’ (Neuwirth, 2014) were used. The data were tested for normal distribution with the Shapiro-Wilk test, an analysis of variance (ANOVA) was calculated to assess main treatment and genotype effects and interactions, and means were compared pairwise by Tukey tests at $p < 0.05$. To minimise unwanted site effects, a randomised complete block design with 24 blocks was used. When normal distribution was not given, the data was either log-transformed or further analysed by a Kruskal-Wallis-Test with Bonferroni adjustment. Packages used for statistical analyses were ‘emmeans’ (Lenth, 2022), ‘multcomp’ (Hothorn et al., 2008) and ‘agricolae’ (Mendiburu, 2021).

Results

Growth parameters under drought stress after seven and 14 days

Noticeable differences between treatments in the morphology and growth of all genotypes were observed in two experiments over time. Plants after seven days of water withdrawal showed lower height, darker leaves that began to wilt, and overall poorer growth than control plants. These observations were even more pronounced after 14 days of stress (Fig. 2). There were significant differences in the biomass data between the two experiments. This might be due to temperature differences in the week before the start of the drought treatment as well as in the first seven days of stress between the experiments and higher sum of global radiation throughout the first experiment (Tables S3, S4 and Figure S1). In experiment 1, which took place in June 2021, temperature peaks were detected on days -5/-4 (31.2/31.4 °C daily mean temperature measured in the canopy). On these days in experiment 2, which took place in July 2021, the daily mean temperature was considerably lower (26.5/22.8 °C). Another peak in experiment 1 was observed on day 4 (32.2 °C) of the experiment whereas in experiment 2 the temperature was rather moderate (24.8 °C).

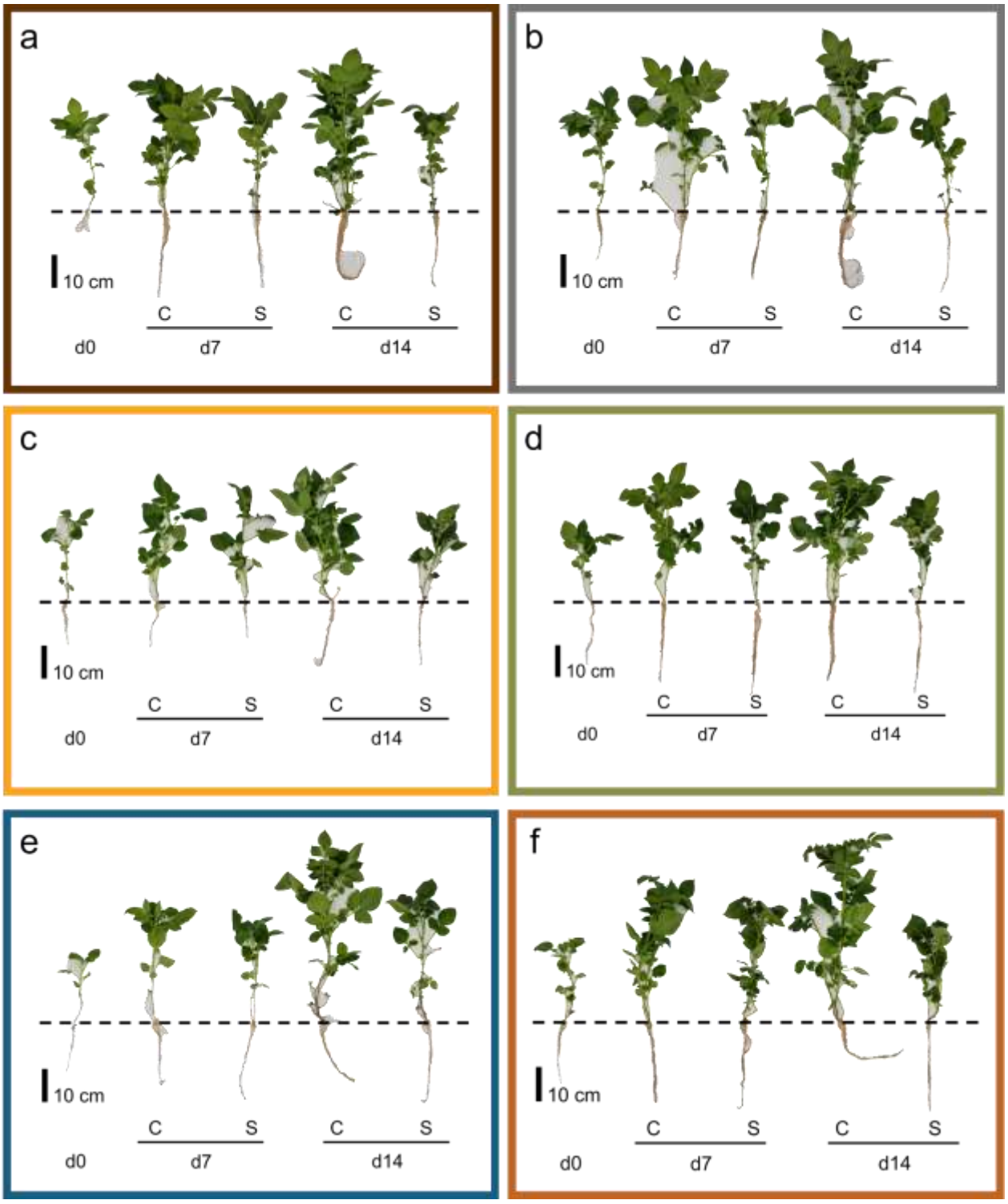


Fig. 2 Plants at the start of the experiment (d0) and after seven (d7) or 14 days (d14) at either control (C) or drought stress (S) conditions. **a**: 'Eurobravo', **b**: 'Eurostarch', **c**: 'Kiebitz', **d**: 'Maxi', **e**: 'Ramses', **f**: 'Tomba. C: control plants (60 % WHC), S: stressed plants (15 % WHC).

Since the genotypes 'Kiebitz' (experiment 1 0.22 g/experiment 2 0.53 g), 'Ramses' (0.23 g/0.38 g), and 'Tomba' (0.29 g/0.58 g) entered the experiments with lower shoot dry mass compared to the other genotypes ('Eurobravo': 0.5 g/1.11 g, 'Eurostarch': 0.53 g/0.93 g, 'Maxi': 0.43 g/0.58 g), the growth data are shown as increments, to account for these differences (Fig. 3 and 4). Absolute mass data can be found in Supplementary Table S5.

After seven days of water withdrawal, the plants of all genotypes showed a lower increase in shoot dry mass under drought stress than under control conditions. For genotype 'Maxi', this difference was significant in both experiments (reduction of 54.6 % and 43.2 % in experiments 1 and 2, respectively), as well as for 'Eurobravo' (53.2 %) and 'Eurostarch' (54.5 %) in experiment 2 (Fig. 3 a, b). In experiment 1, 'Eurobravo' gained significantly more shoot mass than all other genotypes (1.0 ± 0.14 g). In experiment 2, there were no significant differences between the genotypes. High variation and no significant differences in root dry mass increase between control and drought-stressed plants were recorded after seven days for all genotypes (Fig. 3 c, d).

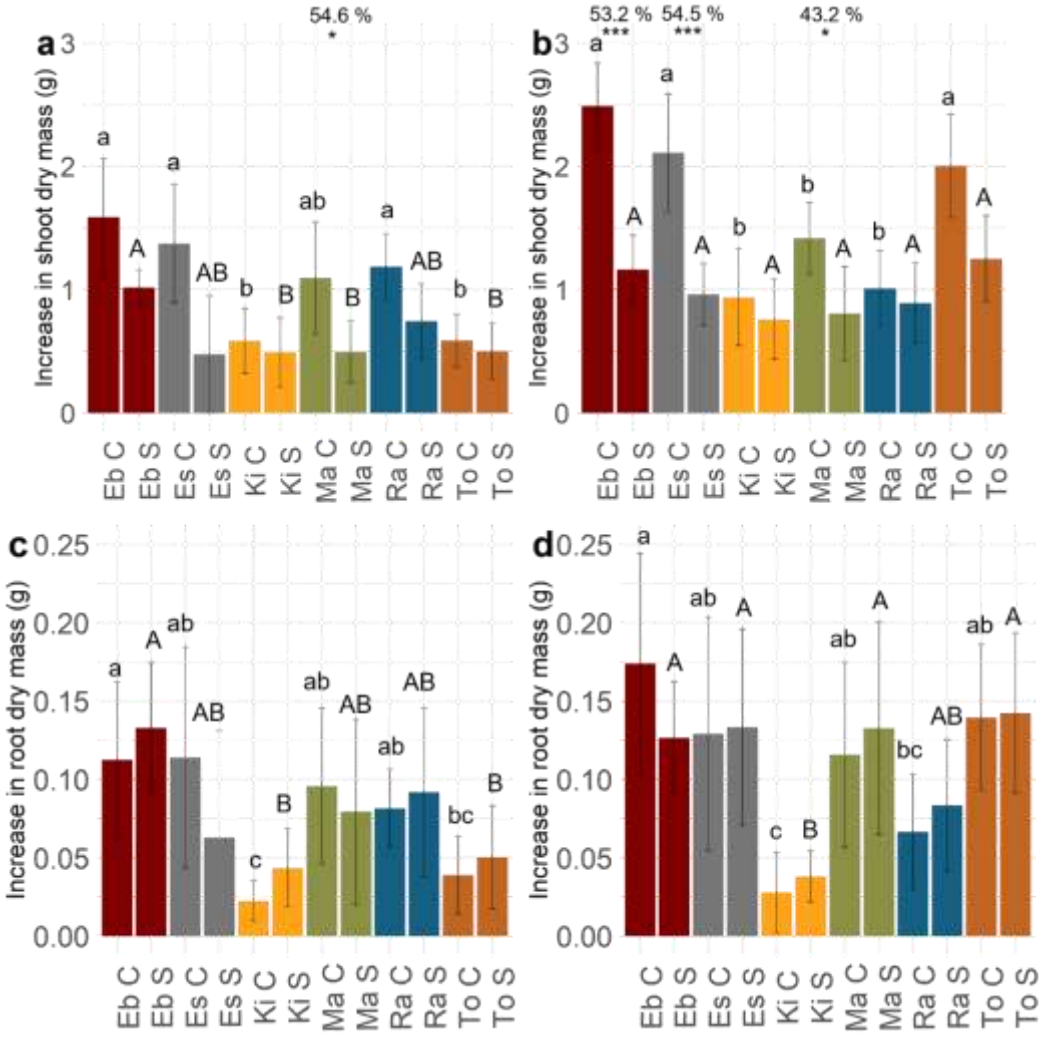
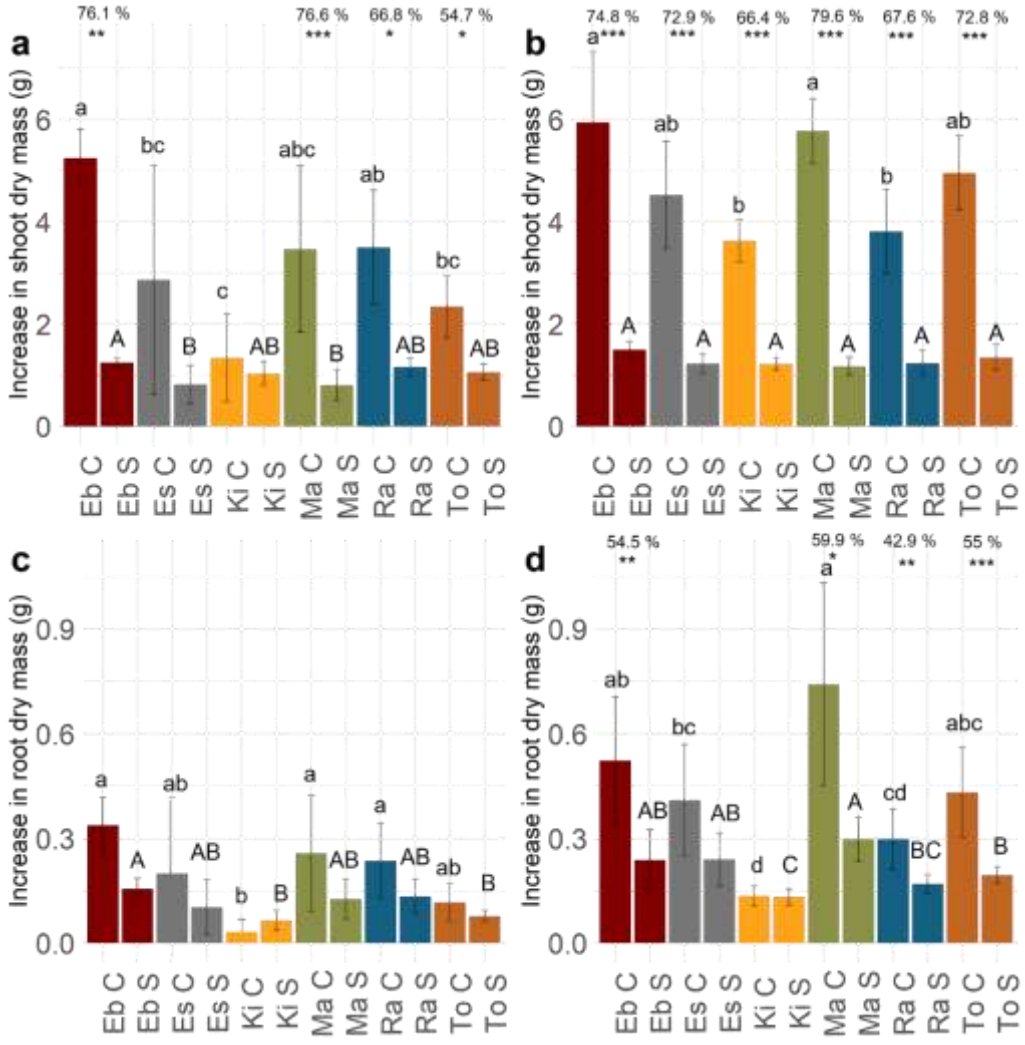


Fig. 3 Increase in shoot (**a**,**b**), and root dry mass (**c**,**d**) in gram after seven days of drought stress with standard deviation, n=8. **a**/**c**: experiment 1, **b**/**d**: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ki: 'Kiebitz', Ma: 'Maxi', Ra: 'Ramses', To: 'Tomba'. C: control, S: stress. Statistical analysis: Kruskal-Wallis test with Bonferroni correction. Significance codes: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

After 14 days of drought stress, a significantly reduced increase in shoot mass was noticed for all genotypes in experiment 2 (74.8 % 'Eurobravo', 72.9 % 'Eurostarch', 66.4 % 'Kiebitz', 79.6 % 'Maxi', 67.6 % 'Ramses' and 72.8 % 'Tomba', see Fig. 4 a, b). In experiment 1, this was only observed for 'Eurobravo' (76.1 %), 'Maxi' (76.6 %), 'Ramses' (66.8 %), and 'Tomba' (54.7 %). The shoot mass increase in drought-stressed plants in experiment 1 was significantly different between 'Eurobravo' (1.52 ± 0.09 g), 'Eurostarch' (0.82 ± 0.37 g) and

275 'Maxi' (0.81 ± 0.3 g), whereas there were no significant differences among the genotypes for drought-stressed
276 plants in experiment 2. For the increase of root mass, no significant differences between control and drought stress
277 variants were recorded in experiment 1 (Fig. 4 c, d). In experiment 2, however, for 'Eurobravo' (54.5 %), 'Maxi'
278 (59.9 %), 'Ramses' (42.9 %), and 'Tomba' (55.0 %) the root dry mass increment of drought stressed plants was
279 significantly lower than that of control plants.



280

281 **Fig. 4** Increase in shoot (**a**,**b**), and root dry mass (**c**,**d**) in gram after 14 days of drought stress with standard
282 deviation, n=8. **a**/**c**: experiment 1, **b**/**d**: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ki: 'Kiebitz', Ma: 'Maxi',
283 Ra: 'Ramses', To: 'Tomba'. C: control, S: stress. Statistical analysis: Kruskal-Wallis test with Bonferroni
284 correction. Significance codes: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

285

286

287 ***INH1, POD* and *SBT1.7* displayed consistent changes of gene expression in all genotypes after seven days of**
288 **drought stress**

289 The normalized expression of the candidate genes was analysed in leaf material at the start of the two experiments
290 (day 0) and after seven days under drought stress (day 7) to determine the early stress response of the analysed
291 potato genotypes (Table 2).

292

Table 2 Mean values of normalized expression of eight genes of interest (GOIs) in leaf tissue of four potato genotypes at the start of the experiment (day 0) and after seven days (day 7) of cultivation under control conditions or drought stress (± SD). Letters a-c display significant differences in between a box of four genotypes in one variant and one gene of interest (Tukey Test or Kruskal-Wallis Test with Bonferroni correction, n=5). Heat map colors reach from green (lowest value) to red (highest value) and were calculated for every column separately. *Glyx: Lactoylglutathione lyase / glyoxalase I, ZBD: Zinc-binding dehydrogenase family protein, RPT5a: regulatory particle triple-A ATPase 5A, 13-LOX: lipoxygenase, SHMT: serine transhydroxymethyltransferase, POD: Protein peroxidase 51-like, SBT1.7: Subtilase family protein, INH1: cell wall / vacuolar inhibitor of fructosidase.* For *INH1*, *POD* and *SBT1.7* see also Fig. 5.

Experiment	Day	Genotype	Variant	Glyx	ZBD	RPT5a	13-LOX	SHMT	POD	SBT1.7	INH1
1	0	Eurobravo	Start	0.082 ± 0.037 a	0.162 ± 0.026 a	0.361 ± 0.052 a	0.490 ± 0.135 a	10.656 ± 1.480 a	0.025 ± 0.007 a	0.100 ± 0.027 a	0.077 ± 0.017 a
		Eurostarch	Start	0.064 ± 0.032 a	0.150 ± 0.023 a	0.363 ± 0.069 a	0.483 ± 0.129 a	8.029 ± 3.208 a	0.019 ± 0.004 a	0.120 ± 0.022 a	0.099 ± 0.039 a
		Maxi	Start	0.061 ± 0.027 a	0.151 ± 0.029 a	0.248 ± 0.045 b	0.261 ± 0.068 b	4.042 ± 1.560 b	0.021 ± 0.005 a	0.074 ± 0.017 a	0.076 ± 0.028 a
		Tomba	Start	0.034 ± 0.012 a	0.169 ± 0.028 a	0.229 ± 0.070 b	0.416 ± 0.186 ab	3.348 ± 0.623 b	0.031 ± 0.018 a	0.094 ± 0.048 a	0.087 ± 0.044 a
	7	Eurobravo	Control	0.053 ± 0.033 a	0.156 ± 0.069 b	0.422 ± 0.084 a	0.400 ± 0.284 a	7.023 ± 4.801 a	0.038 ± 0.014 ab	0.315 ± 0.205 a	0.184 ± 0.063 a
		Eurostarch	Control	0.039 ± 0.015 a	0.141 ± 0.065 b	0.392 ± 0.087 a	0.272 ± 0.210 a	5.533 ± 3.895 a	0.031 ± 0.016 b	0.186 ± 0.126 a	0.253 ± 0.096 a
		Maxi	Control	0.049 ± 0.024 a	0.204 ± 0.042 ab	0.424 ± 0.063 a	0.161 ± 0.042 a	4.033 ± 1.495 a	0.095 ± 0.041 a	0.125 ± 0.042 a	0.340 ± 0.155 a
		Tomba	Control	0.079 ± 0.018 a	0.296 ± 0.050 a	0.336 ± 0.047 a	0.284 ± 0.167 a	7.576 ± 1.286 a	0.070 ± 0.016 a	0.187 ± 0.076 a	0.177 ± 0.028 a
		Eurobravo	Drought stress	0.035 ± 0.007 c	0.116 ± 0.021 c	0.240 ± 0.045 b	0.286 ± 0.206 a	4.665 ± 1.418 b	0.004 ± 0.003 b	0.104 ± 0.062 b	0.768 ± 0.444 ab
		Eurostarch	Drought stress	0.067 ± 0.021 b	0.145 ± 0.047 bc	0.236 ± 0.040 b	0.326 ± 0.154 a	7.444 ± 2.947 ab	0.004 ± 0.003 b	0.118 ± 0.053 ab	1.090 ± 0.193 ab
		Maxi	Drought stress	0.076 ± 0.025 b	0.242 ± 0.051 b	0.317 ± 0.036 ab	0.189 ± 0.139 a	6.398 ± 2.162 ab	0.028 ± 0.013 a	0.137 ± 0.046 ab	1.283 ± 0.329 a
		Tomba	Drought stress	0.141 ± 0.023 a	0.390 ± 0.081 a	0.374 ± 0.046 a	0.563 ± 0.273 a	9.966 ± 1.679 a	0.054 ± 0.016 a	0.233 ± 0.076 a	0.247 ± 0.029 b
2	0	Eurobravo	Start	0.115 ± 0.021 a	0.232 ± 0.056 bc	0.366 ± 0.053 a	0.579 ± 0.094 a	9.721 ± 1.276 a	0.020 ± 0.003 b	0.205 ± 0.052 a	0.270 ± 0.054 b
		Eurostarch	Start	0.073 ± 0.024 a	0.161 ± 0.012 c	0.309 ± 0.007 ab	0.474 ± 0.073 ab	9.130 ± 1.543 a	0.019 ± 0.005 b	0.177 ± 0.021 ab	0.303 ± 0.049 b
		Maxi	Start	0.105 ± 0.027 a	0.260 ± 0.021 b	0.271 ± 0.012 b	0.324 ± 0.067 b	8.575 ± 0.382 a	0.016 ± 0.005 b	0.135 ± 0.011 b	0.617 ± 0.114 a
		Tomba	Start	0.124 ± 0.045 a	0.359 ± 0.048 a	0.369 ± 0.039 a	0.301 ± 0.126 b	8.424 ± 1.013 a	0.033 ± 0.002 a	0.136 ± 0.010 b	0.316 ± 0.033 b
	7	Eurobravo	Control	0.082 ± 0.038 a	0.207 ± 0.023 b	0.322 ± 0.039 ab	0.494 ± 0.128 ab	10.211 ± 1.323 a	0.014 ± 0.005 b	0.373 ± 0.029 a	0.383 ± 0.063 a
		Eurostarch	Control	0.049 ± 0.006 a	0.211 ± 0.018 b	0.307 ± 0.014 b	1.076 ± 0.260 a	9.944 ± 1.220 a	0.019 ± 0.002 b	0.351 ± 0.069 a	0.303 ± 0.110 ab
		Maxi	Control	0.073 ± 0.048 a	0.279 ± 0.033 a	0.370 ± 0.067 ab	0.332 ± 0.127 b	14.354 ± 4.918 a	0.022 ± 0.007 b	0.233 ± 0.049 b	0.419 ± 0.123 a
		Tomba	Control	0.054 ± 0.012 a	0.335 ± 0.022 a	0.400 ± 0.026 a	0.329 ± 0.086 b	14.197 ± 2.387 a	0.054 ± 0.015 a	0.211 ± 0.042 b	0.196 ± 0.050 b
		Eurobravo	Drought stress	0.071 ± 0.159 a	0.214 ± 0.067 b	0.361 ± 0.139 a	0.274 ± 0.114 a	7.948 ± 2.877 a	0.001 ± 0.000 b	0.042 ± 0.019 a	3.122 ± 5.634 a
		Eurostarch	Drought stress	0.057 ± 0.008 ab	0.210 ± 0.022 b	0.344 ± 0.026 a	0.171 ± 0.029 a	9.851 ± 1.346 a	0.001 ± 0.000 c	0.028 ± 0.007 a	3.146 ± 0.525 a
		Maxi	Drought stress	0.046 ± 0.012 ab	0.309 ± 0.014 a	0.338 ± 0.024 a	0.120 ± 0.038 a	6.737 ± 2.800 a	0.001 ± 0.000 bc	0.029 ± 0.009 a	2.642 ± 0.334 a
		Tomba	Drought stress	0.042 ± 0.020 a	0.297 ± 0.038 a	0.358 ± 0.032 a	0.364 ± 0.255 a	5.725 ± 0.909 a	0.003 ± 0.001 a	0.045 ± 0.015 a	1.721 ± 0.162 b

Expression of *Glyx* (*lactoylglutathione lyase / glyoxalase I family protein*), did not show significant changes after seven days between control and stress (Table 2). *13-LOX* (*lipoxygenase*), *RPT5a* (*regulatory particle triple-A ATPase 5A*), *SBT1.7* (*subtilase family protein*) and *SHMT* (*serine transhydroxymethyltransferase*) differed in their regulation of expression between experiments 1 and 2. While no changes in gene expression was detected in experiment 1 for *13-LOX* and *SHMT*, this changed in experiment 2 as the expression in 'Eurobravo', 'Eurostarch' and 'Maxi' decreased for *13-LOX* and decreased in 'Maxi' and 'Tomba' for *SHMT* (Table 2). While in experiment 1 the gene expression was reduced under stress for all genotypes except 'Tomba' for *RPT5a*, no alteration was detected in experiment 2. Expression analysis for *ZBD* displayed no alteration in level, except for 'Tomba' in experiment 1 where it was significantly upregulated. Furthermore, a reduction in expression was detected for *POD* after 7 days of water withdrawal for all genotypes, except 'Tomba' in experiment 1, where there was no visible change (Fig. 5 a,b). Highest expression levels of *POD* were observed in 'Maxi' and 'Tomba' in experiment 1 and in 'Tomba' in experiment 2. The lowest fold change (stress/control) showed 'Eurostarch' in experiment 2 (0.03). For the gene *SBT1.7*, a gene for a subtilase family protein, a significantly lower expression in stressed plants was detected in 'Eurobravo' in experiment 1, while a reduction to the same level took place in the stressed variants of all genotypes in experiment 2 (Fig. 5 c,d). After 7 days of water withdrawal, genotypes in experiment 1 and 2 displayed a higher expression of INH1 (*cell wall / vacuolar inhibitor of fructosidase*), except for genotype 'Tomba' in experiment 1 (Fig. 5 e,f). Fold changes (stress/ control) reached from 3.77 ('Maxi') to 4.3 ('Eurostarch') in experiment 1 and were more pronounced in experiment 2 (from 6.31 in 'Maxi' to 15.51 in 'Eurobravo') (Table S6).

If the normalized gene expression at day 0 before starting the experiments was considered, all genotypes showed a higher expression level of *INH1* and *SBT1.*7 in experiment 2 than in experiment 1 (Table S7). Furthermore, 'Tomba' displayed a higher gene expression of *Glyx, RPT5a, ZBD* and *SHMT*, on day 0 in experiment 2 than in experiment 1. This was also the case for 'Maxi', except for *RPT5a.* 'Eurobravo' also showed higher gene expression of *ZBD* in experiment 2. Expression of *POD* and *13-LOX* was on a similar level in both experiments in the respective genotypes (Table S7).

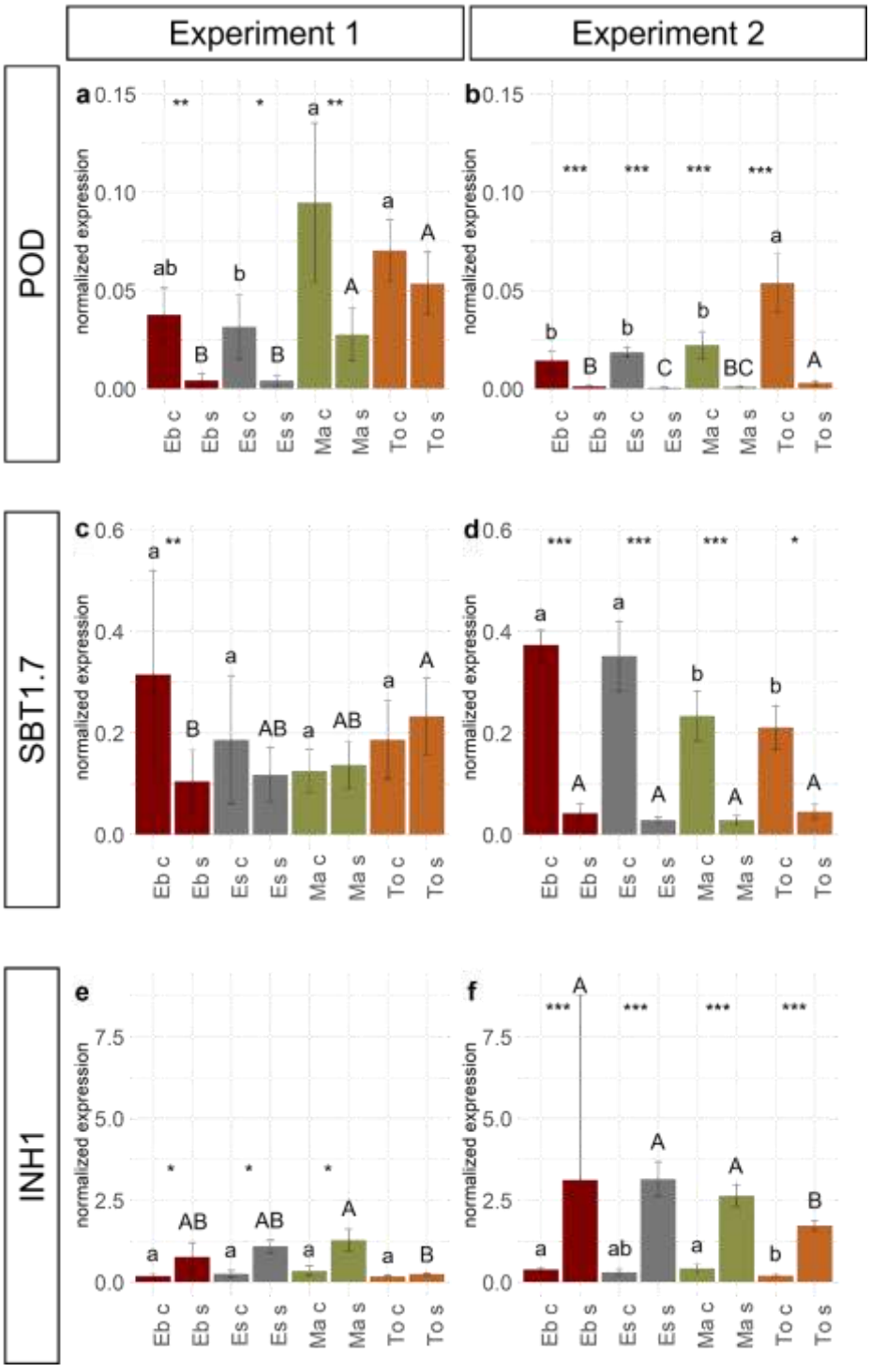


Fig. 5 Normalized expression of the genes *protein peroxidase 51-like* (*POD;* **a,b**), *subtilase family protein* (*SBT1.7;* **c,d**), and *cell wall / vacuolar inhibitor of fructosidase* (*INH1;* **e,f**) after seven days under drought stress or controlled conditions in four potato genotypes with standard deviation, n=5. **a,c,e**: experiment 1, **b,d,f**: experiment 2. Eb: 'Eurobravo', Es: 'Eurostarch', Ki: 'Kiebitz', Ma: 'Maxi', Ra: 'Ramses', To: 'Tomba'. c: control, s: stress. For *INH1* Eb s only positive SD is given. Statistical analysis: Kruskal-Wallis test with Bonferroni correction. Significance codes: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

Discussion

In the early growth phases of potato, drought has a huge impact on quality and quantity of the later yield. Therefore, in this study, early responses to drought stress in late vegetative or early tuber initiation phases of potato were analysed.

Drought decreases overall plant growth after 7 and 14 days of stress

After 7 days (commencing stress) a reduction in plant height, reduced increase in shoot dry mass, darker leaves, and wilting was determined. After 14 days (intensified stress) these changes became more pronounced, but the permanent wilting point was not reached. This can be seen in the data of the recovery plants. All re-watered plants of all genotypes recovered from drought stress and resumed growth (Tab. S4, 'Rec 1' and 'Rec 2'). A reduction in shoot growth under abiotic stress is well described and is among the first visible signs of plant responses to stress (Dahal et al., 2019). Cells enter a status of growth arrest until stress relieve, therefore reducing the leaf area and minimizing water loss through the leaf area (Takahashi et al., 2019). However, a recent study also implies, that a shorter transport way for water and nutrients might also play a role (Aliche et al., 2020).

No significant effect on root growth could be detected after 7 days of drought stress for both experiments (Fig. 3 c,d). After 14 days of drought stress, still no alteration in root growth was observed in experiment 1 (Fig. 4 c,d). However, for experiment 2, a significant reduction in root dry mass was observed for 'Eurobravo', 'Maxi', 'Ramses' and 'Tomba'. This is in agreement with previous results by Boguszewska-Mańkowska et al. (2020) and Lahlou & Ledent (2005), who reported that root growth reduction took place under drought stress in a genotype-specific manner. More tolerant genotypes were shown to have constant root biomass under stress compared to control plants. Based on our data, this was observed for all genotypes in experiment 1 and for 'Eurostarch' and 'Kiebitz' in experiment 2, indicating,

The overall difference in growth between the experiments was striking. Three of six analysed genotypes ('Eurobravo', 'Eurostarch', and 'Maxi') showed significantly higher shoot increment in control plants than in stressed plants after 14 days of drought stress in the first experiment, while in the second experiment the shoot dry mass increased similarly in all genotypes. One important difference between the experiments, which may explain the differences, were the temperature peaks before the beginning of the drought stress phase in experiment 1. Additional heat stress, or more generally double stress, leads to a series of reactions in the plant, which do not mirror the responses under single stress (Meise et al., 2018; Pandey et al., 2015). Mittler (2006) displayed potential correlation effects based on a metadata search of potential double stressors, were heat and drought stress were described as potential negatively correlated. In addition, the differences between genotypes that Meise et al. (2019) or Sprenger et al. (2015) showed, could not be reproduced in the growth data with our setup. However, there are major differences between our experimental setup and those conducted so far. First, in the present study, a large amount of sand was used in the substrate (50 %), as this corresponds more closely to the soil properties in Lower Saxony (Goffart et al., 2022). Also, 2 l containers were chosen instead of larger pots because the plants were not cultivated to natural maturity as in other studies, where yield was analysed. The open greenhouse is a rigid structure with an immovable roof. This contrasts with a rain-out shelter or closed greenhouse as were used in previous studies. This suggests that external circumstances such as pot/ container size, substrate, and environment play an important role in plant response and tolerance groups can only be named within a setup.

This points to the importance of recording and considering physical growth conditions in stress experiments, especially under the semi-controlled settings of open greenhouse and field experiments.

Stable expression of *Glyx* and *ZBD* under commencing drought stress, *RPT5a* expression differs between experiments

The candidate genes in this study were selected based on differentially abundant proteins identified in Wellpott et al. (2021) after drought stress. Significantly higher protein abundances under drought stress were shown for *RPT5a*, *ZBD*, *INH1*, *SHMT* and *13-LOX*, whereas lower abundances under drought stress were detected for *Glyx*, *POD* and *SBT1.7*.

No alteration in gene expression was recorded for *Glyx*, a protein of the glyoxalase system. The protein detoxifies methylglyoxal (MG) in the first step of the glyoxalase system, which was proposed as a signaling molecule under abiotic stress (Hoque et al., 2016; Kaur et al., 2014). Likewise, expression of *ZBD* was not altered during commencing drought stress after seven days, the only exception being 'Tomba' in experiment 1, where *ZBD* expression was significantly increased. Zinc-finger proteins are a family of diverse proteins containing the zinc-finger motif. Comparing the obtained *ZBD* sequence in the SpudDB database showed that the most likely protein was an allyl alcohol dehydrogenase (Soltu.DM.03G015960) (Spud DB, 2022). Alcohol dehydrogenases (ADH) are encoded by a multigene family in plants and have been reported to play a critical role in plant growth, development, and adaptation (Jörnvall et al., 2010; Strommer, 2011). As allyl alcohol dehydrogenases generate NADPH, which can be used as a coenzyme in photosynthesis, no alteration in gene expression might indicate a steady need for reducing agents.

RPT5a was shown to be down-regulated in commencing drought stress after seven days in experiment 1, the exception again being 'Tomba' where no alteration in gene expression was detected. However, in experiment 2 differences were detected for all genotypes between control and stressed plants. RPT represent a large family of regulatory particles for ATPases that have a conserved AAA-motif. They are associated with the 26S proteasome and are essential for the unfolding of the substrates for degradation through mechanical shift (Bar-Nun & Glickman, 2012). The neighbors RPT5/6 within the RPT complex were reported to be essential for the binding of ubiquitin chains from marked proteins to the proteasome (Lam et al., 2002). The decrease in gene expression after seven days of drought stress compared to control plants in *RPT5a* might be explained by phases of high temperature before the sampling of leaves in experiment 1. High temperatures might have led to sort of priming or stress memory effect and a subsequent drop in gene expression at the sampling date (H. Liu et al., 2022).

***LOX* activity is connected to light and temperature**

Expression of *13-LOX* (*lipoxygenase*) was downregulated under drought stress in experiment 2 in 'Eurobravo', 'Eurostarch', and 'Maxi'. In contrast, in experiment 1 there was no alteration in expression after stress, but the gene expression level of *13-LOX* in experiment 1 was similar to the expression level after stress in experiment 2. Lipoxygenases could be correlated positively to ABA synthesis after drought stress and are linked to plant development

and stress adaption (Deluc et al., 2009; Liavonchanka & Feussner, 2006). They can be divided into *9-LOX* and *13-LOX* based on their position of fatty acid oxygenation (Bae et al., 2016). *13-LOX* genes are expressed mainly in the above-ground plant organs, whereas *9-LOX* genes are produced mostly in roots and tubers. *13-LOX* genes play a role in the oxylipin biosynthesis through the lipoxygenase (LOX) cascade in the plant. Well-studied oxylipins are jasmonates, which activate transcription of genes involved in plant defense (Royo et al., 1996). *LOX* activity is also associated with tuberization in potato and their expression can be directly correlated to light range and temperature (Nam et al., 2005). The occurring temperature peaks in experiment 1 and the correlation between light, temperature and *LOX* expression indicate that *13-LOX* was downregulated by both stresses, heat/oxidative stress and drought and can presumably be linked to postponing of tuber formation.

Results indicate a rapid stress response for *SHMT*

Stomatal closure causes down-regulation of photosynthesis, due to less available CO_2. This also leads to changes in gene expression of some genes involved in carbohydrate metabolism, such as *SHMT*. SHMT is a pyridoxal-5'-phosphate (PLP)-dependent enzyme which is linked to catalysing the conversion of glycine to serine and vice versa. *SHMT* activity results in one-carbon units, which are important for many cellular processes, including the synthesis of chlorophyll (Jabrin et al., 2003; Ruszkowski et al., 2018). In plants, mitochondrial SHMT enzymes provide these amino acids for chlorophyll biosynthesis and are linked to photorespiration (Douce et al., 2001; Z. Liu et al., 2022). Furthermore, ROS production is increased under stress, leading to damage to cellular components. One strategy of the plant to protect and adapt to oxidative stress is the detoxification of ROS (Demidchik, 2015) which also involves SHMT (Fang et al., 2020). *SHMT* expression was significantly decreased after seven days of commencing drought stress only in experiment 2 in 'Maxi' and 'Tomba'. In experiment 1 *SHMT* expression was increased in 'Eurostarch', 'Maxi', and 'Tomba', but those alterations were not significant. Hourton-Cabassa et al. (1998) also observed a downregulation of *SHMT* after drought stress in potato. Ambard-Bretteville et al. (2003) showed a drastic downregulation of *SHMT* after an upregulation 8 h after the onset of drought stress in potato. These outcomes, and the fact that the enzyme was higher abundant in potato leaves after drought stress in Wellpott et al. (2021) indicate a rapid response of *SHMT* expression, which should be verified by analysing earlier time points after stress.

Commencing drought stress reduces *POD* and *SBT1.7*, but induces *INH1* expression

Goals of the gene expression analyses were to find evidence whether regulation occurred at the transcriptional level for the selected proteins of interest, and to identify possible molecular markers for early drought stress in potato. The genes *POD, SBT1.7* and *INH1* showed very consistent regulation in all genotypes after commencing drought stress after seven days with *INH1* displaying the highest normalized expression levels.

A reduction of gene expression was detected for *POD* and *SBT1.7* in a specific manner regarding the experiments. Reduction of gene expression was evident for *POD*, a peroxidase superfamily protein, in experiment 1 and 2. However,

an exception was ‘Tomba’ in experiment 1, where no significant change in gene expression was detected. Peroxidases function in detoxification of hydrogen peroxide (H_2O_2), which is known to be related to cell wall modifications and is well known as a signaling molecule under oxidative stress (Boguszewska et al., 2010; Kopyra & Gwóźdź, 2003; Mittler, 2002). Most studies published report an increase in *POD* gene expression, or the activity of the enzymes produced after drought stress (for review see: Suzuki et al., 2012). Earlier time points might be more conclusive as for the gene expression of *POD*.

Expression of *SBT1.7* (also referred to as ARA12; Engineer et al., 2014), a calcium-dependent subtilase, was reduced in all genotypes for experiment 2 and in the genotype ‘Tomba’ in experiment 1. Subtilases comprise a diverse group of serine peptidases, most of which are targeted to the cell wall or were predicted to range in the extracellular space of potato plants (Norero et al., 2016; Schaller et al., 2018). They are known to function in cell growth and development through the regulation of the activity of extracellular signaling molecules as well as properties of the cell wall (Schaller et al., 2018). Reduced gene expression of *SBT1.7* might therefore display a reduced cell growth, as also indicated by the growth data of the plants after seven days of commencing drought stress. As protein abundance was also found to be reduced, this gene might comprise a target for further analysis upon drought stress to develop biomarkers (Wellpott et al., 2021).

INH1, an invertase inhibitor, was found to be significantly upregulated under commencing drought stress in both experiments, the only exception again being ‘Tomba’ in experiment 1. In potato, *INH1* was described to be highly expressed in leaves and flowers compared to *INH2*, which was more prominent in tubers and roots (Brummell et al., 2011). *INH1* was previously described upregulated by Aliche et al. (2022) after drought stress and by Yang et al. (2020) to give rise to drought tolerance when overexpressed in sweet potato. However, they also found a trade-off with growth, as overexpression of *INH1* led to growth reduction in mutant lines. Therefore, cell wall and vacuolar invertase inhibitors are important regulators of plant growth. They are also known to be important regulators of sink-source strength and sugar-related signalling and were shown to be involved in stress responses, e.g. cold-induced sweetening of tubers in potato (Brummell et al., 2011; Castrillon-Arbelaez & Delano-Frier, 2011). *INH1* also plays a major role in drought stress-mediated stomatal closure to reduce water loss (Chen et al., 2016; Kulik et al., 2011; Matsuoka et al., 2021). ABA levels increase in plant cells under abiotic stress, activating SnRK2 family proteins and thus, lead to stomatal closure, which is a common response of the plant to drought stress (Mustilli et al., 2002). Gene *INH1* (*cell wall / vacuolar inhibitor of fructosidase*) was shown to specifically inhibit many proteins from the SnRK2 family (Kulik et al., 2011; Matsuoka et al., 2021). Yang et al. (2020) demonstrated, that the gene *INH1* (*cell wall / vacuolar inhibitor of fructosidase*) activates the ABA-regulated pathway and therefore ABA biosynthesis in sweet potato after drought stress, resulting in enhanced drought tolerance. Other than that, invertases hydrolyse sucrose into glucose and fructose and thus *INH1* plays a major role in regulating the primary metabolism and development of the plant (Ruan et al., 2010). An increase of *INH1* gene expression in potato leaves after seven days of commencing drought stress might therefore directly help plants to cope with starting water deficiency. Since *INH1* was found to be higher abundant after drought stress on protein level only in the more tolerant genotypes ‘Eurostarch’ and ‘Tomba’ (Wellpott et al., 2021), the gene comprises a strong candidate for detection of commencing drought stress on a protein level.

Conclusion

In this study, we successfully applied drought stress in all analysed genotypes without passing the permanent wilting point. No trends concerning different levels of tolerance between the genotypes could be detected in the recorded growth data in contrast to results of previous evaluations which took place in different settings. This was likely due to the fact that experiments outside a climate chamber are subject to natural variations in physical growth conditions and in most previous studies, the plants were analysed after natural maturity. This indicates that the setup of stress experiments is of major importance regarding classification in tolerance levels of individual genotypes. We observed additional heat stress and higher radiation in the first experiment, which led to an alteration in response of the potato plants. For this reason, stress priming may have taken place in experiment 1. This can be reinforced by variable gene expression data of *RPT5a*, *13-LOX*, and *SBT1.7*. Out of the eight GOIs investigated in this study, *INH1* was found to comprise a strong candidate for detection of commencing drought stress in early stages of potato development.

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2.3 Liquid in vitro culture system allows gradual intensification of osmotic stress in *Solanum tuberosum* through sorbitol

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Liquid in vitro culture system allows gradual intensification of osmotic stress in *Solanum tuberosum* through sorbitol

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Key Points

- A new, cost-effective in vitro test system is presented, which is suitable for the addition of osmotica after rooting of plants
- Osmotic stress was gradually intensified in four genotypes in an in vitro test system with liquid medium and sorbitol
- *13-LOX*, a gene of the family of lipoxygenases, linked to osmotic adjustment, was upregulated in all analysed genotypes
- Sorbitol was increased in content in shoots and is likely taken up through roots

Abstract

Because of their shallow root system, drought stress is a major problem in potato cultivation. Due to climate change more severe drought periods are expected to occur in the vegetative phase of potato growth. Therefore, there is a great need for drought tolerant potato genotypes. Potato responds to drought stress in the field in various ways, including osmoregulation. Osmotic stress can be induced in vitro by adding an osmoticum and thus lowering the osmotic potential. In this study, a new, cost-effective in vitro test system is presented, in which the osmoticum can be added after root formation to prevent osmotic shock. This is achieved by using liquid medium, to which the osmoticum can be added gradually and at a later stage. This allows to better approach the stepwise drying of the soil in the field. Morphological responses to osmotic stress in four potato genotypes were analysed and an increase in proline under osmotic stress was detected. Moreover, GOIs that were postulated to be linked to drought stress were regulated under osmotic stress, underpinning the optimized test system for use in stress experiments. Furthermore, we propose that sorbitol, which was used as an osmoticum, is probably taken up into the shoots, because sorbitol content was 700- (630-) fold higher for ‘Eurostarch’ (‘Tomba’) after seven days under osmotic stress and 1093- (349-) fold higher in the two genotypes after 14 days. However, whether it was taken up through the roots, is metabolised or stored remains unclear.

Abbreviations:

13-LOX – lipoxygenase

APRT – adeninphosphoribosyltransferase

Cyclo – cyclophilin

DM – dry mass

Ef1α – elongation factor α

FM – fresh mass

Glyx – lactoylglutathione lyase/glyoxalase I

GOI(s) – gene(s) of interest

INH1 – cell wall / vacuolar inhibitor of fructosidase

MS – Murashige and Skoog

PEG – polyethylene glycol

PES – polyester

POD – peroxidase 51-like

PP – polypropylene

RPT5a – regulatory particle triple-A ATPase 5A

SBT1.7 – subtilase family protein

SHMT – serine transhydroxymethyltransferase

ZBD – zinc-binding dehydrogenase family protein

Introduction:

Drought stress is a major limiting abiotic factor for the yield quality and quantity of many crops including potato. In the temperate regions of the world, climate change will lead to more drought periods in spring and early summer, when potato plants are in their vegetative growth phase (Haverkort und Verhagen 2008). Potato is a rather drought sensitive crop due to their shallow root system (Iwama und Yamaguchi 2006). Therefore, it is of utmost importance to pre-select genotypes that display increased drought tolerance and to better understand mechanisms that allow plants to withstand drought stress. Osmotic stress is a term used for a situation, in which plant growth and development is limited by insufficient water availability due to change in the solute concentration around the cell, and is a part of drought stress (Zhu et al. 1997; Chen und Jiang 2010). Plants are able to alleviate drought stress by osmoregulation, i.e., by synthesis of compounds like glycine betaine or polyols (Mullet und Whitsitt 1996). Responses to osmotic stress are therefore indicators for drought stress responses, and osmotic stress can be induced under in vitro culture conditions.

In vitro tests systems are more controlled and less time intensive than field studies. However, the plant responses under the artificial mixotrophic in vitro conditions may not fully reflect the responses of plants grown ex vitro. Nevertheless, since potato breeders establish in vitro cultures of their important germplasm for reasons of sanitation and breeding, the important potential new genotypes are available as in vitro shoot cultures. Therefore, an in vitro pre-test to determine osmotic stress tolerant genotypes would be helpful for breeders to narrow the number of genotypes which have to be tested for drought tolerance in field. In earlier in vitro studies, potato plants were mostly grown on solid medium to which an osmoticum was added. Dobránszki et al. (2003) used four concentrations of mannitol to induce osmotic stress in vitro. They managed to group five potato genotypes into three osmotic tolerance groups. But a severe disadvantage of using mannitol as an osmoticum was reported by Lipavsk und Vreugdenhil (1996). The authors showed in an in vitro study with wheat, rape, and potato, that mannitol was taken up by the plants, transported to the shoots and accounted for up to 20 % of shoot dry mass. Another osmoticum used for inducing osmotic stress in vitro is polyethylene glycol (PEG). Stefan et al. (2020) tested several potato breeding lines with different concentrations of PEG6000 in solid MS-medium for their osmotic stress tolerance. However, Gopal und Iwama (2007) stated that PEG might limit O_2 movement due to its high viscosity. The most widely used osmoticum in potato to date is sorbitol (Gopal und Iwama 2007; Bündig et al. 2016a; Mawia et al. 2020; Hanász et al. 2022). Sorbitol is nontoxic to

plants and not as viscous as PEG. However, Bündig et al. (2016b) reported a possible uptake of sorbitol through the freshly cut surface of the shoots after their cultivation on solid sorbitol-containing medium.

In addition to the production of table potatoes, starch potatoes with high starch contents are grown for adhesives, cosmetics, and the paper industry. In this study, we tested four starch potato genotypes for their responses to osmotic stress in a liquid MS-medium (Murashige und Skoog 1962). This allowed the shoots to form roots prior to being exposed to the osmoticum. Thus, the stress could be intensified gradually through the stepwise addition of sorbitol. The intact roots were expected to prevent the uptake of sorbitol through the Casparian strip (Łotocka et al. 2016). We investigated fresh and dry mass of shoots and roots, proline content in shoots, and normalised gene expression of candidate genes, which had been selected based upon a proteomic study of drought stressed potatoes (Wellpott et al. 2021). Also, the sorbitol content in the shoots was measured by LC-MS to determine whether sorbitol might be taken up through the intact roots in vitro.

Material and methods

Plant material

Four starch potato genotypes with contrasting responses to osmotic stress were used in this study. ‘Eurobravo’, ‘Eurostarch’, and ‘Tomba’ originate from EUROPLANT Pflanzenzucht GmbH, Lüneburg, Germany. ‘Maxi’ was bred by Bayerische Pflanzenzuchtgesellschaft eG &Co KG, Hamburg, Germany. In vitro material was kindly provided by the Julius Kühn-Insitute (JKI), Federal Research Centre for Cultivated Plants – Institute for Resistance Research and Stress Tolerance, Groß Lüsewitz and cultivated in 500 ml polypropylene (PP) vessels (plastikbecher.de GmbH, Giengen, DE) on 80 ml solid MS (Murashige und Skoog 1962) medium (3 % (w/v) sucrose, 7.5 g/l Plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands), pH 5.8) at 18 °C in a long day photoperiod (16 h light/8 h dark) with a photon flux density of ~35 µmol m^{-2} s^{-1}. Subcultures were done using nodal cuttings every four to five weeks.

Osmotic stress experiment

Plants were grown for three weeks before five shoot tips were placed in each experimental vessel, which contained 45 ml liquid MS-medium each (Murashige und Skoog 1962). The plant holders were made in-house in order to establish the liquid culture test system: Through a screen mesh,

which was melted onto a polypropylene tube section, the shoot tips were fixed ensuring that the stem base accessed the medium (Fig. 1). The holder was made of a 1.5 cm pipe ring (Ostendorf Kunststoffe GmbH, Vechta, Germany) and an attached PES (polyester) screen mesh (⌀ 1600-1800 µm, Franz Eckert, Waldkirch, Germany).

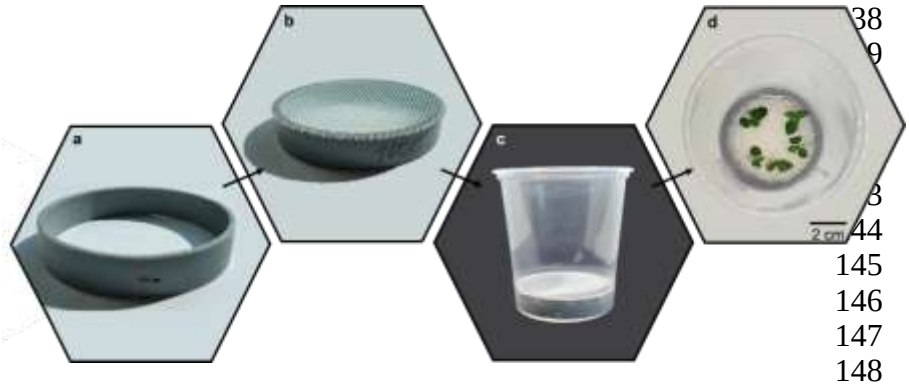

Fig. 1 Details of the established liquid medium stress system in vitro. a: plastic pipe is cut into rings of 1.5 cm height and deburred with sandpaper. b: sieve mesh (1600 µm diameter) is melted onto pipe ring. c: sieve fits into 500 ml PP vessels. d: 45 ml medium is filled into the vessel up to sieve level. e: top view of cultivated potato plants in liquid medium after seven and 14 days of osmotic stress.

Based on the in vitro test system using solidified medium by Bündig et al. (2016a) the aim was to establish a protocol for osmotic stress tests in vitro in a liquid culture system where stress could be applied to rooted plants and gradually increased over time. The stress response of the plants was measured through growth parameters, as well as by proline content, and candidate gene expression. During a series of experiments, the following parameters were altered in order to optimise the system (compare Table S1 and S2):

- Rooting time was varied between seven and eleven days. Nine days of rooting provided the plants with enough initial roots to continue root growth and secure stability.

- The final concentration of the osmoticum (here: sorbitol) tested in the medium ranged from 0.3 M to 0.6 M. The concentration in the medium of 0.3 M displayed first differences between control and stressed plants without causing excessive damage to the plants.

- Stress was applied exponentially over 4 application time points (0.1 M, 0.13 M, 0.28 M and 0.6 M) or linearly over three time points (0.1 M, 0.2 M, 0.3 M) to determine optimal application intervals. Both application schemes resulted in differences of growth (FM and DM of shoots and roots) between the variants, however, linear application was chosen for simplicity.

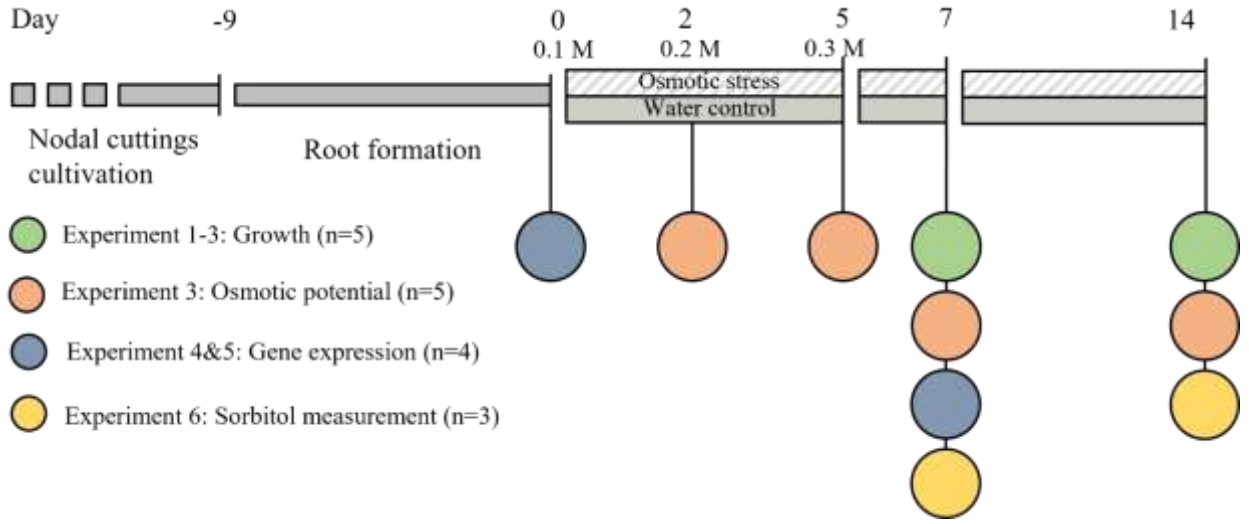

Fig 2 Timeline of osmotic stress experiments in vitro. Four starch potato genotypes were cultivated in vitro through nodal cuttings for three weeks. Osmotic stress treatment received sorbitol as osmoticum in three steps until 0.3 M end concentration in the medium. Control treatment received deionised water instead. Samples were taken after nine days of root formation (day 0), on day 2, day 5, day 7, and day 14 from different experiments for different analyses (see colors). Green: Experiment 1&2 for growth data (n=5). Red: Experiment 1&2 for osmotic potential (n=5). Blue: Experiment 3&4 for gene expression analysis (n=4). Yellow: Experiment 5 for sorbitol measurement (n=3).

Based on these findings, the optimised osmotic stress test system was applied in the experiments reported here: After nine days of root formation in 45 ml of MS-medium, liquid sorbitol was added as osmoticum in three steps (0.76 ml on day 0, 0.79 ml on day 2, and 0.82 ml on day 5) of 0.1 M until an end concentration of 0.3 M sorbitol in the medium was reached (Fig. 2). Addition of the same amount (0.76/0.79/0.82 ml) of autoclaved deionised water served as a control (2.37 ml in total). The experiment was conducted two times with ten replicates (one replicate corresponds to one vessel with 5 shoots each) per genotype. Samples were collected from three experimental replications (hereafter termed experiment 1, experiment 2, and expreiment 3) for growth and proline and two additional experiments (4 and 5) for gene expression analysis. A sixth experiment was conducted to measure sorbitol content in the shoots. Shoot length, as well as shoot and root fresh mass was measured from plants of five vessels per variant and genotype after seven and 14 days, respectively. After 48 h at 70 °C in an oven, shoot and root dry mass were examined. Samples were kept at RT in tubes until further use. For gene expression analysis around 100 mg of fresh shoot material from four vessels per variant was collected, blotted dry with sterile paper and immediately frozen in liquid nitrogen. For sorbitol measurement, shoots were washed in deionised

191 water to remove sorbitol which might be adhering in condensation water on the shoot surface. One
192 hundred mg were collected, blotted dry, frozen in liquid nitrogen and stored at -80 °C until further
193 use.

194 **Gene expression analyses**

195 RNA isolation and cDNA synthesis

196 Frozen shoot material from four vessels (corresponds to 4 replicates) per variant (control, C;
197 osmotic stress, S) and genotype ('Eurobravo', 'Eurostarch', 'Maxi', and 'Tomba') was ground in
198 a mixer mill at 25 Hz for 2 min (MM400, Retsch, Haan, Germany) and RNA was extracted by
199 following the manufacturer's instructions for the InviTrap Spin Plant RNA Mini Kit (Stratec,
200 Birkenfeld, Germany) using the DCT lysis buffer. Genomic DNA was removed with DNase I
201 according to the manufacturer (Thermo Scientific, Waltham, MA, USA). The integrity of RNA
202 was examined in a 1 % (w/v) agarose gel before cDNA was synthesised by using the RevertAid
203 First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with the oligo-dT
204 primer and 1 µg RNA as a template. The cDNA was diluted 1:10 (v/v) and stored at -20 °C.

205 Primer selection

206 Eight candidate genes were selected based on proteins found in genotypes 'Tomba' and
207 'Eurostarch', which are hypothezised to be rather drought tolerant genotypes based on a previous
208 rain-out shelter experiment (Wellpott et al. 2021). Primers were designed upon the following
209 criteria: 18-24 bp length, GC content 40-60 %, 80-250 bp amplification product, melting
210 temperature 60 °C. The primers were tested for specificity with BLAST (Basic Local Alignment
211 Search Tool, https://blast.ncbi.nlm.nih.gov) aligning it to the *Solanum tuberosum* subsp. *tuberosum*
212 genome (NCBI: txid4113). Sequence information was obtained from Spud DB using *Solanum*
213 *tuberosum* group Phureja Dm1-3 v6.1. Primers were tested in a standard PCR with cDNA of
214 'Eurostarch', with T_A= 60 °C on a 1.5 % (w/v) agarose gel. Amplification products were sequenced
215 by Sanger sequencing (Sanger et al. 1977).

216

RT-qPCR

RT-qPCR was performed by Applied Biosystems QuantStudio 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). All primers were tested with a pool of all cDNAs for efficiency. Primer efficiencies were calculated with the software QuantStudio™ Real-Time PCR Software v1.3. *EF1a* (elongation factor α), *APRT* (adeninphosphoribosyltranferase), and *Cyclo* (cyclophilin) served as reference genes (Nicot et al. 2005). After a test for stability in RStudio (2022.07.1 Build 554) based on R version 4.1.3 using the NormFinder algorithm (Andersen et al. 2004) *EF1a* was excluded from calculations of the normalised gene expression because of a stability value > 0.25. Four biological and three technical replicates were measured for experiments 3 and 4 on day 0 and day 7. Overall, diluted cDNA of 96 samples was mixed with Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) diluted 1:4 (v/v) for analysis with every primer pair (final concentration in reaction: 0.2 µM). Following PCR conditions were used: one cycle at 95 °C for 60 s, 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Hereafter, melting curve analysis (60 °C to 95 °C with an increment of 0.5 °C/15 s) was conducted to determine specificity of amplification. Data were further processed with QuantStudio™ Real-Time PCR Software v1.3. Data are shown as normalised gene expression (Pfaffl 2001).

Sorbitol measurement

The extraction of sorbitol from plant material was performed acccording to Salem et al. (2016) with minor modifications. In detail, approximately 100-mg plant material was weighed into a 2-ml safe-lock centrifuge-vial and frozen in liquid nitrogen together with five 5-mm steel beads. The exact sample weight was noted (Table S3) and used for calculating analyte concentrations. The tissue was disrupted using a MM 400 beadmill (Retsch, Haan, Germany) at 30 Hz for 3 min. A mixture of precooled methyl tert-butyl ether (MTBE) and methanol (3:1, v:v; 1 ml per sample) was added and the disruption step was repeated. Samples were incubated on a tube rotator (20 rpm) for 15 min at 4°C and subsequently sonicated in an ice-cooled sonication bath for 15 min. The samples were centrifuged for 10 min at 4°C and 10,000 × g and 800 µl of the supernatant was transferred to a new reaction tube. A mixture of water and methanol (3:1, v:v; 800 µl per sample) was added and mixed by vortexing. The samples we centrifuged for 10 min at 4°C and 10,000 × g and the lower phase was collected in a new reaction tube. Samples were dried in a vacuum concentrator

until no liquid was left and reconstituted in mobile phase A (0.1% formic acid in water) prior to LC-MS analysis.

The method for the chromtaographic separation of sorbitol was inspired by a protocol from Antonio et al. (2007) also using a porous graphitic carbon column for the anaylsis of sugars. An Agilent 1290 Infinity II LC System coupled with an Agilent 6460 triple quadrupole mass spectrometer was used. Chromatographic separations employed a 50 × 4.6 mm Hypercarb column with 5-µm particle size (Thermo scientific, Waltham, MA, USA). The column was operated at a flowrate of 0.2 ml min−l and a temperature of 30°C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The following gradient was employed (Table 1):

Table 1 Gradient for chromatographic separation of sorbitol.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
5.00	92	8
7.00	75	25
10.00	75	25
12.00	50	50
16.00	50	50
18.00	92	8
28.00	92	8

The injection volume was 5 µl and analysis was carried out in negative mode employing the multiple-reaction-monitoring (MRM) mode. Transitions (precursor ions and product ions) as well as collision energies, fragmentor energies and retention time were as following (Table 2):

Table 2 Transitions (precursor ion and product ion), fragmentor, collision energy, and retention time.

Analyte		precursor ion [M-H]- (m/z)	product ion	fragmentor	collision energy (V)	retention time (min)
Sorbitol	Quantifier	181.1	71	127	21	3.96
	Qualifier	181.1	89	127	5	3.96

The in-source parameters were: gas temperature 150°C, gas flow 11 L min−1, nebulizer pressure 40 psi, sheath gas temperature 300°C, sheath gas flow 11 l min−1, capillary voltage 2,000 V, and nozzle voltage 2,000 V. The analyte eluted in a single peak with a full width at half maximum (FWHM) between 0.2 and 0.4 and a signal to noise ratio (SNR) over 500. Pure D-sorbitol (Sigma Aldrich, St. Louis, MO, USA) as a standard eluted with the same retention time and a similar ratio

of product ion abundances was observed for the pure standard and the analyte in matrix. Different concentrations of the standard dissolved in water were used for external calibration (tentative absolute quantification). The signal obtained for the quantifier product ion was converted to a concentration with the help of a standard calibration curve. The concentration was normalized to the measured weight of the respective sample. Measured values are shown in Table S3.

Osmotic potential

The measurement of the liquid medium's osmotic potential took place after insertion of the shoots in the mesh (day 0), after the first sorbitol addition (day 2), after the second sorbitol addition (day 4), and on both days on which the evaluation took place (day 7 and day 14) for samples from both, control and stress variants. Medium was analysed by vapor pressure osmometry (VAPRO 5600; Wescor, Logan, UT). Distilled water and medium without plants were measured in addition. Measurements were carried out against three osmolality standards (Opti-Mole 100 mmol kg^{-1}, 290 mmol kg^{-1}, and 1000 mmol kg^{-1}). Three biological replicates and two to three technical replicates were measured. Osmolality was transformed into osmotic potential (Bündig et al. 2016a).

Proline analysis

Proline analysis was performed according to (Bates et al. 1973). To 25 mg of dried and ground shoot material, a total of 1.8 ml of sulphosalicylic acid (3 %) was added in two steps (2 x 900 µl). The samples were incubated on ice for 30 min., mixed, and centrifuged at 14,800 rpm for 15 min. The supernatant (150 µl) was transferred into new tubes per sample and 90 µl glacial acid and 90 µl ninhydrin reagent were added and mixed. The samples were placed in boiling water for 45 min before they were cooled down on ice. After the addition of 1.5 ml toluene, three technical replicates containing 200 µl of the toluene phase were put on a microtiter plate. Absorption was measured at 520 nm. Toluene served as a blank. For each sample, five biological replicates were measured.

Statistical analysis

Illustration of data and statistical analysis were performed in R version 4.1.3 (R Core Team 2022) using Rstudio v. 2022.07.1 Build 554 (RStudio Team 2022). Packages used for figures included 'ggplot2' (Wickham 2016), 'ggpubr' (Kassambara 2020), 'ggsci' (Xiao 2018) 'cowplot' (Wilke 2020), and 'Rcolorbrewer' (Neuwirth 2014). An analysis of variance (ANOVA) was calculated to assess treatment and genotype effects and interactions. Means were compared pairwise by Tukey's

296 test ($p < 0.05$). When normal distribution was not given, data were log transformed or analysed by
297 a Kruskal-Wallis-Test with Bonferroni adjustment. Packages used for statistics were ‘agricolae’
298 (Mendiburu 2021), ‘emmeans’ (Lenth 2022), and ‘multcomp’ (Hothorn et al. 2008).

299

Results

Osmotic potential of the liquid medium

The measured osmotic potential of the liquid medium was -0.5 MPa in the liquid MS medium without plants and did not differ significantly when plants had been cultured in it for two, four, seven, and 14 days in ‘Eurobravo’ and ‘Eurostarch’ (control variant). In the genotype ‘Maxi’ the osmotic potential was increased slightly after 7 days. In the stress variant of all genotypes, the osmotic potential decreased gradually to -1.5 MPa in MS-medium with 0.3 M sorbitol. At day 14 there was a significantly lower osmotic potential than on day seven in ‘Eurobravo’ and ‘Tomba’ (Fig. 3).

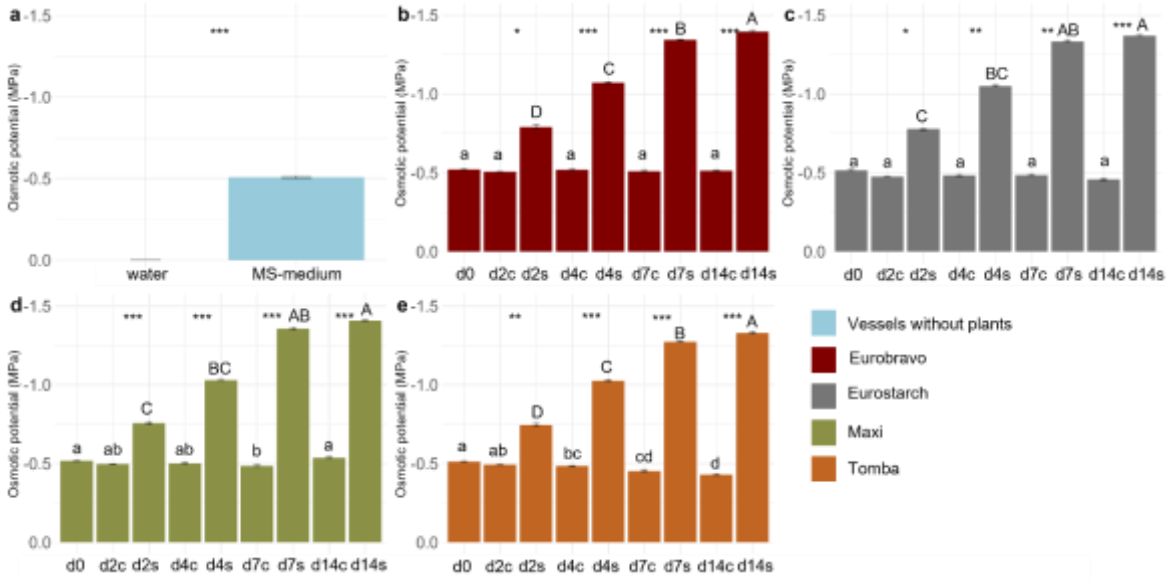

Fig. 3 Osmotic potential in MPa. The vapor pressure of liquid MS medium to which sorbitol was added with or without plants was measured. The osmotic vapor pressure was converted to osmotic potential (MPa). H_2O: water. MS medium: liquid MS medium without plants with different concentrations of sorbitol. a= ‘Eurobravo’ on day 0 (d0), day 2 control (d2c) and under stress (d2s), day 4 control (d4c) and stress (d4s), day 7 control (d7c) and stress (d7s), and day 14 control (d14c) and stress (d14s). b= ‘Eurostarch’. c= ‘Maxi’. d= ‘Tomba’. Lower case letters compare control variants between the days, whereas upper case letters compare stress variants between days using Kruskal-Wallis Test with Bonferroni-correction. Asterisks compare control and stress variant within one day. Significance codes after Kruskal-Wallis test: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Given are means and standard deviations of n=3 replicates.

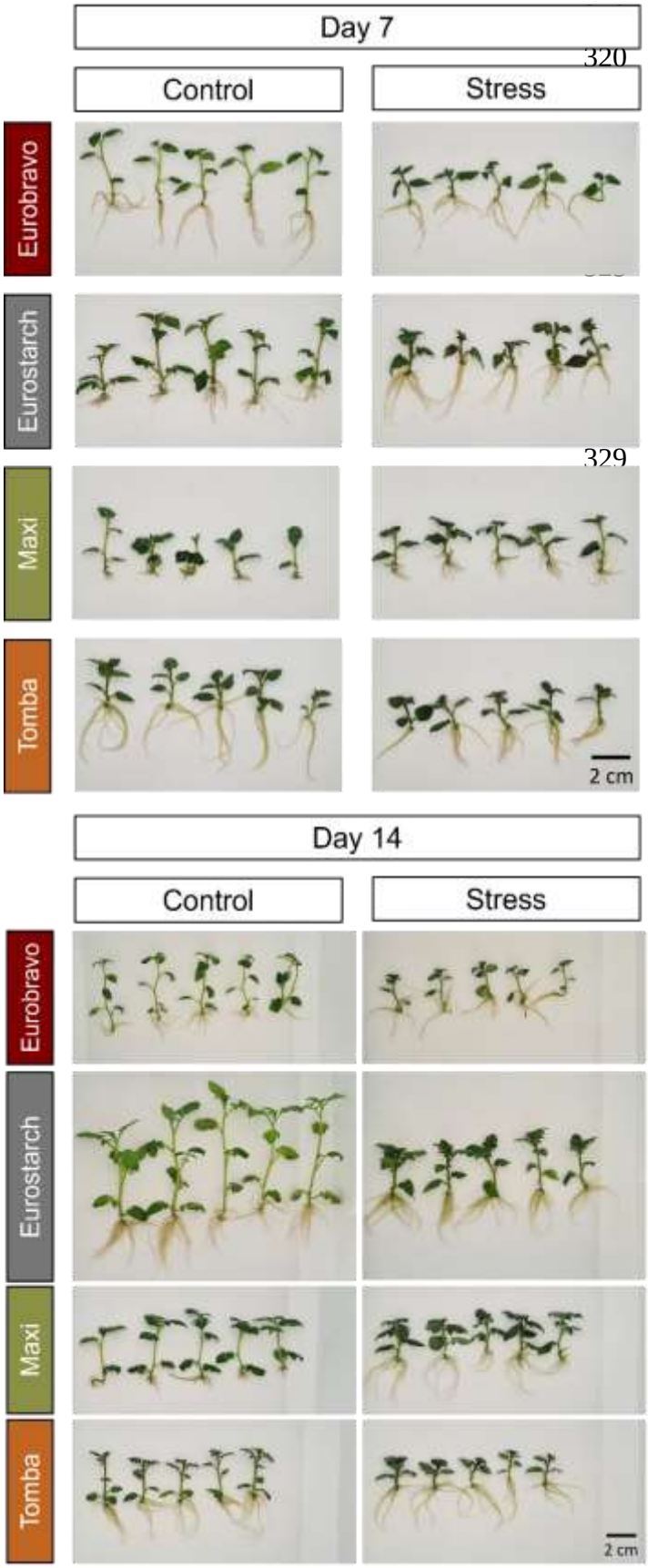

Fig. 4 Morphology of the plants after 7 and 14 days of culture under control or osmotic stress conditions. Control = Medium with addition of water, Stress = Medium with addition of sorbitol up to an end concentration of 0.3 M sorbitol. Day 7 = seven days after first addition of sorbitol, Day 14 = 14 days after first addition of sorbitol. Side view of all plants of an representative culture vessel.

Shoot mass decreased in all genotypes after 14 days of osmotic stress

After seven days of osmotic stress in liquid medium the plants displayed decreased shoot length in all genotypes and overall more roots in the three genotypes ‘Eurobravo’, ‘Eurostarch’, and ‘Maxi’. After 14 days of stress these changes became more pronounced (Fig. 4).

The decrease in shoot dry mass after seven days of osmotic stress ranged between 12.1 % (‘Tomba’) and 17.1 % (‘Maxi’) in experiment 2. In experiment 1 and 3, the shoot dry mass was not decreased after seven days. Shoot mass of the genotype ‘Tomba’ was significantly lower in experiment 2 when compared to the other three genotypes, while the other three genotypes performed similar under stress(Fig. 5). This was also the case for the root mass of ‘Tomba’ in experiment 2 and 3. Root masses increased in experiment 1 and 3 significantly for the genotypes ‘Eurostarch’ (experiment 1: 53 %, experiment 3: 28 %), and ‘Maxi’ (212 % and 33.6 %).

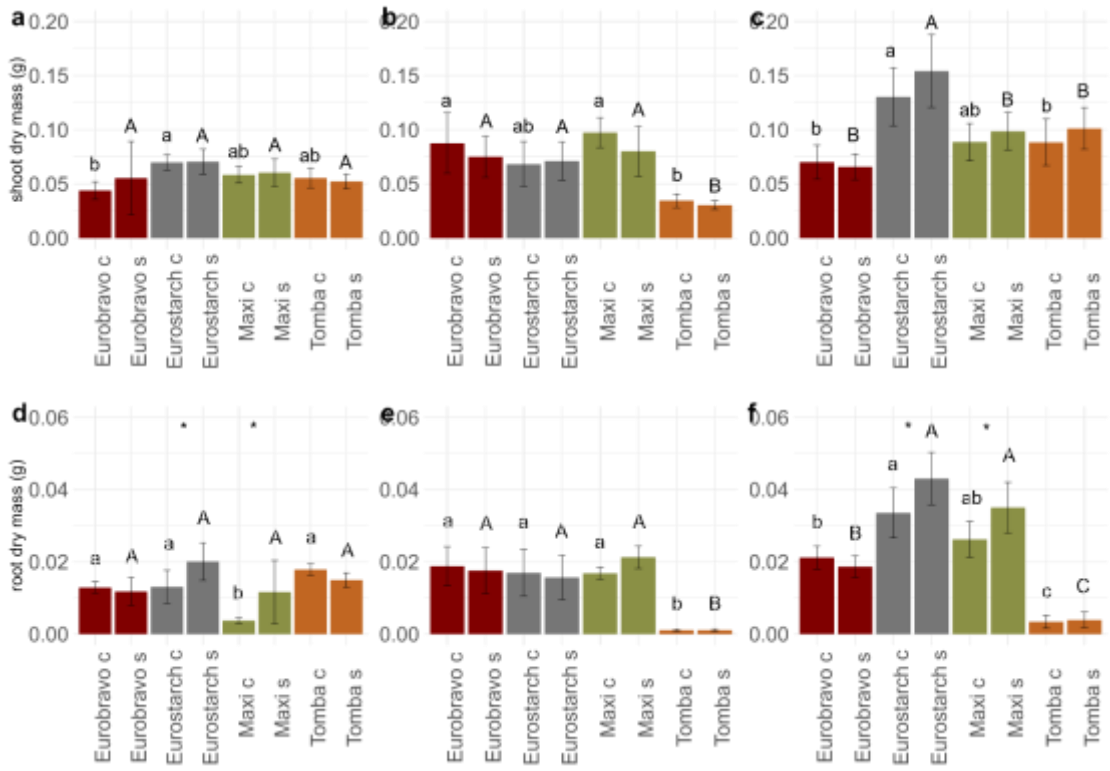

Fig. 5 Shoot and root dry mass after 7 days of osmotic stress. a-c: shoot dry mass from experiment 1, 2, 3, d-f: root dry mass from experiment 1, 2, and 3. c: control, s: stress. Lower case letters compare control variants between the genotypes, whereas upper case letters compare stress variants between genotypes using Tukey’s test (Kruskal-Wallis Test with Bonferroni-correction for root DM of experiment 2). Asterisks compare control and stress variant within one genotype. Significance codes after Tukey’s test or Kruskal-Wallis test: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Given are means and standard deviations of n=5 replicates.

After 14 days, the plants displayed significantly lower shoot mass in genotype ‘Eurobravo’ in experiment 1 and in ‘Eurostarch’ in experiment 3. In experiment 2 all genotypes showed lower shoot mass under stress compared to the growth under control conditions. ‘Eurobravo’ showed the greatest decrease among all genotypes (56.9 %), followed by ‘Tomba’ (54.0 %) and ‘Eurostarch’ (41.5 %), whereas ‘Maxi’ showed the smallest decrease in shoot mass (34.7 %). The root mass difference between control and stressed variant were only significant for ‘Eurobravo’ (44.6 %) in experiment 2 (Fig. 6).

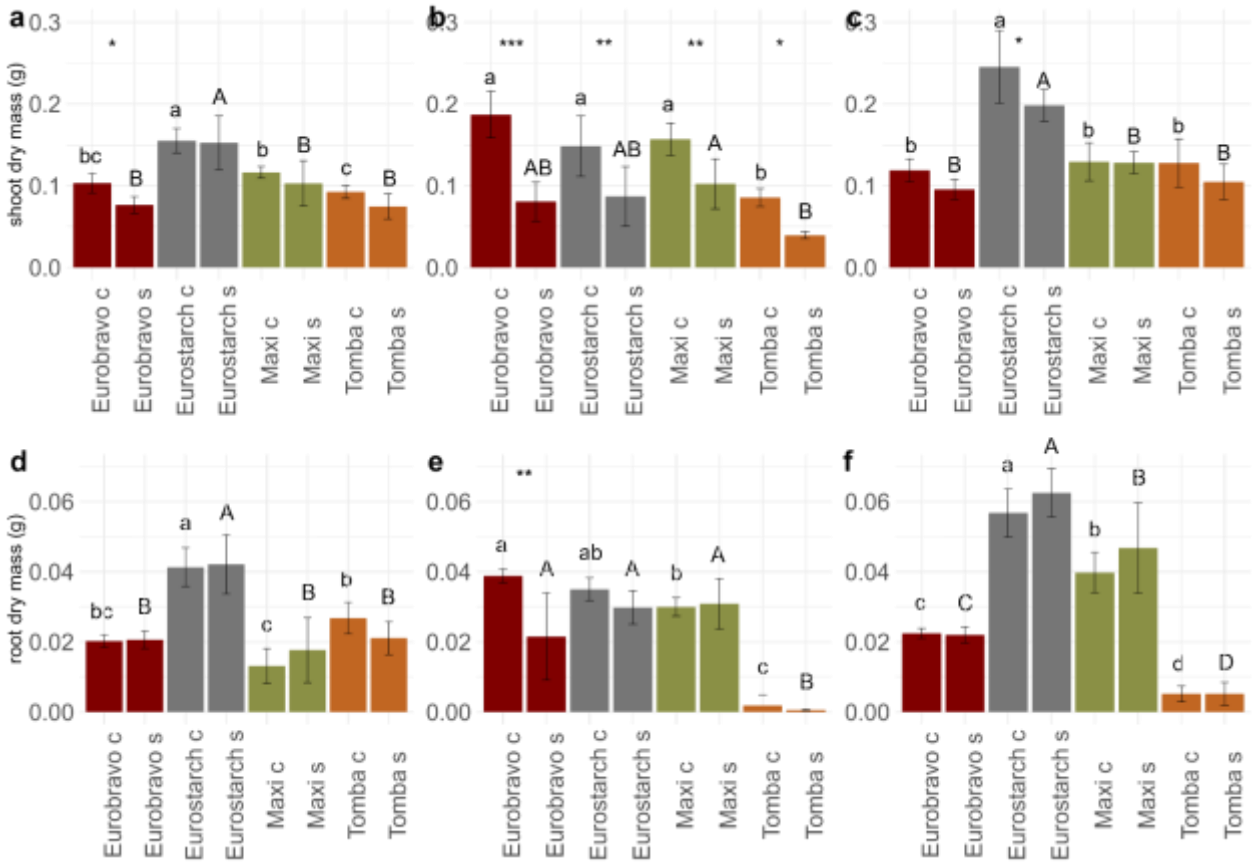

Fig. 6 Shoot and root dry mass in gram after 14 days of osmotic stress with standard deviation. a-c: shoot dry mass from experiment 1, 2, 3, d-f: root dry mass from experiment 1, 2, and 3. c: control, s: stress. Statistical analysis: ANOVA and Tukey’s test (Kruskal-Wallis Test with Bonferroni-correction for root DM of experiment 2). Lower case letters compare control values between the genotypes. Upper case letters compare stress values between genotypes. Asterisks compare control and stress variant within one genotype. Significance codes after Tukey’s test or Kruskal-Wallis test: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. n=5.

The root-shoot ratio based on the dry mass (DM) was similar under control and stress conditions in all experiments after seven days (Fig. S1). After 14 days the ratio under osmotic stress was higher compared to the ratio under control conditions in 'Eurobravo' in experiment 1 (0.2 ± 0.01/0.27 ± 0.03, 'Maxi' in experiment 2 (0.19 ± 0.04/0.30 ± 0.03), and 'Eurostarch' (0.23 ± 0.0/0.32 ± 0.05) in experiment 3.

Gene expression

Two independent experiments (further termed as experiment 4 and 5), in which plants were treated with osmotic stress for seven days, were conducted to show early responses of the plants to osmotic stress by selected candidates for drought stress indicator genes from Wellpott et al. (2021). Results for *RPT5a*, *POD*, and *SBT1.7* are shown separately for each experiment (Table 4), whereas statistical analyses allowed the presentation of combined data from both experiments for *Glyx*, *ZBD*, *INH1*, *SMHT*, and *13-LOX* (Table 5 a,b).

Expression of *RPT5a* was not regulated significantly after seven days of stress. *Glyx* showed downregulation in all genotypes. The gene expression of this gene showed high variations between vessells for the genotypes 'Eurobravo' and 'Maxi', however, leading to a significant alteration in 'Eurostarch' with a fold change (stress/control) of 0.39 and 'Tomba' with a fold change of 0.42. 'Tomba' (fold change 0.61) and 'Maxi' (0.42) also displayed a downregulation of *13-LOX*. Downregulation was significant in all genotypes for *ZBD*. The fold changes of *ZBD* were 0.56 ('Eurobravo'). 0.60 ('Eurostarch'), 0.66 ('Maxi'), and 0.54 ('Tomba').

Expression of *SBT1.7* was lower in genotype 'Tomba' than in the other genotypes on day 0 (Tab. 1 a). After seven days of osmotic stress *SBT1.7* was downregulated in all genotypes in experiment 4 (fold changes 'Eurobravo' 0.22, 'Eurostarch' 0.27, 'Maxi' 0.32, 'Tomba' 0.33), as well as in 'Eurostarch' (0.03) and 'Tomba' (0.09) in experiment 5 (Table 4 b).

POD expression was similar in all genotypes on day 0 (Table 4 a). The gene was significantly lower expressed after seven days of osmotic stress in 'Eurobravo' (FC experiment 4: 0.08 and experiment 5: 0.03), 'Eurostarch' (FC 0.09 and 0.03), and 'Tomba' (FC 0.16 and 0.01). Gene expression was also reduced in 'Maxi', however, this was not statistically significant (Table 4 b).

Table 4 Mean values under control conditions or osmotic stress and fold changes (S/C) of normalised expression of *RPT5a, POD, SBT1.7* of four potato genotypes at the start of the experiment (day 0) and after seven days (day 7). a: mean values. Data are means of 4 biological replicates ± SD and are displayed for experiment 4&5 separately. Lower and upper case letters compare values of one variant between the genotypes within one gene of interest in experiment 4 and 5, respectively. Heat map colors reach from white (lowest value) to dark orange (highest value) and were calculated for each column, separately. b: Fold changes (stress/control). Asterisks display significant differences in mean normalised expression between control and stress variants, significance codes: *** = p < 0.001; ** = p < 0.01; * = p < 0.05.). Significant upregulation is marked by dark grey, significant downregulation is marked by light grey cells. Statistical analysis: Kruskal-Wallis test with Bonferroni correction (for all genes day 7 of experiment 5 and *POD* day 7 of experiment 4) or Tukey's test (for remaining comparisons), $p < 0.05$, n=4. *RPT5a*: regulatory particle triple-A ATPase 5A, *POD*: Protein peroxidase 51-like, *SBT1.7*: Subtilase family protein

a

Day	Variant	Genotype	Experiment	*RPT5a*	*POD*	*SBT1.7*
0	Start	Eurobravo	3	0.352 ± 0.049 a	0.034 ± 0.022 a	0.079 ± 0.016 a
			4	0.413 ± 0.029 A	0.023 ± 0.002 A	0.039 ± 0.003 AB
		Eurostarch	3	0.334 ± 0.014 a	0.022 ± 0.011 a	0.080 ± 0.027 a
			4	0.381 ± 0.023 AB	0.023 ± 0.004 A	0.056 ± 0013 A
		Maxi	3	0.369 ± 0.060 a	0.014 ± 0.005 a	0.052 ± 0.007 ab
			4	0.329 ± 0.021 B	0.051 ± 0.005 A	0.057 ± 0.013 A
		Tomba	3	0.423 ± 0.107 a	0.039 ± 0.020 a	0.042 ± 0.005 b
			4	0.376 ± 0.026 AB	0.079 ± 0.047 A	0.030 ± 0.006 B
7	Control	Eurobravo	3	0.308 ± 0.017 a	0.026 ± 0.007 a	0.073 ± 0.018 a
			4	0.299 ± 0.041 A	0.050 ± 0.025 A	0.031 ± 0.015 A
		Eurostarch	3	0.340 ± 0.050 a	0.020 ± 0.017 a	0.067 ± 0.026 a
			4	0.273 ± 0.088 A	0.033 ± 0.017 A	0.039 ± 0.006 A
		Maxi	3	0.379 ± 0.033 a	0.020 ± 0.008 a	0.054 ± 0.015 a
			4	0.222 ± 0.022 A	0.029 ± 0.013 A	0.027 ± 0.006 A
		Tomba	3	0.359 ± 0.045 a	0.037 ± 0.015 a	0.036 ± 0.007 a
			4	0.405 ± 0.035 A	0.038 ± 0.016 A	0.034 ± 0.014 A
	Stress	Eurobravo	3	0.356 ± 0.039 a	0.002 ± 0.001 a	0.016 ± 0.004 a
			4	0.254 ± 0.085 A	0.001 ± 0.001 AB	0.013 ± 0.008 A
		Eurostarch	3	0.376 ± 0.075 a	0.002 ± 0.000 a	0.018 ± 0.004 a
			4	0.311 ± 0.066 A	0.001 ± 0.000 AB	0.008 ± 0.002 A
		Maxi	3	0.406 ± 0.041 a	0.006 ± 0.004 a	0.017 ± 0.004 a
			4	0.316 ± 0.053 A	0.005 ± 0.003 A	0.010 ± 0.003 A
		Tomba	3	0.361 ± 0.020 a	0.006 ± 0.009 a	0.012 ± 0.007 a
			4	0.293 ± 0.045 A	0.001 ± 0.000 B	0.003 ± 0.002 A

b

Genotype	Eurobravo		Eurostarch		Maxi		Tomba	
Experiment	3	4	3	4	3	4	3	4
RPT5a	1.15	0.85	1.11	1.14	1.07	1.42	1.01	0.72
POD	0.08 **	0.03 ***	0.09 *	0.03 **	0.30	0.18	0.16 ***	0.01 ***
SBT1.7	0.22 ***	0.42	0.27 ***	0.21 ***	0.32 **	0.38	0.33 *	0.09 ***

Table 5 Mean values under control conditions or osmotic stress and fold changes (S/C) of normalised expression of *Glyx*, *ZBD*, *INH1*, *SHMT*, and *13-LOX* of four potato genotypes at the start of the experiment (day 0) and after seven days (day 7). a: mean values. Data are displayed for experiment 4&5 combined, because of statistical similarity. Letters a-c display significant differences in between a box of four genotypes in one variant and one gene of interest. Statistical analysis: Tukey's test ($p < 0.05$; n=8). Heat map colors reach from white (lowest value) to dark orange (highest value) and were calculated for every column separately. b: Fold changes (stress/control). Asterisks display significant differences in mean normalised expression between control and stress variants (Tukey's test, n=8, significance codes: *** = p < 0.001; ** = p < 0.01; * = p < 0.05.). Significant upregulation is marked by dark grey, significant downregulation is marked by light grey cells. *Glyx*: Lactoylglutathione lyase / glyoxalase I, *ZBD*: Zinc-binding dehydrogenase family protein, *13-LOX*: lipoxygenase, *SHMT*: serine transhydroxymethyltransferase, *INH1*: cell wall / vacuolar inhibitor of fructosidase

a

Experiment	Day	Genotype	Variant	*Glyx*	*ZBD*	*INH1*	*SHMT*	*13-LOX*
3&4	0	Eurobravo	Start	0.365 ± 0.111 a	0.104 ± 0.013 c	0.365 ± 0.093 a	1.871 ± 0.419 b	0.100 ± 0.052 a
		Eurostarch	Start	0.349 ± 0.091 a	0.115 ± 0.027 bc	0.422 ± 0.187 a	2.163 ± 0.570 b	0.084 ± 0.055 a
		Maxi	Start	0.426 ± 0.147 a	0.156 ± 0.043 b	0.492 ± 0.419 a	2.460 ± 0.453 b	0.102 ± 0.056 a
		Tomba	Start	0.382 ± 0.090 a	0.234 ± 0.033 a	0.744 ± 0.526 a	3.490 ± 0.902 a	0.104 ± 0.043 a
	7	Eurobravo	Control	0.282 ± 0.069 b	0.122 ± 0.015 b	0.344 ± 0.182 ab	2.353 ± 0.618 a	0.0510 ± 0.027 b
		Eurostarch	Control	0.389 ± 0.121 ab	0.127 ± 0.050 b	0.190 ± 0.067 b	2.294 ± 0.829 a	0.031 ± 0.017 b
		Maxi	Control	0.453 ± 0.151 a	0.160 ± 0.054 ab	0.343 ± 0.171 ab	3.406 ± 1.071 a	0.112 ± 0.044 a
		Tomba	Control	0.310 ± 0.070 ab	0.224 ± 0.059 a	0.442 ± 0.192 a	2.329 ± 0.651 a	0.146 ± 0.053 a
		Eurobravo	Stress	0.193 ± 0.092 ab	0.068 ± 0.012 b	0.484 ± 0.101 b	0.795 ± 0.222 a	0.055 ± 0.028 ab
		Eurostarch	Stress	0.152 ± 0.060 b	0.076 ± 0.013 b	0.654 ± 0.135 b	0.786 ± 0.248 a	0.034 ± 0.006 b
		Maxi	Stress	0.351 ± 0.197 a	0.105 ± 0.035 ab	0.385 ± 0.158 b	1.282 ± 0.560 a	0.048 ± 0.041 ab
		Tomba	Stress	0.132 ± 0.045 b	0.120 ± 0.044 a	1.068 ± 0.391 a	1.186 ± 0.393 a	0.090 ± 0.040 a

b

Genotype	Eurobravo		Eurostarch		Maxi		Tomba	
Experiment	3&4							
Glyx	0.68		0.39	***	0.78		0.42	**
ZBD	0.56	*	0.60	*	0.66	*	0.54	***
INH1	1.41		3.44	***	1.12		2.42	***
SHMT	0.34	***	0.34	***	0.38	***	0.51	**
13-LOX	1.08		1.11		0.42	***	0.61	**

The expression of *SHMT* differed between the genotypes on day 0 (Table 5 a). 'Tomba' (3.490 ± 0.902) showed a significantly higher expression than 'Eurobravo' (1.871 ± 0.419), 'Eurostarch' (2.163 ± 0.570), and 'Maxi' (2.460 ± 0.453) (Table 5 b). All genotypes showed downregulation of *SHMT* after seven days of osmotic stress. 'Tomba' (0.51) displayed the highest fold change, followed by 'Maxi' (0.38), 'Eurobravo' (0.34) and 'Eurostarch' (0.34) (Table 5 b).

INH1 was the only analysed gene to show upregulation after osmotic stress (Table 5 a). The gene was expressed similarly in all genotypes on day 0 and displayed a significant upregulation in 'Eurostarch' (3.44) and 'Tomba' (2.42) after seven days of stress (Table 5 b).

Proline accumulated in stressed shoots

Proline was analysed in ‘Eurobravo’, ‘Eurostarch’, ‘Maxi’, and ‘Tomba’ based on the shoot dry mass after seven and 14 days under osmotic stress. The results show an increase of proline in all genotypes under osmotic stress after seven and 14 days, except for ‘Eurostarch’ and ‘Maxi’ in experiment 3 after seven days and ‘Tomba’ in experiment 1 after 14 days. After seven days the lowest fold change (stress/control) is diplayed by ‘Tomba’ (0.58) and the highest increase by ‘Eurostarch’ (7.76). After 14 days ‘Tomba’ (1.34) showed the lowest increase and ‘Eurostarch’ (6.67) the highest (Fig. 7).

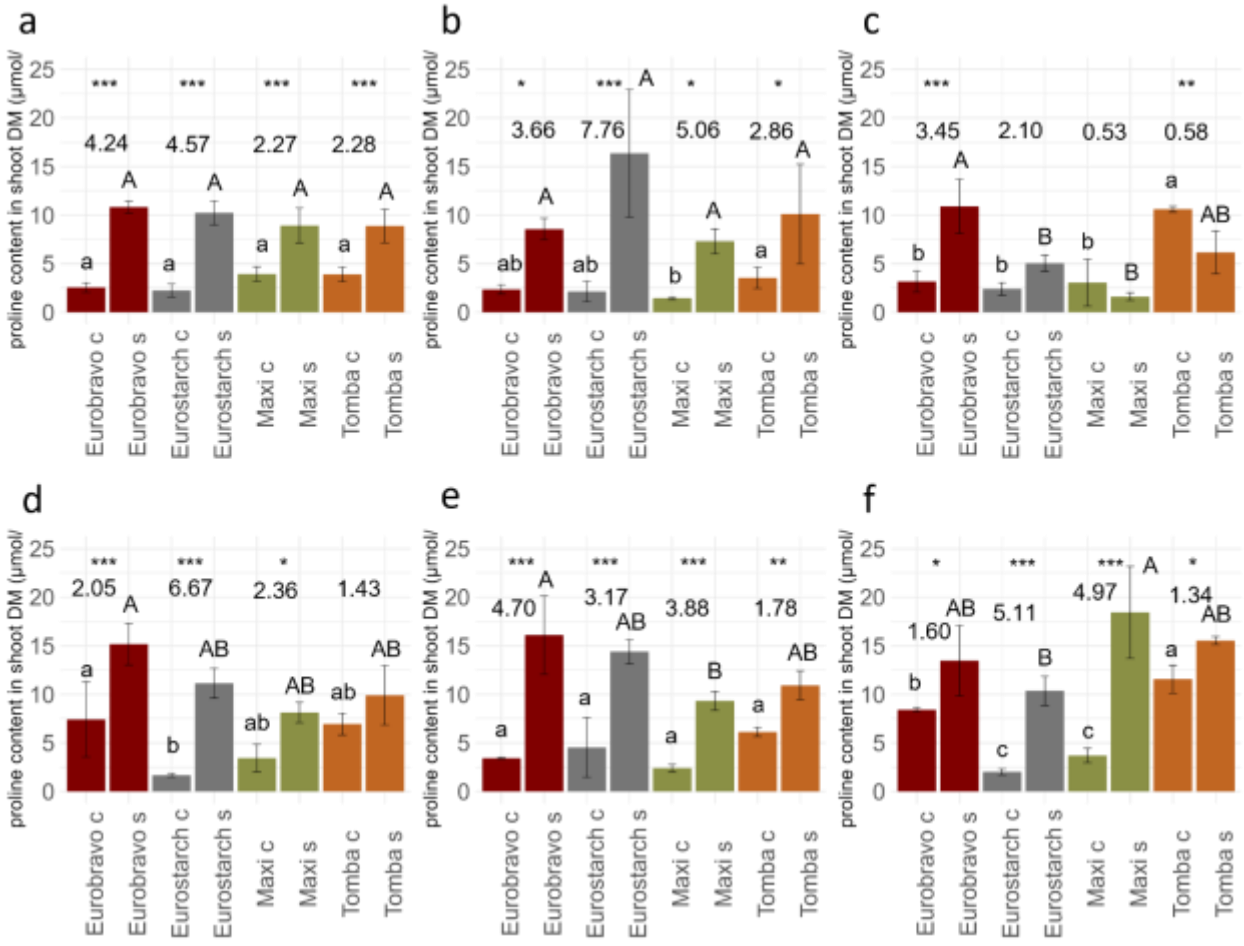

Fig. 7 Prolin content of in vitro shoots after seven and 14 days under osmotic stress in vitro with standard deviation (SD). a-c: proline content in shoot dry mass from experiment 1, 2, 3 after seven days, d-f: proline content in shoot dry mass from experiment 1, 2, and 3 after 14 days. Values above bars represent the fold change (stress/control). c: control, s: stress. Statistical analysis: ANOVA and Tukey’s test. Lower case letters compare control values between the genotypes. Upper case letters compare stress values between genotypes. Asterisks compare control and stress variant within one genotype. Significance codes after Tukey’s test or Kruskal-Wallis test: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. n=5.

Sorbitol was detected in shoots from plants with root growth

Sorbitol was measured in shoot samples of stressed and control plants of genotypes ‘Eurostach’ and ‘Maxi’ to determine whether the osmoticum was taken up by plants after rooting (Table S2). ‘Eurostarch’ showed a sorbitol content in shoots of control plants of 3.8 µg/g fresh mass (FM). After 14 days sorbitol content dropped to 0.9 µg/g. After seven days of stress treatment with sorbitol, the shoot content rose to 2696.5 µg/g shoot FM and to 939.3 µg/g shoot FM after 14 days. This resulted in fold changes (stress/control) of 702 and 1093 for seven and 14 days, respectively. ‘Maxi’ showed a sorbitol content in shoots of control plants of 1.9 µg/g FM after 7 days and 2.2 µg/g FM after 14 days. They increased to 1211.5 µg/g and 769.3 µg/g in plants treated with sorbitol, resulting in fold changes of 630 and 349 after 7 and 14 days, respectively (Table S2).

Discussion

General response to osmotic stress in vitro

With climate change and severe drought periods in temperate regions there is growing need for drought tolerant potato genotypes (Haverkort und Verhagen 2008). Osmotic stress arises ex vitro as part of e.g. drought and salt stress and can be achieved in vitro by adding an osmoticum to the culture medium in vitro. Literature shows that in vitro systems are time- and cost-efficient systems for the detection of tolerance in newly bred genotypes (Gopal und Iwama 2007).

Growth reduction is one of the first responses to osmotic stress (Dobránszki et al. 2003). Likewise, in our study, seven days after the first sorbitol application, the plants showed a visible reduced shoot growth (Fig. 4), which was also detectable in shoot fresh mass in experiment 2 and 3 (Table. S4). Since the difference between control and stress plants is no longer reflected in the dry mass of the shoots (Fig. 5), it can be assumed that the plants without osmotic stress primarily contained more water. Water loss was higher for all stressed genotypes compared to their control. The decrease of water content in the shoots from control to stressed shoots ranges from 14 % (‘Maxi’) to 36.8 % (‘Eurostarch’) in experiment 1, from 50.8 % (‘Tomba’) to 64.6 % (‘Maxi’) in experiment 2 and from 27.1 % (‘Tomba’) to 54.7 % (‘Maxi’) in experiment 3 (Table S5). At day 14, all genotypes expressed a shoot growth reduction also in their dry mass in experiment 2 (Fig. 6 b). ‘Maxi’ displayed the highest DM after osmotic stress in our test set. This is in agreement with previous results that ‘Maxi’ better copes with osmotic stress in vitro than the genotype ‘Eurobravo’ using solidified media (Bündig et al. 2016a). Interestingly, ‘Maxi’ was also rated rather tolerant to

drought stress compared to a test set under greenhouse and rain-out shelter conditions in which ‘Eurobravo’ was also represented and presented as rather sensistive genotype (Sprenger et al. 2015; Meise et al. 2019). However, decreased shoot mass under osmotic stress after 14 days was only shown for ‘Eurobravo’ in experiment 1 and ‘Eurostarch’ in experiment 3. For that reason the osmotic stress intesity should be considered to be increased in future studies.

Overall, root growth was not as severely affected as shoot growth. This reaction of potato to osmotic stress in vitro was also postulated by Dobránszki et al. (2003). The small differences in root mass between stress and control could originate from the previous rooting phase. All plants were able to form roots prior to the stress treatment, which were initially sufficient for them to continue growing. The root/shoot ratio was significantly shifted towards the roots for ‘Eurobravo’ in experiment 1, in ‘Maxi’ in experiment 2, and in ‘Eurostarch’ in experiment 3 (Fig. S1). A shift towards the root may be a sign for stress tolerance (Bündig et al. 2016a). Adaptation of individual genotypes to osmotic stress by shifting their root to shoot ratio towards the roots more consistently may be recorded at a later stage and needs further investigation.

Abiotic stress like drought stress and osmotic stress lead to an accumulation of proline in the plants by both, activation of proline biosynthesis and inhibition of degradation (Hayat et al. 2012). The amino acid acts as an osmoprotectant, as well as prevents damage caused by reactive oxygen species (ROS), and stabilises DNA, membranes and proteins (Ben Rejeb et al. 2014). In several studies on osmotic stress, proline showed to be higher abundant in the stress treated plant materials (Bündig et al. 2016a; Mawia et al. 2020). This correlates with the results presented. With accumulated proline in stressed potato shoots, we can prove the successful application of osmotic stress in vitro for experiment 1 and 2, as well as for ‘Eurobravo’ and ‘Tomba’ in experiment 3. However, a statement on the stress level and the difference in tolerance cannot be made on the basis of the results.

Normalised gene expression indicated osmotic stress reactions for all genotypes

Normalised gene expression was analysed seven days after the first sorbitol addition. This time point was chosen based on the visible alteration in growth (Fig. 4) in order to analyse rather early responses to osmotic stress. Early molecular responses can occur even minutes, hours, or days after onset of stress (Kollist et al. 2019). Response to osmotic stress was therefore visible for most GOIs despite no significant alteration after seven days based on shoot dry mass.

Plants can induce osmoregulation in vitro (Dobránszki et al. 2003). This was demonstrated by expression analyses of genes linked to osmotic adjustment, like *lipoxygenase* and *subtilisin* (Ueda et al. 2004). Upregulation of *lipoxygenase* was assigned to osmotic stress: Daneshmand et al. (2010) showed that NaCl, as well as PEG6000 led to osmotic stress leading to increased activity of a *lipoxygenase* in *Solanum stoloniferum* in vitro. The lipoxygenase cascade in plants is linked to oxylipin biosynthesis, which includes jasmonates, that are involved in plant defense mechanisms (Royo et al. 1996; García-Marcos et al. 2013). In our study, *13-LOX* was downregulated in 'Maxi' and 'Tomba', but not in 'Eurobravo' and 'Eurostarch'. There are genotypic differences in early regulation of *13-LOX*. Expression should be investigated by studies including earlier and later sampling time points under osmotic stress.

Expression of *ZBD* was downregulated in all genotypes under osmotic stress. The most likely protein for *ZBD* found in Wellpott et al. (2021) is an allyl alcohol dehydrogenase (Soltu.DM.03G015960), which is part of a family that can be linked to plant growth, development, and to adaption (Jörnvall et al. 2010; Strommer 2011). Allyl alcohol dehydrogenases are known to be $NADP^+$ dependent (Ying et al. 2014), which is a cofactor for e.g. photosynthesis and the calvin cyle. Downregulation of *ZBD* can therefore point to reduced photosynthesis rate, which is a common response to abiotic stress (Sharma et al. 2020). It is also important to consider the plants growing mixotrophic in vitro. Mixotrophy describes the ability to use different carbon sources for growth and mixotrophic cultivation is a standard technique for in vitro laboratories as light intesity is usually low. This change in metabolism is mainly due to the addition of sugar as a C source in the culture medium, high relative air humidity, and the decreased gas exchange through vessel lids leading to a less active photosystem of the plants (Kozai und Kubota 2001). Considering this, a less active photosystem and therefore less electron transfer in photosystem II can lead to downregulation of $NADP^+$-dependent alcohol dehydrogenase.

***SBT1.7, POD,* and *SHMT* showed consistent downregulation, whereas *INH1* displayed upregulation in all genotypes after osmotic stress**

*SBT1.*7, a subtilase family gene, was downregulated under osmotic stress in vitro. Subtilases are linked to cell growth and development (Schaller et al. 2018), leading to the conclusion, that reduced expression under osmotic stress can be assigned to reduced cell growth and thus, smaller shoot and root systems.

Another group of proteins, which are linked to osmotic stress, are peroxidases (Csiszár et al. 2012). Peroxidases are involved in detoxification of hydrogen peroxide (H_2O_2), which can be related to oxidative stress (Boguszewska et al. 2010). A gene of the peroxidase family was strongly downregulated in our study in all genotypes. The same gene was strongly downregulated in a study in an open greenhouse (Wellpott et al., submitted manuscript). Sprenger et al. (2016) also showed a gene of the peroxidase family to be downregulated in potato after drought stress, linking the response of drought and osmotic stress. However, this was not the same peroxidase as found in this study, pointing to the fact, that not all peroxidases were adressed under osmotic or drought stress. A consequence of oxidative stress is ROS production and thereafter damage of the plant cells. A strategy to overcome this damage and protect the plants is the detoxification of ROS. *SHMT* is also involved in this response (Hourton-Cabassa et al. 1998; Ambard-Bretteville et al. 2003). Gene expression of *SHMT* was significantly reduced in all genotypes under osmotic stress. This, paired with the higher abundant associated enzyme observed under drought stress in Wellpott et al. (2021), may point to a more rapid response of *SHMT*. This should be investigated further by analysing earlier time points after stress onset and by linking this to ROS production.

Genotype-specific responses were recorded for *Glyx*, a protein of the glyoxalase system (Kaur et al. 2014; Hoque et al. 2016) which was downregulated and *INH1*, an invertase inhibitor, which was upregulated in 'Eurostarch' and 'Tomba'. *Glyx* is known to detoxify methylglyoxal, which is potentially cytotoxic (Upadhyaya et al. 2011). A downregulation could have happened after a quick upregulation early after stress onset. This has to be clarified in future studies with further sampling dates. Meanwhile, *INH1* was found to be upregulated after drought stress treatment in potato by Aliche et al. (2022) linked to reduced growth. *INH1* plays a role in drought stress-mediated stomatal closure ex vitro (Kulik et al. 2011; Matsuoka et al. 2021) and the primary metabolism by hydrolysis of sucrose into glucose and fructose (Ruan et al. 2010). A significant response of this gene in two genotypes points to a more rapid response and thus a probably better coping mechanism of osmotic stress in vitro. A test-set including diverse genotypes and knock-down mutants of these genes would be helpful to understand the role of these genes in osmotic stress response.

The demonstrated regulation of genes, that are linked to a general stress response of potato to abiotic stress (*13-LOX*, *SBT1.7*, *POD*, *ZBD*, and *SHMT*), we were able to show that the studied plants indicated osmotic stress before it was measurable in the shoot dry mass. *Glyx* and *INH1*, reacted genotype-specific in our test setup, which therefore might be interesting GOIs for the

identification of biomarkers for osmotic stress tolerance. Further studies under osmotic, drought and other abiotic stress should be conducted to show if this is a general or osmotic and drought stress specific response and could yield suitable biomarker genes for stress tolerance in potato.

Sorbitol concentration drastically increased in rooted shoots under osmotic stress

Osmotica for an in vitro stress test should fullfill several criteria, such as reducing the osmotic potential in the medium, being inert, non-toxic to the plant and plants should not take up the osmoticum, as it is unknown which internal interactions the osmoticum affects. Several studies reported experiments using mannitol or PEG to induce osmotic stress in vitro (Gopal and Iwama 2007, Sahoo et al. 2020, Jiroutova et al. 2021; Hanász et al. 2022). However, both of these substances have disadvantages that complicate the evaluation of tolerance to osmotic stress. Lipavsk und Vreugdenhil (1996) showed that mannitol was taken up by potato, influencing growth. Gopal und Iwama (2007) proposed PEG to limit O_2 movement resulting in O_2 deficiency in roots, and therefore postulated that PEG might not be an ideal osmoticum.

Being less viscous, sorbitol is an often used osmoticum to induce osmotic stress in potato in vitro (Gopal und Iwama 2007; Bündig et al. 2016a; Mawia et al. 2020; Sajid und Aftab 2022). Bündig et al. (2016b) stated that nodal cuttings presumably take up sorbitol through cut surfaces of shoots. In our study, plants in the osmotic stress experiments were rooted prior to the stress treatment to avoid an uptake of sorbitol through the wound. Measurement of sorbitol in the shoots of ‘Eurostarch’ and ‘Maxi’ displayed much higher concentration of sorbitol in shoots after sorbitol treatment than in control shoots after treatment with water. This leads to the conclusion that sorbitol was probably taken up through roots from stressed plants in vitro. If the osmoticum is actively taken up from the medium and transported to the shoots, metabolised or stored in the shoots, remains unclear and should be analysed in future experiments using isotope labeling. *Solanum lycopersicum* is classified as a non-usual sorbitol producer (Pleyerová et al 2022) and consistently we only detected small amounts of sorbitol in the related species *Solanum tuberosum* even in the absence of a sorbitol treatment. Furthermore, it was shown that drought stress in tomato results in the increased production of sorbitol (Nosarzewski et al. 2021). Since we cannot distinguish the sorbitol supplied by the stress treatment from the endogenously formed sorbitol we cannot exclude that the increase of sorbitol in the shoots upon stress treatment is not the result of its formation in the plant upon sensing drought-like conditions in the in vitro culture. It would be interesting to study the sorbitol content upon treatment with a different osmoticum such as PEG to see whether

598 this also results in a high sorbitol content in stress-treated plants. The treatment with isotopically
599 labeled sorbitol would enable us to distinguish both sorbitol pools by mass spectrometry and give
600 a conclusive answer to the possibility of sorbitol uptake by rooted potato plants in vitro.

601 ## Conclusion

602 In this study, we introduce a test system with liquid medium, in which the plants were allowed to
603 form roots prior to the stress treatment with sorbitol. In addition to osmotic stress, it is possible to
604 add substances to trigger other abiotic stresses like salt stress, at the desired time. Unlike in solid
605 medium, it is also possible to transfer the plants with roots in the plastic sieves to new medium and
606 continue the test system while the conditions in the medium may change. Finding a suitable
607 osmoticum is crucial for the induction of osmotic stress with the purpose of classification of potato
608 genotypes according to their tolerance level. In this study, we propose that sorbitol is probably
609 taken up by plants into the shoot. Whether sorbitol is taken up through the roots, metabolised or
610 stored in the plants remains unclear, and should be further analysed. If the osmoticum is taken up
611 through the shoots, sorbitol has to be replaced in future osmotic stress studies. However, we could
612 show that in vitro plants show morphological responses to osmotic stress in vitro and gene
613 expression was altered for the majority of the analysed GOI. In the liquid medium we were able to
614 apply the osmoticum gradually. To a point, this leads to the possibility to mimic the development
615 of drought stress in the field more closely.

616

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Statements and Declarations

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Competing Interests

The authors have no financial or non-financial competing interests to disclose.

Author contribution statement

Material preparation and data collection were performed by KW and CB. MH performed and supervised part of the data collection. KW and CB contributed to data analysis. CB and TW conceived and coordinated the project. The first draft of the manuscript was written by KW. The manuscript was revised by MH, CB and TW. All authors have read and approved the final document.

Data availability statement

The data that support the findings of this study are openly available in Research Data Repository of the Leibniz University Hannover at https://doi.org/10.25835/u5gj5bdx

2.4 Combined nitrogen and drought stress leads to overlapping and unique proteomic responses in potato

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ORIGINAL ARTICLE

Combined nitrogen and drought stress leads to overlapping and unique proteomic responses in potato

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Abstract

Main conclusion **Nitrogen deficient and drought-tolerant or sensitive potatoes differ in proteomic responses under combined (NWD) and individual stresses. The sensitive genotype 'Kiebitz' exhibits a higher abundance of proteases under NWD.**

Abstract Abiotic stresses such as N deficiency and drought affect the yield of *Solanum tuberosum* L. tremendously. Therefore, it is of importance to improve potato genotypes in terms of stress tolerance. In this study, we identified differentially abundant proteins (DAPs) in four starch potato genotypes under N deficiency (ND), drought stress (WD), or combined stress (NWD) in two rain-out shelter experiments. The gel-free LC–MS analysis generated a set of 1177 identified and quantified proteins. The incidence of common DAPs in tolerant and sensitive genotypes under NWD indicates general responses to this stress combination. Most of these proteins were part of the amino acid metabolism (13.9%). Three isoforms of S-adenosyl methionine synthase (SAMS) were found to be lower abundant in all genotypes. As SAMS were found upon application of single stresses as well, these proteins appear to be part of the general stress response in potato. Interestingly, the sensitive genotype 'Kiebitz' showed a higher abundance of three proteases (subtilase, carboxypeptidase, subtilase family protein) and a lower abundance of a protease inhibitor (stigma expressed protein) under NWD stress compared to control plants. The comparably tolerant genotype 'Tomba', however, displayed lower abundances of proteases. This indicates a better coping strategy for the tolerant genotype and a quicker reaction to WD when previously stressed with ND.

Keywords Abiotic stress · Combined stress · Label-free quantification · LC–MS · Protease · *Solanum tuberosum* · Stress response

Abbreviations

NWD	Nitrogen and water deficiency
ND	Nitrogen deficiency
WD	Water deficiency
DAP	Differentially abundant protein
SAMS	S-adenosyl-L-methionine synthase

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Introduction

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide with a production of 359 million tons in 2020 (FAO 2020). In addition to table potato as a food source, starch potatoes are grown for industrial purposes such as paper, adhesives, or bioplastics due to their high starch content (Röper 2002).

With the growing world population and an increase in extreme weather conditions due to climate change, there is an urgent need to improve potato genotypes to ensure stable yields. Abiotic stresses, such as drought, are climate change-related problems in agriculture. In potato, such stresses can result in reduced plant growth and poor tuber yield and quality (Aliche et al. 2018; Hill et al. 2021). Due to their shallow root system, potato plants are more susceptible to drought stress than other crops. Therefore, irrigation is mostly essential for optimal yield (Zarzyńska et al. 2017). Furthermore, potato yield depends highly on sufficient N in the soil. N fertilization is unavoidable during periods of high vegetative growth in spring and early summer (Bélanger et al. 2000). Especially on sandy soils, where potatoes are mainly cultivated, the risk of N loss is high. Since the irrigation and fertilization phases fall into the same period and potato plants only take up 30–60% of the fertilized N from the soil, a high risk arises that N in form of nitrate (NO_3^-) leaches into the groundwater (Zerbarth and Rosen 2007). Therefore, N-efficient and drought-tolerant potato genotypes could mitigate these ecological problems and would be highly desired by farmers and breeders.

In the past, many transcriptomic studies have been performed to display the plant response to high and low levels of N as well as to drought stress. They demonstrated that numerous biological processes, such as amino and nucleic acid synthesis, protein folding, RNA processing, secondary metabolism and hormone biosynthesis are rapidly affected when nitrate is depleted or resupplied (Wang et al. 2003; Scheible et al. 2004; Gutiérrez et al. 2007). Carbohydrate metabolism, lipid metabolism, heat shock proteins and secondary metabolism are affected under drought stress (Evers et al. 2010; Aliche et al. 2022). In proteomic and transcriptomic studies on individual abiotic stressors such as salt (Legay et al. 2009), heat (Hancock et al. 2014), drought (Vasquez-Robinet et al. 2008; Boguszewska-Mankowska et al. 2020), or N deficiency (Jozefowicz et al. 2017; Meise et al. 2017; Tiwari et al. 2020a, 2020b) proteins and genes involved in the stress response were identified. Boguszewska-Mankowska et al. (2020) detected proteins that could be assigned to carbohydrate or amino acid metabolism to appear in higher abundance under drought stress conditions in a proteomic approach. Moreover, Vasquez-Robinet et al. (2008) found chaperones in a higher abundance under drought stress. Under N deficiency, Tiwari et al. (2020b) presented genes of protease inhibitors upregulated in a N-efficient potato cultivar. When abiotic stressors like drought and heat were applied in combination, evidence for divergently affected metabolic pathways and proteins was reported (Mittler 2006; Pandey et al. 2015; Demirel et al. 2020). However, knowledge about metabolic pathways and specific proteins for the combined stress of water deficit and N deficiency is absent for potato.

This study aimed to identify differentially abundant proteins (DAPs) in control and stress treatments to highlight general biochemical responses of potato to combined stress (NWD) as well as specific responses of genotypes with differing tolerance level to the provided stresses. This intended to get a deeper insight into the processes of abiotic stress tolerance and lead to identification of marker proteins. We chose a comprehensive proteomic approach to decipher the final metabolic adjustments rather than initial cellular responses. To pursue this aim, we selected two varieties, 'Tomba' and 'Kiebitz', among others, showing specific and contrasting reaction to either single or combined stress. With particular consideration of NWD, we showed both, general proteomic responses observed in both analyzed genotypes and divergent genotype dependent reactions to NWD. Differentially affected metabolic pathways were identified and related to the level of genotypes' stress tolerance. Moreover, we emphasized differences in the responses to NWD as compared to the reactions to N deficiency (ND) and drought stress (WD).

Materials and methods

Plant materials

Plant material used for this study was sampled from two experiments in a rain-out shelter which took place in the Federal Research Centre for Cultivated Plants, Institute for Resistance Research and Stress Tolerance, Julius Kühn-Institute (JKI), Sanitz, Germany, in 2013 and 2015. Among 14 starch potato cultivars and 3 table potato cultivars tested in these experiments, the most divergently responding cultivars (hereafter: genotypes) 'Eurostarch', 'Kiebitz', 'Kolibri', and 'Tomba' were selected for this study based upon tuber and starch yield (Meise et al. 2019). Plants were grown under N deficiency (ND, supplied with a total of 260 mg N) and control conditions (C, cultivated at a continuous 60% water capacity, supplied with a total of 1040 mg N). Drought stress was applied at the beginning of tuber initiation. For this purpose, plants were kept 12–13 days without watering (WD), while the control treatment received water to

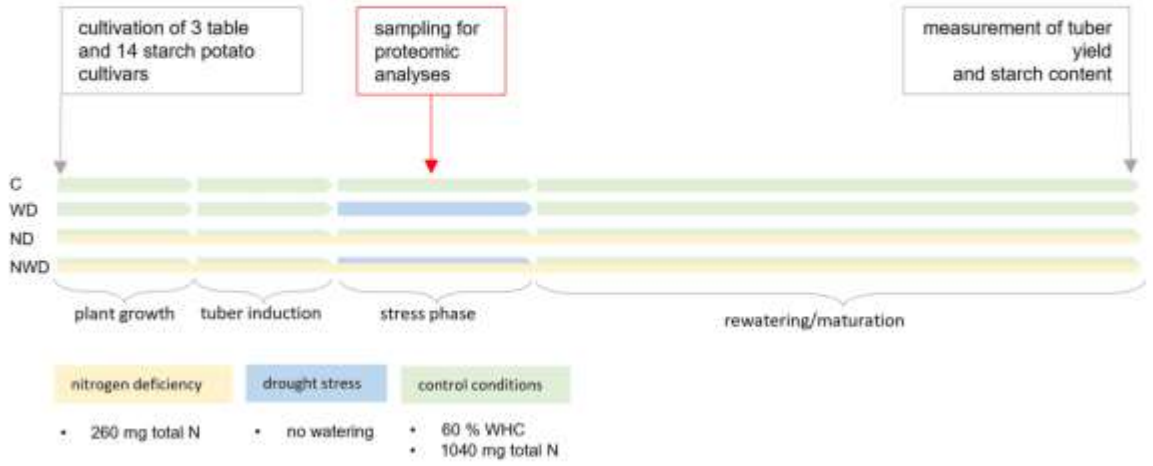


Fig. 1 Timeline of pot trials with drought stress and N deficiency. Three table and 14 starch potato genotypes were cultivated until tuber formation (ND + NWD: 260 mg total N; C (control) and WD: 1040 mg total N). ND and C treatment received water up to 60% WHC, WD and NWD were not watered. At the first sign of wilting (5 d after stress onset), samples for proteomic analyses were taken. After the stress period, all plants were rewatered until maturity (60% WHC)

maintain 60% water capacity during the whole experiment. Combined stress included both, drought and N deficiency (NWD). Details of the experimental setup are described in Meise et al. (2018, 2019) and an outline is given in Fig. 1. Samples were taken 5 days after drought stress initiation. The fourth and fifth leaflets of the youngest fully developed pinnate leaf were sampled and immediately frozen in liquid nitrogen (LN). Samples were stored at − 80 °C until analysis. Based on the calculation of the SSI (stress susceptibility index after Fischer and Maurer 1978) of the tuber and starch yield at harvest, the genotype 'Tomba' was found to be more tolerant to both, drought stress and N deficiency, as well as the stress combination in comparison to all other genotypes of the test set. The genotype 'Kiebitz' was found to be more sensitive under both stress situations compared to the other genotypes of the test set. Therefore, we will refer to the genotype 'Tomba' as 'tolerant' and to the genotype 'Kiebitz' as 'sensitive' hereafter. The genotypes 'Eurostarch' and 'Kolibri' showed contrasting responses depending on the stress type: 'Eurostarch' was assigned to the more tolerant genotypes, according to the SSI based on tuber yield under ND, whereas 'Kolibri' belonged to the more sensitive genotypes under NWD (Meise et al. 2019).

Protein extraction and digestion

The protein processing and measurement were performed separately for single stress treatments ND and WD and the combination of stresses NWD. Control condition samples were measured in both analyses.

Frozen plant material was ground to a fine powder under LN using a mixer mill (MM400, Retsch, Haan, Germany; steel beads Ø 3 mm). A maximum of 100 mg of ground material was used for protein extraction. Leaf proteins were extracted using a trichloroacetic acid/acetone protocol (Tsugita and Kamo 1999) with some modifications. TCA (trichloroacetic acid) solutions A and B contained 20 mM DTT (dithiothreitol) instead of 0.07% 2-mercaptoethanol. The resulting dried pellets (25–35 mg) were resuspended in 100 µl lysis buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), 5 mM DTT; pH 8.0), incubated for 1 h at 37 °C and centrifuged for 15 min at 17.000 g.

The concentration of protein in the solution was estimated using a 2-D Quant Kit (GE Healthcare, Munich, Germany) as previously described by Jozefowicz et al. (2020). Aliquots containing 10 µg of proteins were subjected to filter-based digestion, following Jozefowicz et al. (2017), which consisted of overnight digestion at 37 °C in a 1:50 dilution of sequencing grade modified trypsin (Promega, Mannheim, Germany). Before LC–MS analysis, peptides were suspended in 50 µl of 2% acetonitrile (ACN) and 0.1% (v/v) formic acid (FA).

Label-free quantification of proteins

Peptides were analyzed by LC–MS, using Dionex UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific, Dreieich, Germany) coupled with an Impact II (Bruker Daltonics, Bremen, Germany). Digested protein

samples were separated using a nano trap column (Acclaim PepMap100 C18, 5 µm, 100 Å) and an analytical column (Acclaim PepMap RSLC C18, Thermo Fisher Scientific, 50 cm × 75 µm).

600 µg of peptides were separated through a 2–40% acetonitrile gradient (buffer A: 0.1% FA in LC–MS grade water; buffer B: 0.1% FA in LC–MS grade ACN) over 120 min applying a flow rate of 300 nl/min. Due to loading and washing steps, the total time for an LC–MS/MS run was prolonged to 160 min.

The CaptiveSpray ion source with a nanoBooster device was used to connect the LC system to the MS instrument. The source was operated in positive ion mode at 150 °C dry temperature, 1300 V capillary voltage, 0.2 bar nanoBooster, and a dry gas flow of 0.3 l/min. For the MS and MS/MS acquisition, the predefined 'Instant Expertise' method was used (Compass 1.9, Bruker). Briefly, the *m/z* data were acquired in the range of 150 to 2200 and the fixed total cycle time was set to 3.0 s. The instrument settings were as follows: hexapole radio frequency (RF) voltage of 350 V peak-to-peak (Vpp), a funnel 1 RF of 400 Vpp, a funnel 2 RF of 600 Vpp, a pre-pulse storage time of 10 µs, a transfer time of 90 µs and a collision cell RF of 2000 Vpp. For the MS spectra, the acquisition speed was 2 Hz with a collision energy of 7 eV. For the MS/MS, the acquisition speed was dependent on the precursor signal intensities and was set to 4 Hz for lower (2500 cts) and 16 Hz for higher (25,000 cts) intensities with linear adjustment for the precursors between low and high. The collision energy was adjusted between 23 and 65 eV as a function of the *m/z* value. The instrument was calibrated using 10 mM sodium formate.

Data analysis

The acquired spectra were processed for label-free quantifications using Progenesis QI software for proteomics (Version 3.0, Nonlinear Dynamics, Newcastle upon Tyne, UK) as recommended by the manufacturer, thereby enabling mass correction, alignment, normalization, peak picking, quantification, and statistics. MS/MS spectra were exported from the Progenesis QI software as Mascot generic files and used for peptide identification with Mascot v2.5.1. The potato database based on the sequences from *Solanum tuberosum* group Phureja DM1-3 (PGSC_DM_v3.4_pep_representative, 39,031 entries) (Xu et al. 2011) was annotated by matching against available NCBI entries with Blast2GO software (09.2014) (Conesa and Götz 2008) and merged with the sequences of human keratin and trypsin. The search parameters applied were as follows: 15 ppm peptide mass tolerance, 0.05 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation as fixed modification, and methionine oxidation as variable modification. A Mascot integrated peptide decoy database search was performed and searches were processed with the Percolator machine-learning algorithm (Käll et al. 2008). The false discovery rate was < 1% and ion score cut-off 13. For subsequent analysis, the set of identified sequences was re-imported into Progenesis QI. Quantification was performed for proteins identified with at least two unique peptides. The results of protein quantification were exported and further analyzed in MS Excel.

Statistics and selection of differentially abundant proteins (DAPs)

The protein data obtained for experiment 1 (2013) and experiment 2 (2015) for each genotype were analyzed separately, due to the different weather conditions in both years, particularly very high temperatures during the 5 days of water withdrawal in experiment 1 (mean temperature 2013: 22.03 °C; 2015: 19.03 °C) (Meise et al. 2018). Only proteins that were of significantly changed abundance in both experiments (student's *T* test $P < 0.05$ and fold change stress/control < 0.66 or > 1.5) were considered DAPs. Proteins of differential abundance in single experiments only were considered as altered due to additional factors such as fluctuations in the weather conditions and were therefore withdrawn from further analysis. Venn diagrams were created using Venny 2.0 tool (Oliveros 2007).

Additional annotation for selected proteins was sought by referring to the UniProt server (www.uniprot.org). Proteins were functionally classified according to KEGG orthology using BlastKoala or manual classification in case functions could not be assigned automatically. Principal component analysis, Z-score normalization, and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework (Tyanova et al. 2016). A full listing of the differentially expressed proteins has been archived, together with all of the raw data, in the IPK Gatersleben system e!DAL (Arend et al. 2014), available at: https://doi.org/10.5447/IPK/2023/4.

Results and discussion

Genotype 'Tomba' was selected as more tolerant to ND, WD, and NWD

A previous study, in which the performance of 14 starch and 3 table potato genotypes was compared under N deficiency (ND), water deficiency (WD), and a combination of stresses (NWD) in two rain-out-shelter experiments (Meise et al. 2018), was the basis for the proteomic analysis in the present investigation. Out of the 14 starch genotypes, 4 genotypes with the most contrasting response to a combination of drought and N deficiency were selected. Genotype 'Tomba'

exhibited the highest tuber yield under two of the three applied stress conditions (WD, NWD), whereas 'Eurostarch' had a slightly higher yield under N deficiency (ND). On the contrary, genotype 'Kiebitz' produced the lowest tuber biomass under control, N deficiency, and water deficiency conditions within the experiments. Genotype 'Kolibri' produced the lowest yield when combined N and water deficiency (NWD) was applied. When both stresses were combined, genotype 'Kiebitz' produced only 38%, whereas genotype 'Tomba' still produced 68% of the tuber fresh weight under control conditions (Meise et al. 2018). The changes in the growth (Fig. 2) and the nutritional status of the potato plants were displayed by measuring $N_{Kjeldahl}$, total protein content, soluble sugars, and proline content upon stress application of the genotypes analyzed (Suppl. Table S1). 'Tomba' showed a higher N content with 30.8 ± 6.4 mg N/g DM than 'Kiebitz' with 19.5 ± 1.1 mg N/g DM after NWD. Pure protein content, as well as proline content, were also higher in 'Tomba' (26.7 ± 5.0 mg/g DM; 4.0 ± 3.2 µmol/g DM) than in 'Kiebitz' (18.9 ± 1.8 mg/g DM; 2.2 ± 0.7 µmol/g DM). Relative water content was 80.1 ± 3.8% for 'Tomba', while it dropped to 76.1 ± 3.9% for 'Kiebitz'. Plant height also differed between the two genotypes. With 16.9 ± 1.8 cm, 'Kiebitz' was the shortest genotype of the tested genotypes after combined stress. 'Tomba' reached a plant height of 22.7 ± 2.2 cm.

Different numbers of proteins are changed in potato genotypes after NWD treatment

The label-free LC-MS analysis generated a set of 1177 identified and quantified proteins, based on 6,060 non-conflicting peptides (the full list of identified proteins is stored together with raw data in the e!DAL system of the IPK-Gatersleben). The differences in the protein profiles were elucidated by a principal component analysis (PCA) for both experiments independently (Fig. 3). The four potato genotypes and the treatments were separated by PC1 and

Fig. 2 Condition of genotype 'Tomba' in control, ND, WD and NWD treatment (left to right) in experiment 1 (**a**) and experiment 2 (**c**), and of genotype 'Kiebitz' in experiment 1 (**b**) and 2 (**d**)

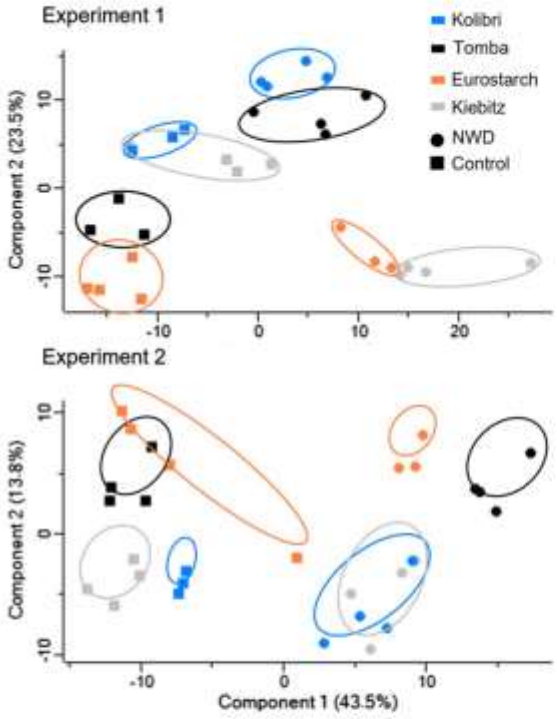


Fig. 3 Principal component analysis showed a clear separation of the NWD treatment from controls for all four potato genotypes. The shape of data points indicates combined N and water deficiency (NWD, circle) or control conditions (square). The colors indicate the genotype: 'Tomba' (black), 'Kiebitz' (grey), 'Eurostarch' (orange), 'Kolibri' (blue)

PC2, with PC1 accounting for 44.7% and 43.5% of the explained variance in experiments 1 and 2, respectively, and PC2 for 23.5% and 13.8%. The clustering of protein profiles showed differences in experiments 1 and 2. For instance, genotypes 'Kiebitz' and 'Kolibri' behaved similarly under control conditions in experiment 1 and under NWD treatment in experiment 2. Thus, additional factors (temperature, air humidity) might have influenced the protein profiles during both experiments (Georgii et al. 2017), and therefore, only proteins with significantly changed abundance in both years were considered differentially abundant proteins (DAPs). Applying this filter, for the NWD stress treatment 234, 199, 199, and 74 DAPs were identified in genotypes 'Tomba', 'Eurostarch', 'Kiebitz', and 'Kolibri', respectively. These will be discussed in detail in the following sections with emphasis on common (shared) general responses to NWD seen in all four genotypes, before the specific responses of the tolerant genotype 'Tomba' and the sensitive genotype 'Kiebitz will be elaborated. Finally, the response to the combined stress will be compared to both single stresses, N deficiency and drought.

General response of potato genotypes to NWD stress

The focus of this study was set on the comparison of DAPs in the genotypes 'Tomba' and 'Kiebitz', as these genotypes showed the most contrasting response to ND, WD, and NWD (Meise et al. 2019). Most of the proteins considered in the following paragraph showed a very similar trend in abundance alteration in the other two genotypes 'Kolibri' and 'Eurostarch' but did not fulfill the criteria of significance as stated in Material and methods (e.g. student's T test *P* and FC stress/control, Suppl. Table S2). The comparison of DAPs in 'Tomba' and 'Kiebitz' revealed that 86 proteins were significantly changed in both genotypes, whereas 148 were specific to 'Tomba' and 113 to 'Kiebitz' (Suppl. Fig. S1). Functional assignment of the proteins according to KEGG orthology was performed to gain a first understanding of processes commonly regulated in response to NWD stress (Fig. 4a). The hierarchical clustering analysis revealed five clusters of DAPs with similar regulation in response to NWD stress (Fig. 4b, Suppl. Table S2). Only one DAP belonged to cluster I (ribosomal protein S10), which reacted with an increase in relative abundance to NWD in both genotypes in experiment 1 but decreased in 'Kiebitz' in experiment 2. Cluster II comprised three DAPs (vacuolar processing enzyme 1, snakin-2, and remorin) with an increase in abundance due to the NWD stress. Cluster III grouped three DAPs (cytochrome C oxidase polypeptide, cell wall invertase, and cysteine peptidase 3). Those proteins increased in the stress response, with the exception of 'Tomba' in experiment 1. Cluster IV captured three DAPs (ATP-dependent Clp protease, lysine-tRNA ligase, and ribulose bisphosphate carboxylase large chain), that decreased in the stress response, with exception of 'Tomba' in experiment 1. Finally, cluster V contained the majority of NWD-responding DAPs, which decreased in relative abundance in response to stress in both genotypes. The overrepresented processes and pathways in cluster V were: TCA cycle and glycolysis (fructose-bisphosphate aldolase, pyruvate dehydrogenase E1 and E2 component, diphosphate-dependent phosphofructokinase, dihydrolipoamide dehydrogenase, and pyruvate kinase), chlorophyll synthesis (Mg-protoporphyrin IX chelatase, delta-aminolevulinic acid dehydratase, uroporphyrinogen decarboxylase, protoporphyrinogen oxidase, glutamate-1-semialdehyde 2,1-aminomutase and glutaminase), ethylene biosynthesis (S-adenosylmethionine synthetase (SAMS)-3 isoforms, adenosylhomocysteinase, and aminocyclopropane carboxylate oxidase) and cytoskeleton proteins (Ase1/PRC1/MAP65 family protein, katanin p60 ATPase, tubulin alpha and beta). The 86 DAPs and their functional classification are accessible in detail in Suppl. Table S2, in the same order as presented in the heat map (Fig. 4b).

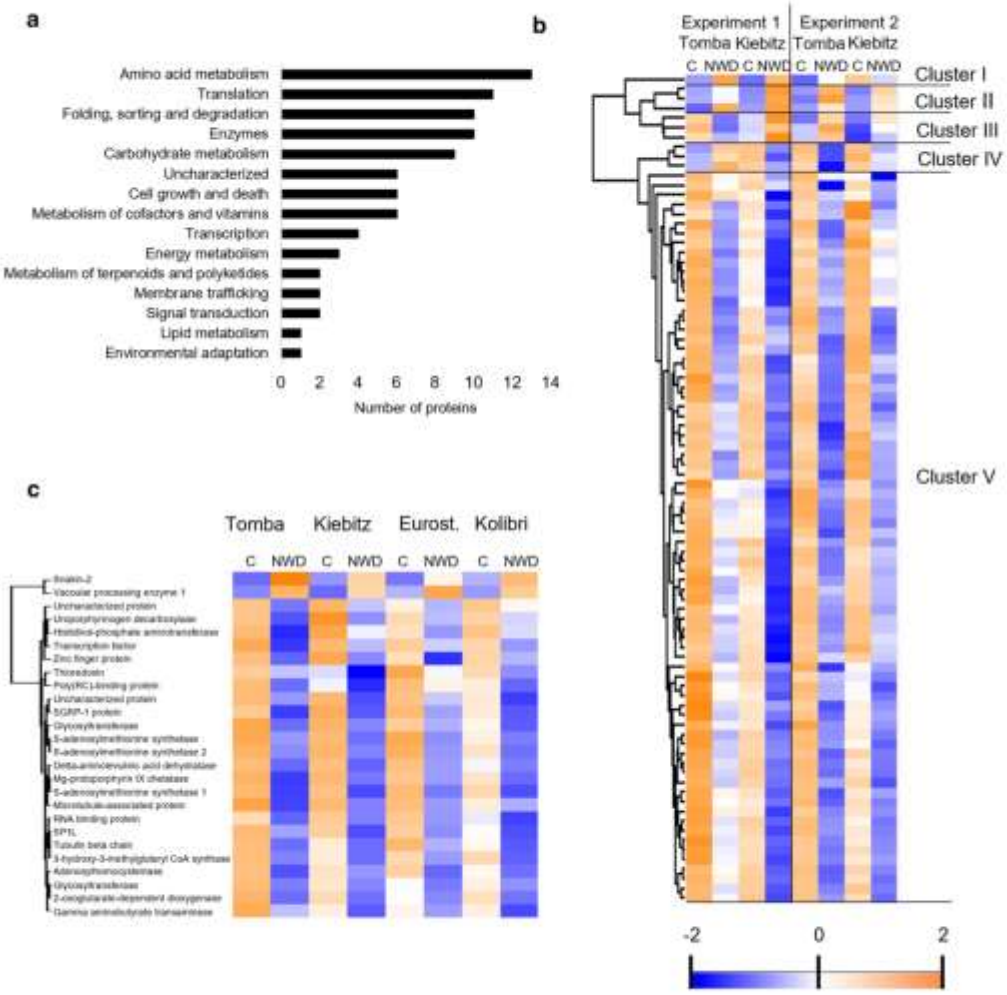


Fig. 4 Eighty-six proteins showed a similar response to NWD stress in 'Tomba' and Kiebitz'. **a** Functional classification of proteins responding to NWD stress in both genotypes. The classification was performed according to the KEGG orthology. **b** Heat map representation of proteins with a similar response to NWD in 'Tomba' and 'Kiebitz' divided into five clusters with similar abundance profiles. Hierarchical clustering was carried out with k-means preprocessing and was based on average Euclidean distance linkage. Relative abundance in the heatmap has been color-coded following Z-score normalization. Each column represents one treatment. *NWD* N and water deficiency; *C* control conditions. **c** Heat map representation of proteins with a similar response to NWD in all four potato genotypes. Eurost, 'Eurostarch'

Amino acid metabolism was assigned the largest group of DAPs (12 DAPs; 13.9%), which differed significantly in abundance in both genotypes, especially cysteine and methionine metabolism. As many as three isoforms of SAMS were significantly less abundant under NWD in both genotypes and experimental years (Cluster V) (Suppl. Table S2). S-adenosyl-l-methionine (SAM) is a key metabolite for different processes such as polyamine biosynthesis as well as lignin biosynthesis and is catalyzed by SAMS from methionine and ATP. SAMS is also known to have a function in the response to environmental stressors (Heidari et al. 2020). Kim et al. (2015) were able to link SAMS in wild potato (*S. brevidens*) to the upregulation of ABA and ethylene metabolic pathway genes. They detected higher salt and drought stress tolerance when a full-length cDNA of SAMS from *S. brevidens* was overexpressed in *Arabidopsis thaliana*. Because SAM is the precursor for ethylene (Amir 2010), the expression of SAMS is important for ethylene biosynthesis and, therefore, for the regulation of growth and senescence (Khan et al. 2015). Zhang et al. (2020) reported overexpression of *SlSAMS1* to influence the reaction to drought, salt stress, low temperature, and ABA

treatments in *Solanum lycopersicum*. They showed increased abiotic stress tolerance in *SlSAMS1*-overexpressing plants by improved water retention and photosynthesis capacity as well as higher levels of ROS-scavenging enzymes. In contrast, in our study, we found a lower abundance of SAMS in NWD. Furthermore, SAMS was also observed to be lower abundant in WD in all genotypes and ND in the more tolerant genotypes 'Tomba' and 'Eurostarch'. ND was applied since the beginning of the experiment (5 weeks in total). Subsequently, 5 days before sampling of the leaves, water deficiency in the corresponding variants started. Due to the prolonged nitrogen deficiency, it could be assumed that components such as methionine were used up by the plants at the time of protein analysis. Methionine should be measured in a future trial to provide information on methionine content in potato leaves.

Ten DAPs out of the 86 DAPs under NWD were associated with folding, sorting, and degradation of proteins. Two proteins were found to be higher abundant in both genotypes after combined stress was applied (Cluster II): vacuolar processing enzyme (VPE) and cysteine proteinase 3-like. Both proteins are known to be key factors in programmed cell death and thus related to abiotic stress (Solomon et al. 1999; Teper-Bamnolker et al. 2021). While programmed cell death is a way for plants to selectively eliminate damaged cells and recycle nutrients (Wingler et al. 2004), a higher abundance of VPE and cysteine proteinase 3-like might be a strategy for the plant to cope with abiotic stresses. VPE is an enzyme that is stimulated by various stressors like heat, oxidative, and salt as well as biotic stressors. Besides being involved in PCD, vacuolar processing enzymes are also described to be responsible for processing protein precursors of chitinases and proteinase inhibitors to evoke their active forms (Yamada et al. 2020).

In general, abiotic stress reduces photosynthesis efficiency either directly due to decreased CO_2 availability by stomatal closure or indirectly by oxidative stress (Chaves et al. 2009; Golldack et al. 2014). Therefore, plants are facing a reduced energy supply in form of C products during abiotic stress. Nine DAPs were identified, being associated with C metabolism. Especially proteins linked to glycolysis, the pentose phosphate pathway, and the TCA cycle were found to be less abundant under combined stress. Pyruvate dehydrogenase (PDH) is the first enzyme of the PDH complex, which enables the entry of C into the TCA cycle and thus energy production (Ohbayashi et al. 2019). With less PDH available, less carbon is fed into the TCA cycle, which is, therefore, unavailable for respiration. Thus, the higher abundance of cytochrome c oxidase subunit 6b, which was determined for 'Kiebitz' in both years and for 'Tomba' in 2015, might help to sustain respiration, as it is part of complex IV and a terminal electron acceptor of the mitochondrial respiratory chain (Chen et al. 2009). The increased respiration could be used to generate ATP for nutrient recycling and export during senescence and PCD (Mayta et al. 2019). Metabolic and functional studies would be needed in future studies to shed light on the amino acid and carbohydrate metabolism under combined stress.

Biosynthesis of special cofactors e.g. ascorbate (ABA biosynthesis) can be linked to abiotic stressors such as light and drought (Smith et al. 2007) as well as to drought stress tolerance in maize and soybean (Krannich et al. 2015). In our study, six DAPs were lower abundant in NWD and identified to be related to the metabolic pathways of cofactors and vitamins. Most of them were found to be associated with chlorophyll biosynthesis, which is essential for functional photosynthesis. This process was likely reduced under NWD stress as indicated by the lower abundance of porphobilinogen synthase (also 5-aminolevulinate dehydrogenase or delta-aminolevulinic acid dehydratase), which combines two molecules of 5-aminolevulinic acids to form porphobilinogen, and magnesium chelatase, which catalyzes the reaction of protoporphyrin IX to Mg-protoporphyrin IX in a later step (Ohmiya et al. 2014). This leads to less efficient photosynthesis, which has also been described in potato by Li et al. (2016) under NWD and by Aliche et al. (2018) under WD. Magnesium chelatase has also been linked to ABA-mediated signaling and ABA-induced stomatal closure. In *Arabidopsis thaliana* mutants, overexpressing the Mg-chelatase H subunit, a higher tolerance to drought stress was observed (Tsuzuki et al. 2013). In line with this observation, when Meise et al. (2017) applied ND in an in vitro test system, they found magnesium chelatase to be higher abundant after ND in a tolerant potato genotype. In the present study, however, magnesium chelatase was lower abundant in all genotypes under NWD.

Proteins specific to the tolerant genotype 'Tomba'

148 proteins were differentially abundant only in the genotype 'Tomba' (Suppl. Table S3). The revision of the DAPs, however, showed that 93 of them displayed similar trends in the genotype 'Kiebitz' but did not meet the criteria of significance in one of the experiments (student's T test *P* and FC stress/control). This is because drought stress started earlier in 'Tomba' than in 'Kiebitz' and there were high temperatures in experiment 1 when the plants were stressed (Meise et al. 2018). Consequently, the substrate dried faster in that experiment. Additionally, the substrate dried out more quickly in 'Tomba' pots due to their greater biomass. For that reason, those proteins should be rather considered a common response to NWD stress. We decided to exclude those proteins from further analysis, to focus on the DAPs differentially abundant only in the tolerant genotype. Among these remaining 55 DAPs specific to the genotype 'Tomba',

14 were assigned to the category carbohydrate metabolism (Fig. 5). Other overrepresented categories included protein folding, sorting and degradation (nine DAPs), energy metabolism (seven DAPs), and lipid metabolism (four DAPs).

Only four of the 55 DAPs showed a higher abundance in the NWD treatment compared to the control (photosystem II 11 kDa protein, oligopeptidase, cell division inhibitor, and ATP synthase, Fig. 6). Three of them (except cell division inhibitor) were assigned to energy metabolism. One of the first responses to abiotic stress in plants is down-regulation of energy metabolism (Romero et al. 2017; Dahal et al. 2019). The fact that the tolerant genotype 'Tomba' contained proteins of energy metabolism in higher abundance may indicate that—after dealing with the stress—it was already able to upregulate its energy metabolism to return to a normal physiological state. However, this hypothesis cannot be verified with the current setup, as further earlier samples of N deficiency would have to be analyzed.

Interestingly, the lactoylglutathione lyase (synonyme: glyoxalase) was lower abundant under NWD stress in the genotype 'Tomba', but higher abundant in genotype 'Kiebitz' in experiment 1. Lactoylglutathione lyase regulates methylglyoxal, which is a cytotoxic compound inhibiting cell proliferation and leading to degradation of proteins, thus affecting the antioxidant defense system negatively (Upadhyaya et al. 2011). Because genotype 'Tomba' was categorized as tolerant to NWD based on the tuber yield and starch content, a lower abundance of lactoylglutathione lyase might help to maintain normal metabolism. Likewise, nucleoside diphosphate kinase was lower abundant under NWD in genotype 'Tomba', but higher abundant in genotype 'Kiebitz' in experiment 1. This protein is a housekeeping enzyme, which can be associated with ROS scavenging (Moon et al. 2003). Jozefowicz et al. (2017) presented an altered protein composition in potato roots under ND. Lactoylglutathione lyase was higher abundant in the tolerant genotype in the study of Jozefowicz et al. (2017), whereas in our study, the protein showed higher abundance in the sensitive genotype. These DAPs deserve further analysis involving earlier time points and gene expression analyses to determine their role in stress response.

Fourteen proteins (25.5%) were assigned to the functional group of carbohydrate metabolism. The metabolic pathways of the pentose phosphate pathway, glycolysis, and the TCA cycle were affected, but proteins of the starch metabolism were also less abundant after NWD. The aconitate hydratases from the TCA cycle and the glyoxylate cycle (Moeder et al. 2007) are cycle-maintaining proteins. The mitochondrial aconitate hydratase also provides 2-oxoglutarate for amino acid synthesis and ammonia assimilation (Araújo et al. 2014; Eprintsev et al. 2021). Due to the lower N availability in the NWD treatment, the lower abundance of this protein could indicate a stress response toward N deficiency. Also, in the tolerant genotypes 'Eurostarch' and 'Tomba', there was a lower abundance in both individually applied stresses.

Three proteins were assigned to nucleotide metabolism (nucleoside diphosphate kinase, OMPdecase, and beta-ureidopropionase). Proteins of the nucleotide metabolism are needed in several energetic reactions such as the TCA cycle (nucleoside diphosphate kinase), as well as in the de novo biosynthesis of pyrimidines (Witte and Herde 2020), which can be connected to the pentose phosphate pathway. Since pyrimidines also contain N, a lower abundance of related proteins could indicate this to be part of the N deficiency response.

Proteins and enzymes involved in proteolysis are responsible, amongst others, for the degradation of proteins (van Wijk 2015). Three proteins from the category folding, sorting, and degradation (ubiquitin-conjugating enzyme E2, proteasome subunit alpha, proteinase inhibitor) were identified to be less abundant under NWD stress compared

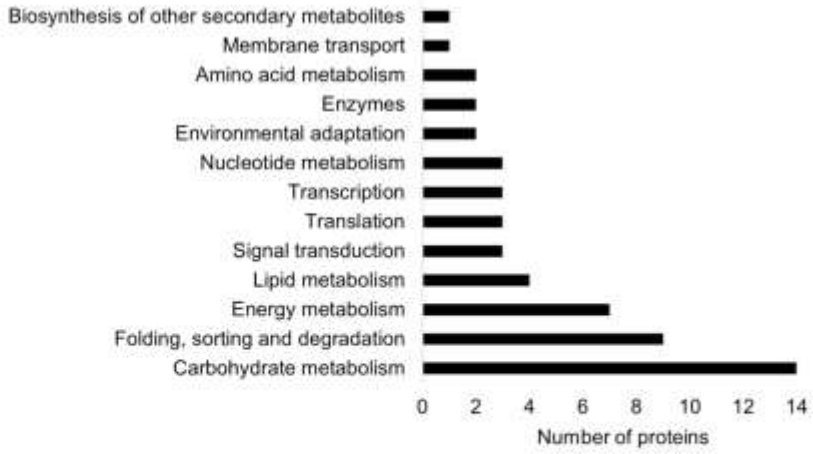
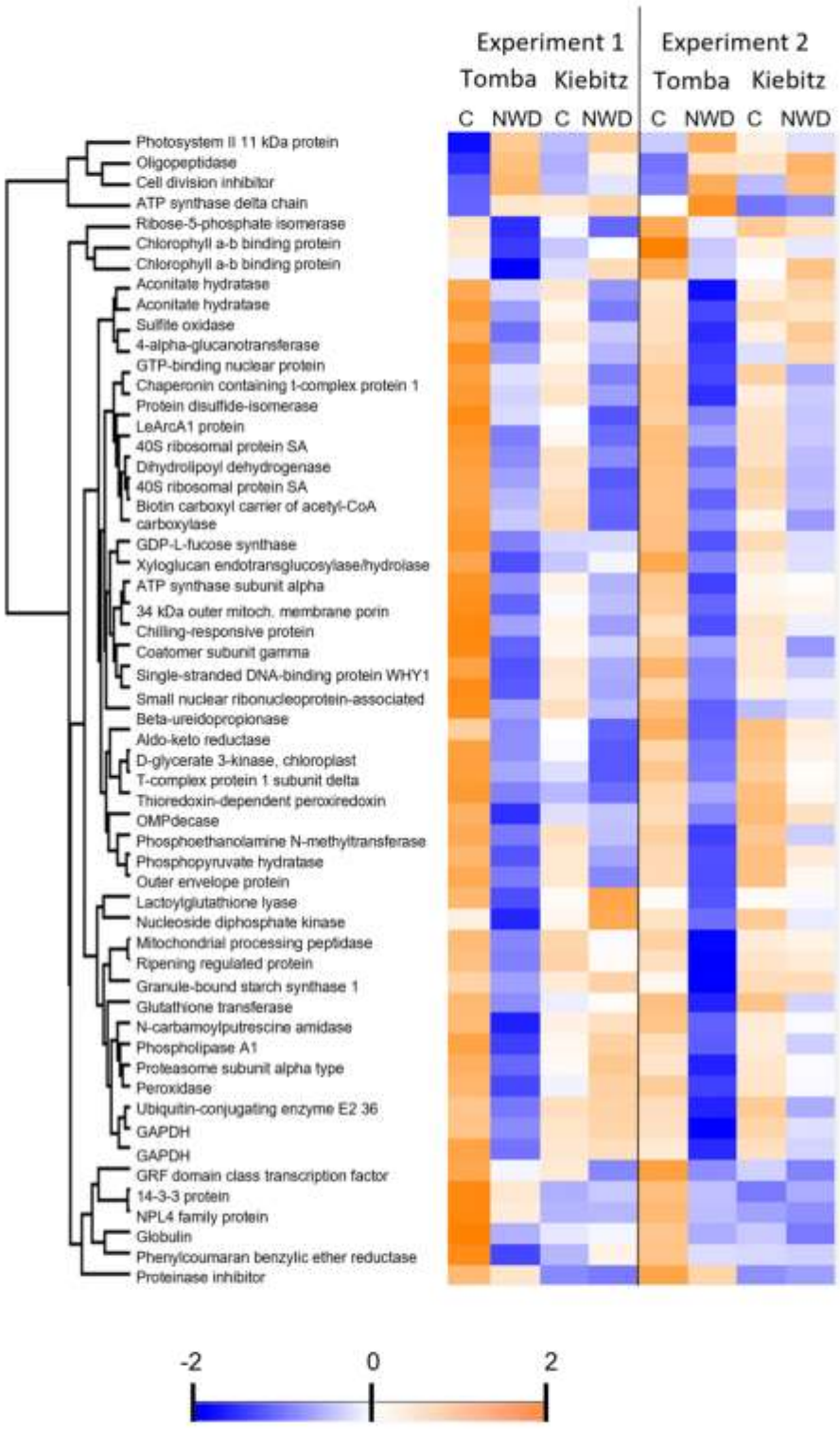


Fig. 5 Functional categories of DAPs specific for the tolerant genotype 'Tomba'. Most proteins responsive to NWD in the genotype 'Tomba' belonged to categories carbohydrate and energy metabolism. The classification was performed according to the KEGG orthology


Experiment 1
Experiment 2
Tomba
Kiebitz
Tomba
Kiebitz
C NWD C NWD C NWD C NWD
Photosystem II 11 kDa protein
Oligopeptidase
Cell division inhibitor
ATP synthase delta chain
Ribose-5-phosphate isomerase
Chlorophyll a-b binding protein
Chlorophyll a-b binding protein
Aconitate hydratase
Aconitate hydratase
Sulfite oxidase
4-alpha-glucanotransferase
GTP-binding nuclear protein
Chaperonin containing t-complex protein 1
Protein disulfide-isomerase
LeArcA1 protein
40S ribosomal protein SA
Dihydrolipoyl dehydrogenase
40S ribosomal protein SA
Biotin carboxyl carrier of acetyl-CoA carboxylase
GDP-L-fucose synthase
Xyloglucan endotransglucosylase/hydrolase
ATP synthase subunit alpha
34 kDa outer mitoch. membrane porin
Chilling-responsive protein
Coatomer subunit gamma
Single-stranded DNA-binding protein WHY1
Small nuclear ribonucleoprotein-associated
Beta-ureidopropionase
Aldo-keto reductase
D-glycerate 3-kinase, chloroplast
T-complex protein 1 subunit delta
Thioredoxin-dependent peroxiredoxin
OMPdecase
Phosphoethanolamine N-methyltransferase
Phosphopyruvate hydratase
Outer envelope protein
Lactoylglutathione lyase
Nucleoside diphosphate kinase
Mitochondrial processing peptidase
Ripening regulated protein
Granule-bound starch synthase 1
Glutathione transferase
N-carbamoylputrescine amidase
Phospholipase A1
Proteasome subunit alpha type
Peroxidase
Ubiquitin-conjugating enzyme E2 36
GAPDH
GAPDH
GRF domain class transcription factor
14-3-3 protein
NPL4 family protein
Globulin
Phenylcoumaran benzylic ether reductase
Proteinase inhibitor
-2
0
2

◂**Fig. 6** Fifty-five DAPs were specific to the tolerant genotype 'Tomba'. Hierarchical clustering was carried out with k-means preprocessing and was based on average Euclidean distance linkage. Relative abundance in the heatmap has been color-coded following Z-score normalization. Each column represents one treatment. NWD, N and water deficiency

to the control. This could indicate that 'Tomba', as a tolerant genotype, was able to adapt to N deficiency which had been applied since the beginning of the experiment. This genotype could better cope with the additional drought stress and thus protect its resources. Meise et al. (2019) showed similar levels of protein content under NWD stress and in the control treatment in genotype 'Tomba'. Genotype 'Kiebitz' on the other hand showed lower protein contents in NWD than in the control (Meise et al. 2018). This might indicate, that genotype 'Tomba' decreased the proteolysis to maintain or return to a normal level of metabolism after the initial stress response.

Proteins specific to sensitive genotype 'Kiebitz'

In our proteomic analysis, 113 proteins significantly changed due to NWD stress in the sensitive genotype 'Kiebitz' (Suppl. Table S4). However, the number of DAPs decreased drastically when proteins, which showed significant differences in abundance in 'Tomba' in experiment 2 as well, were excluded. We could observe that the abundance of many proteins changed in the same way in 'Kiebitz' and 'Tomba' in experiment 2 but not in experiment 1. The differences were probably driven by additional high temperatures in the five-day drought stress treatment during experiment 1 as explained earlier (Meise et al. 2018). After the exclusion of those proteins, there were 19 DAPs highly specific to the more sensitive genotype (Table 1). Eleven of them increased in abundance in response to NWD stress, whereas eight showed a decrease.

Among these DAPs, four proteins were assigned to the category of proteases/protease inhibitors. The three proteases (subtilase, carboxypeptidase, subtilase family protein) were higher abundant in the NWD treatment than in the control treatment. The protease inhibitor found in this study (stigma expressed protein) was less abundant in the NWD treatment. This protein showed a similar pattern of abundance in genotype 'Kolibri', which was also considered sensitive to NWD stress (Suppl. Table S4). Proteases are involved in diverse cellular processes such as photoinhibition in the chloroplast, defense mechanisms, PCD, and thus protein denaturation, which is triggered by different abiotic stresses, such as drought stress (Estelle 2001). Protease inhibitors can prevent the dismantling of proteins by proteases and their decreased abundance under abiotic stress can thus result in free N that can be used for recycling (Folgado et al. 2013). Thus, our results indicate that sensitive potato genotypes responded to NWD with increased protein degradation. During senescence and ND, proteases like subtilisin and the proteasome were reported to degrade soluble proteins and recycle RuBisCO in oilseed rape indicating a response specific to ND (Poret et al. 2019).

Four DAPs were assigned to the carbohydrate metabolism, of which three proteins [aldehyde dehydrogenase (NAD(+)), pectin esterase, aldose 1-epimerase] were higher abundant in the stress treatment. Aldehyde dehydrogenase (NAD(+)) is an initial stress response protein that occurs during water deficiency, N deficiency, and salt stress (Kirch et al. 2005; Meise et al. 2017) and supports the vegetative growth of the plants (Tola et al. 2020). It was also found in NWD in all other genotypes but only in 1 year (experiment 2, year 2015), which might suggest a common response mechanism to NWD stress among the genotypes. The peroxidase 3-like protein, which was higher abundant under NWD in this study, is also classified as a protein of the initial stress response. It is striking that this protein was altered only in the sensitive genotype. Pectin esterases are involved in cell wall formation, specifically in plasticity of the cell wall. A higher abundance of pectin esterase in stressed plants can be linked to higher plasticity of the cells and, therefore, better maintenance of the cell turgor. The formation and architecture of the cell wall are of great importance for signal transduction and stress sensing, so cell wall-related proteins can be linked to stress response (Le Gall et al. 2015).

Response of potato genotypes to individual stresses: N deficiency (ND) and water deficiency (WD)

To investigate potential differences in the response to combined NWD stress compared to the single stress factors, we also analyzed the proteomic response of all four potato genotypes to individually applied ND and WD. The LC–MS runs were separately done for NWD/control and ND/WD/control. The proteome analysis of ND and WD samples allowed the identification of 699 proteins based on 2,354 non-conflicting peptides. Protein profiles were investigated using PCA plots (Suppl. Fig. S2) independently for ND and WD treatments. The four potato genotypes and the treatments clustered distinctly from the control in both, ND and WD treatments in experiment 1, a clear grouping was, however, not observed in experiment 2. In response to ND 38, 14, 5, and 29 DAPs were found in genotypes 'Tomba', 'Kiebitz', 'Kolibri', and 'Eurostarch', respectively. WD caused significant changes in abundance of 38, 7, 19, and 23 proteins in genotypes 'Tomba', 'Kiebitz', 'Kolibri', and 'Eurostarch', respectively. The relatively low number of DAPs was caused by the weak proteomic response of plants in experiment 2, resulting in a reduced overlap between

Table 1 List of differentially abundant proteins in the sensitive potato genotype 'Kiebitz' induced by N deficiency combined with drought stress (NWD)

Accession	Protein description (according to Uniprot)	KEGG classification 2nd dimension	Fold change stress/control			
			Experiment 1		Experiment 2	
			Tomba	Kiebitz	Tomba	Kiebitz
400029393	Plasma membrane polypeptide	Signal transduction	1.33	1.54	1.44	1.74
400058896	Aldehyde dehydrogenase (NAD(+))	Carbohydrate metabolism	0.58	2.14	1.45	2.17
400078034	NAD(P)H dehydrogenase (quinone)	Metabolism of cofactors and vitamins	0.94	2.07	1.47	2.03
400065518	Peroxidase	Biosynthesis of other secondary metabolites	0.64	2.48	1.42	1.64
400046584	Aldose 1-epimerase	Carbohydrate metabolism	0.66	1.68	1.21	1.54
400088012	Subtilase family protein	Folding, sorting and degradation/signaling molecules and interaction	0.86	1.83	1.40	1.78
400031890	Purple acid phosphatase	Protein phosphatases and associated proteins	0.88	1.92	1.21	1.57
400009216	Alpha-mannosidase	Glycan biosynthesis and metabolism	0.73	1.99	0.91	1.70
400017451	Subtilase	Folding, sorting and degradation/signaling molecules and interaction	0.92	1.66	0.63	2.05
400066639	Carboxypeptidase	Peptidases and inhibitors	1.00	1.59	0.93	1.54
400081312	Pectinesterase	Carbohydrate metabolism	0.68	1.86	1.48	1.50
400039443	Plastid RNA-binding protein	Environmental adaptation	1.16	0.51	0.75	0.59
400016844	Pyruvate kinase	Carbohydrate metabolism	0.80	0.62	0.82	0.50
400026666	Assimilatory sulfite reductase	Energy metabolism	1.22	0.51	0.70	0.27
400051668	Poly(RC)-binding protein	Messenger RNA biogenesis	0.68	0.46	0.71	0.64
400057203	RNA Binding Protein 45	Transcription machinery/Messenger RNA biogenesis	0.79	0.47	0.69	0.62
400078506	Fruit protein PKIWI502	Signaling molecules and interaction	0.78	0.62	0.68	0.61
400026271	Stigma expressed protein	Peptidases and inhibitors	0.76	0.38	0.65	0.38
400055527	Single-stranded DNA binding protein	Ribosome biogenesis	0.68	0.63	0.68	0.54

The abundance is presented in the form of fold change. Accession numbers are given without the PGSC003DMT prefix. Full details of the protein identification are stored together with raw data

experiments 1 and 2. Proteins responding to ND and WD are presented in Suppl. Tables S5 and S6.

The purpose of analyzing the response to ND and WD was to find similarities and differences in the response of potato genotypes to NWD stress and individually applied stresses. This distinction between single and double stress is extremely important. Demirel et al. (2020) found differences in the regulation of biochemical pathways depending on the stress combination. Venn diagrams display the overlap of DAPs in the four potato genotypes in response to ND, WD, and NWD stresses (Fig. 7). For all genotypes, the highest number of DAPs was found for the NWD treatment, whereas much lower numbers were recorded under the single stresses and even fewer DAPs were detected in the overlaps. In Tables 2, 3, proteins overlapping and specific to ND, WD, and NWD are presented for the most contrasting genotypes 'Tomba' and 'Kiebitz', respectively. Proteins specific to NWD are not included in Tables 2, 3, this information is to be found in Suppl. Tables S2 and S3. The data for genotypes 'Eurostarch' and 'Kolibri' is presented in Suppl. Tables S7 and S8.

While in genotype 'Kiebitz', no overlapping DAPs were identified between ND, WD, and NWD, in genotype 'Tomba', seven DAPs were shared between all three applied stresses (3-hydroxy-3-methylglutaryl CoA synthase, glycosyltransferase, 2-oxoglutarate-dependent dioxygenase, SAMS, phospho-2-dehydro-3-deoxyheptonate aldolase 2, katanin p60 ATPase-containing subunit, and UDP-glucose 6-dehydrogenase; Table 2). Those shared DAPs might indicate genotype-specific proteins for a general abiotic stress response. Especially 2-oxoglutarate-dependent dioxygenase and 3-hydroxy-3-methylglutaryl coenzyme A synthase are known to be part of an abiotic stress response (Meng et al. 2017; Tiwari et al. 2020a). Tiwari et al. (2020a) showed up- and down-regulation of 2-oxoglutarate-dependent dioxygenase to ND in roots and stolons of potato.

Four DAPs were overlapping for ND and NWD stress in genotype 'Tomba' (vacuolar processing enzyme (VPE) 1, oxidoreductase, cysteine proteinase 3, and dihydrolipoyl dehydrogenase) and nine for WD and NWD stress (e.g. granule-bound starch synthase 1, small heat shock protein, phospholipase A1, ribulose-phosphate 3-epimerase, and

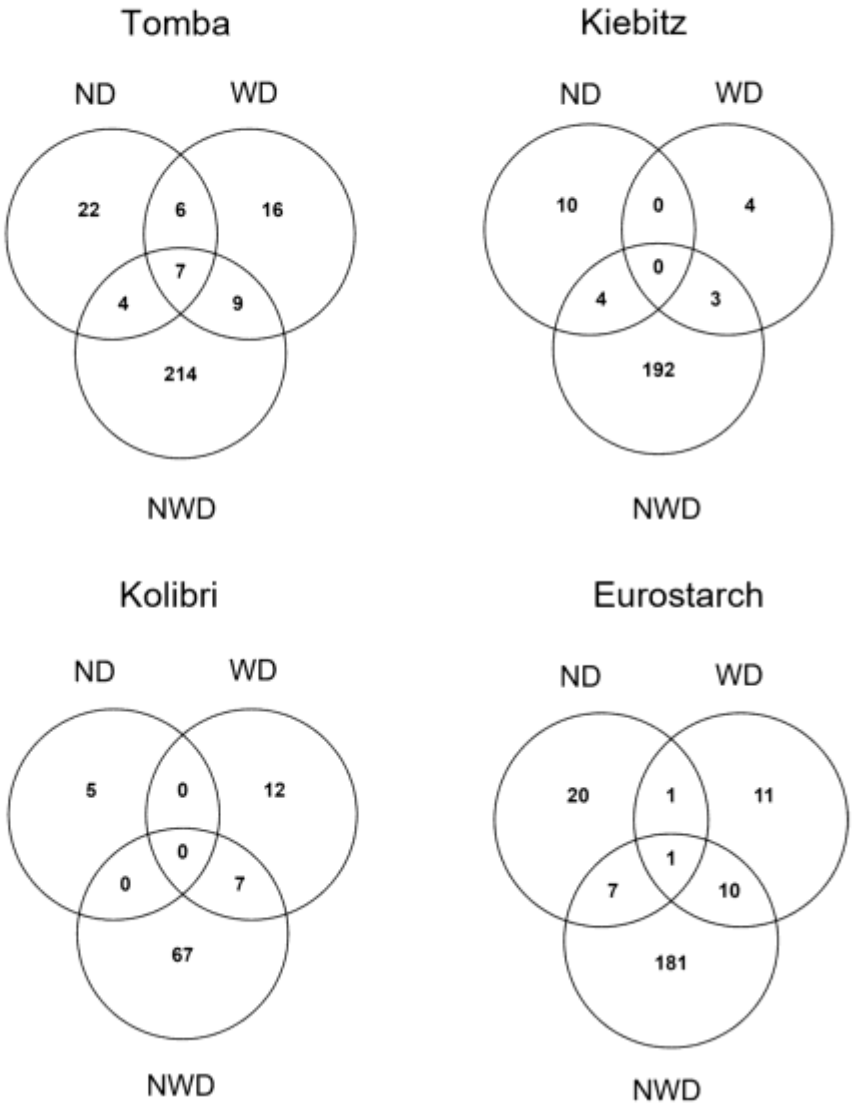


Fig. 7 Number of differentially abundant proteins in the potato genotypes under ND, WD as well as combined NWD. The Venn diagrams show the number of proteins specific to examined conditions or shared between them. *ND* N deficiency; *WD* drought stress; *NWD* N and water deficiency

SAMS). Six proteins (light-induced protein, rhamnose biosynthetic enzyme 1-like, D-3-phosphoglycerate dehydrogenase, early tobacco anther 1, heat shock protein, and phenylalanine ammonia-lyase) were shared for ND and WD but were not responsive to NWD stress in genotype 'Tomba'. In 'Kiebitz', four DAPs were common for ND and NWD (vacuolar processing enzyme 1, plasma membrane polypeptide, glycerophosphodiester phosphodiesterase, cysteine proteinase 3) and three for WD and NWD (aldehyde dehydrogenase (NAD(+)), SAMS and tubulin beta chain). The fact that VPE and cysteine proteinase 3 were also higher abundant in genotype 'Kiebitz' is supporting the idea of a general and not a tolerance-dependent stress response.

The DAPs for ND that appeared in the more tolerant variety 'Tomba' and the more sensitive variety 'Kiebitz' differed mainly in number. 'Tomba' (38 DAPs) had more DAPs overall than 'Kiebitz' (14 DAPs). The pathways affected were equivalent (e.g. carbohydrate metabolism, energy metabolism, environmental adaptation). Both genotypes shared a higher abundance of five DAPs (vacuolar processing enzyme 1, light-induced protein, hairpin binding protein 1, cysteine proteinase 3, and receptor protein kinase) under ND stress. What is striking, however, is that all DAPs of 'Kiebitz' were higher abundant in ND, while the ND treatment of 'Tomba' also showed some less abundant proteins. In combination with the morphological and physiological performance of 'Tomba', which included

Table 2 List of differentially abundant proteins in the tolerant potato genotype 'Tomba' induced by N deficiency (ND), drought stress (WD) or combined N deficiency with drought stress (NWD)

Conditions	Accession	Protein description (according to Uniprot)	KEGG classification 2nd dimension	Ratio stress/control					
				Experiment 1			Experiment 2		
				ND	WD	NWD	ND	WD	NWD
ND/WD/NWD	400058564	3-hydroxy-3-methylglutaryl CoA synthase	Carbohydrate metabolism	0.51	0.53	0.29	0.59	0.53	0.34
ND/WD/NWD	400030650	Glycosyltransferase	Enzymes*	0.40	0.54	0.41	0.46	0.50	0.43
ND/WD/NWD	400030676	2-oxoglutarate-dependent dioxygenase	Enzymes*	0.49	0.40	0.35	0.44	0.41	0.24
ND/WD/NWD	400072701	S-adenosylmethionine synthetase	Amino acid metabolism	0.54	0.35	0.35	0.47	0.36	0.33
ND/WD/NWD	400041818	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	Amino acid metabolism	0.53	0.46	0.65	0.40	0.48	0.46
ND/WD/NWD	400054532	Katanin p60 ATPase-containing subunit	Cell growth and death	0.49	0.44	0.46	0.34	0.47	0.08
ND/WD/NWD	400003666	UDP-glucose 6-dehydrogenase	Carbohydrate metabolism	0.50	0.58	0.54	0.55	0.44	0.59
ND/WD	400043112	Light-induced protein	Environmental adaptation*	2.10	1.86	1.26	1.55	1.51	1.86
ND/WD	400018192	Rhamnose biosynthetic enzyme 1-like	Carbohydrate metabolism	0.44	0.38	NA	0.39	0.43	NA
ND/WD	400023675	D-3-phosphoglycerate dehydrogenase	Amino acid metabolism	0.63	0.59	0.45	0.66	0.63	0.67
ND/WD	400053209	Early tobacco anther 1	Uncharacterized*	0.48	0.63	0.51	0.53	0.65	0.84
ND/WD	400003652	Heat shock protein	Chaperones and folding catalysts	0.66	2.71	1.09	0.66	2.05	0.55
ND/WD	400055488	Phenylalanine ammonia-lyase	Amino acid metabolism	0.49	0.24	NA	0.47	0.41	NA
ND/NWD	400035925	Vacuolar processing enzyme 1	Peptidases and inhibitors	2.50*	1.21	1.75*	1.94	1.01	5.11
ND/NWD	400008936	Oxidoreductase, 2OG-Fe(II) oxygenase family	Enzymes*	0.49	0.80	0.28	0.62	0.75	0.50
ND/NWD	400048984	Cysteine proteinase 3	Peptidases and inhibitors	1.68	0.75	0.66	2.59	0.79	1.66
ND/NWD	400007182	Dihydrolipoyl dehydrogenase	Carbohydrate metabolism	0.59	0.67	0.55	0.62	0.67**	0.65
WD/NWD	400031568	Granule-bound starch synthase 1	Carbohydrate metabolism	1.23	0.47	0.58	1.55	0.32	0.40
WD/NWD	400008351	Small heat shock protein	Folding, sorting and degradation	0.76	8.14	2.94	0.46	21.66	7.47
WD/NWD	400081247	Phospholipase A1	Lipid Metabolism	0.90	0.49	0.47	1.11	0.53	0.63
WD/NWD	400057147	Plastid-dividing ring protein	Chromosome and associated proteins	1.21	0.64	0.58	0.88	0.62	0.61
WD/NWD	400007216	Uncharacterized protein	Uncharacterized*	0.66	0.52	0.49	0.73	0.50	0.49
WD/NWD	400050256	Ribulose-phosphate 3-epimerase,	Carbohydrate metabolism	1.41	1.60	0.57	2.09	2.24	0.60
WD/NWD	400047146	S-adenosylmethionine synthetase 1	Amino acid metabolism	0.84	0.45	0.56	0.66	0.41	0.25
WD/NWD	400087679	S-adenosylmethionine synthetase 2	Amino acid metabolism	0.65	0.18	0.23	0.60**	0.24	0.25
WD/NWD	400078206	Tubulin beta chain	Cytoskeleton proteins	0.95	0.54	0.47	0.81	0.65	0.37
ND	400039851	Subtilisin-like protease preproenzyme	Folding, sorting and degradation/ Signaling molecules and interaction*	1.61	1.19	0.98	2.79	1.30	1.89
ND	400044209	Harpin binding protein 1	Environmental adaptation*	1.81	0.99	0.85	2.14	1.36	1.31
ND	400050664	Elongation factor 1-alpha	Translation	0.40	0.49	NA	0.59	1.35	NA
ND	400075611	Catalase isozyme 2	Carbohydrate metabolism	0.26	0.67	0.69	0.64	0.60	0.30

Table 2 (continued)

Conditions	Accession	Protein description (according to Uniprot)	KEGG classification 2nd dimension	Ratio stress/control					
				Experiment 1			Experiment 2		
				ND	WD	NWD	ND	WD	NWD
ND	400022085	Peptidyl-prolyl cis–trans isomerase	Chaperones and folding catalysts*	1.79	0.96	0.88	1.90	0.92	0.64
ND	400064434	Thioredoxin	Enzymes*	1.77	1.39	0.83	1.92	1.66	1.21
ND	400041576	Cinnamyl alcohol dehydrogenase	Biosynthesis of other secondary metabolites*	1.67	1.31	1.02	2.75	1.79	1.08
ND	400069750	Chloroplast sedoheptulose-1,7-bisphosphatase	Energy metabolism	1.70	0.81	0.94	1.79	1.27	1.19
ND	400050234	Geranylgeranyl reductase	Metabolism of cofactors and vitamins	0.43	0.72	0.76	0.38	0.60	0.45
ND	400057522	Suberization-associated anionic peroxidase	Enzymes*	1.68	0.95	0.90	2.11	1.43	2.16
ND	400044818	Glucose-6-phosphate 1-dehydrogenase	Carbohydrate metabolism	0.36	1.09	NA	0.36	0.91	NA
ND	400024090	Phosphoribulokinase	Energy metabolism	1.72	1.21	0.98	1.55	1.30	0.84
ND	400065504	Receptor protein kinase	Signal transduction*	2.03	0.71	NA	1.77	1.18	NA
ND	400000946	Arginine–tRNA ligase	Translation*	0.30	0.85	NA	0.51	1.25	NA
ND	400031351	Fructose-bisphosphate aldolase	Carbohydrate metabolism	1.51	0.82	0.85	1.77	1.03	0.81
ND	400057332	Fructose-bisphosphate aldolase	Carbohydrate metabolism	1.81	0.97	1.23	1.53	1.21	1.02
ND	400083971	Calmodulin-1	Signal transduction	1.81	1.18	0.74	1.83	1.33	1.37
ND	400081752	Uncharacterized protein	Uncharacterized*	1.53	0.99	1.04	2.27	1.37	1.89**
ND	400001149	Glycosyltransferase	Metabolism of terpenoids and polyketides	0.66	0.81	0.93	0.42	0.61	0.53
ND	400011133	Glutamine synthetase	Energy metabolism	1.51	0.92	NA	1.55	1.29	NA
ND	400039222	2-deoxyglucose-6-phosphate phosphatase	Carbohydrate metabolism	1.57	1.50	NA	1.68	1.31	NA
ND	400036729	U2 small nuclear ribonucleoprotein A	Transcription	0.64	1.23	NA	0.54	1.73	NA
WD	400004360	Ascorbate peroxidase	Carbohydrate metabolism	0.67	4.51	1.18	0.77	2.49	1.49
WD	400052308	CBS domain-containing protein	Uncharacterized*	0.65**	1.67	1.18	1.36	1.90	0.77
WD	400079161	Phospho-2-dehydro-3-deoxyheptonate aldolase 1	Amino acid metabolism	0.30	0.25	NA	0.20	0.26	NA
WD	400026271	Stigma expressed protein	Peptidases and inhibitors *	1.01	2.52	0.76	1.09	2.03	0.65**
WD	400071115	(S)-2-hydroxy-acid oxidase	Carbohydrate metabolism	0.90	1.70	1.18	1.35	1.51	0.92
WD	400003356	Granule-bound starch synthase 2	Carbohydrate metabolism	0.85	0.58	0.92	0.70	0.40	0.52
WD	400070986	Heat shock protein 70	Folding, sorting and degradation	1.72	4.29	NA	0.86	3.63	NA
WD	400011762	Invertase inhibitor	Carbohydrate metabolism*	0.74	3.02	NA	0.41	1.60	NA
WD	400021142	Class II small heat shock protein	Folding, sorting and degradation	0.87	11.26	2.89**	0.80	8.04	6.05
WD	400022265	Galactose mutarotase	Carbohydrate metabolism*	0.96	1.81	0.84	1.30	1.90	0.54
WD	400095387	Uncharacterized protein	Uncharacterized*	2.04	1.89	0.92	1.44	1.68	0.67
WD	400073479	Uncharacterized protein	Uncharacterized*	1.26	4.60	0.79	0.84	7.72	0.62
WD	400048880	DUF1995 domain-containing protein	Uncharacterized *	1.47	0.41	NA	0.97	0.48	NA
WD	400074842	Small rubber particle protein	Environmental adaptation*	0.81	1.77	0.99	1.45	2.18	0.93

Table 2 (continued)

Conditions	Accession	Protein description (according to Uniprot)	KEGG classification 2nd dimension	Ratio stress/control					
				Experiment 1			Experiment 2		
				ND	WD	NWD	ND	WD	NWD
WD	400055410	SBT1 protein	Folding, sorting and degradation/ Signaling molecules and interaction*	1.74	0.39	NA	1.20	0.36	NA
WD	400064274	Subtilisin-like protease	Folding, sorting and degradation/ Signaling molecules and interaction*	1.35	0.60	0.71	1.20	0.60	1.34

The abundance is presented in the form of fold change. Accession numbers are given without the PGSC003DMT prefix. Full details of the protein identification are stored together with raw data

*Classification performed manually, ** Fold change within significance limits, but *P* value higher than 0.05

Table 3 List of differentially abundant proteins in the sensitive potato genotype 'Kiebitz' induced by nitrogen deficiency (ND), drought stress (WD) or combined N deficiency with drought stress (NWD)

Conditions	Accession	Protein description (according to uniprot)	KEGG classification 2nd dimension	Ratio stress/control					
				Experiment 1			Experiment 2		
				ND	WD	NWD	ND	WD	NWD
ND/NWD	400035925	Vacuolar processing enzyme 1	Folding, sorting and degradation	5.41	1.34	7.84	2.76	1.90	4.25
ND/NWD	400029393	Plasma membrane polypeptide	Signal transduction*	4.67	1.03	1.54	3.31	2.13	1.74
ND/NWD	400057418	Glycerophosphodiester phosphodiesterase	Lipid metabolism*	1.74	0.90	1.88	1.74	1.15	1.71
ND/NWD	400048984	Cysteine proteinase 3	Folding, sorting and degradation	2.39	0.62	2.04	2.28	1.95**	1.51
WD/NWD	400058896	Aldehyde dehydrogenase (NAD(+))	Carbohydrate metabolism	1.42	1.60	2.14	1.49**	2.32	2.17
WD/NWD	400072701	S-adenosylmethionine synthase	Amino acid metabolism	0.75	0.56	0.43	0.51	0.36	0.32
WD/NWD	400078206	Tubulin beta chain	Cell growth and death	0.68	0.60	0.42	0.73	0.65	0.51
ND	400075915	Desacetoxyvindoline 4-hydroxylase	Biosynthesis of other secondary metabolites*	1.85	0.83	1.26	2.09	1.26	1.64
ND	400043112	Light-induced protein	Environmental adaptation*	2.50	2.83**	1.32	2.36	1.57**	2.22
ND	400044209	Harpin binding protein 1	Environmental adaptation*	1.90	1.06	1.46	1.76	1.24	1.41
ND	400070131	Carboxypeptidase	Folding, sorting and degradation	1.84	0.93	NA	1.78	0.90	NA
ND	400031568	Granule-bound starch synthase 1	Carbohydrate metabolism	2.45	0.71	0.65	2.02	0.35	0.76
ND	400064274	Subtilisin-like protease	Folding, sorting and degradation/ Signaling molecules and interaction*	1.66	0.75	1.55	1.60	0.92	0.97
ND	400025043	Pom14 protein	Membrane transport*	2.22	1.26	1.31	1.63	1.29	1.03
ND	400038370	3-beta hydroxysteroid dehydrogenase/somerase	Lipid metabolism*	2.35	1.29	NA	2.09	1.54	NA
ND	400065504	Receptor protein kinase	Signal transduction*	1.78	1.06	NA	1.75	1.37	NA
ND	400015365	ATP synthase subunit beta	Energy metabolism	1.73	1.10	0.86	1.52	0.88	1.03
WD	400083137	P5CDH1	Amino acid metabolism	1.10	1.58	0.44	1.01	1.90	0.77
WD	400003356	Granule-bound starch synthase 2	Carbohydrate metabolism	1.07	0.52	1.22	1.25	0.37	1.02
WD	400071822	RNA-binding protein	Uncharacterized*	0.71	0.51	NA	0.88	0.57	NA
WD	400006854	Cell division protein FtsZ	Chromosome and associated proteins	1.26	0.53	0.76	0.88	0.61	0.73

The abundance is presented in the form of fold change. Accession numbers are given without the PGSC003DMT prefix. Full details of the protein identification are stored together with raw data

*Classification performed manually, ** Fold change within significance limits, but *P* value higher than 0.05

higher tuber and starch yield, this might display a faster adaptation of the genotype to the stress conditions (Dahal et al. 2019).

The genotype 'Tomba' showed several DAPs with a higher abundance in the WD treatment, especially in the functional class of chaperones (heat shock protein 70, class II small heat shock protein LE-HSP17.6), in environmental adaptation proteins (small rubber particle protein), and carbohydrate metabolism (invertase inhibitor, (S)-2-hydroxy-acid oxidase, ascorbate peroxidase). Interestingly, higher abundant HSPs under WD showed no longer a higher abundance under NWD. This suggests an influence of NWD on HSP biosynthesis. Whether the plant does not find sufficient resources to continue expressing heat shock proteins or whether the plant no longer needs those proteins in large quantities, remains unclear. Ascorbate peroxidase is part of a ROS-scavenging pathway in plants (Aghaei et al. 2009; Dahal et al. 2019).

Conclusions

Potato genotypes grown under ND, WD and NWD displayed many common proteomic responses but also showed reactions specific for tolerant or sensitive genotypes, respectively (Fig. 8). (i) Increase of DAPs related to protein folding and decrease of amino acid metabolism participating DAPs was a general stress response to the combination of N deficiency and drought. (ii) Adaptions of the tolerant genotype 'Tomba' towards restructuring of the plant processes most likely led to a better NWD tolerance by higher abundance of DAPs participating in energy metabolism and a protease inhibitor, decrease of DAPs related to carbohydrate metabolism and proteases, and higher abundance of DAPs for amino acid and carbohydrate metabolism after ND. (iii) Proteins related to proteolysis were higher abundant

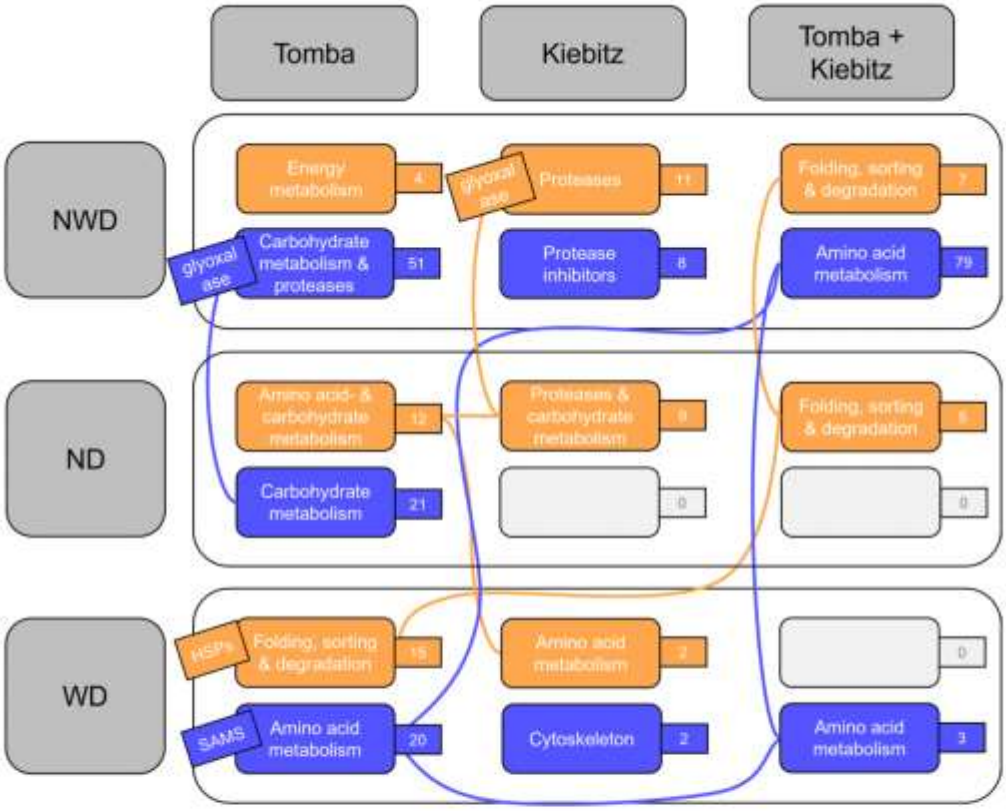


Fig. 8 Overview of major changes in protein abundance after nitrogen deficiency (*ND*), water deficiency (*WD*) and combined stress (*NWD*) in the genotypes 'Tomba' and 'Kiebitz'. The most important biochemical metabolic pathways are presented in the large tiles and the number of identified proteins in the small tiles. Orange: higher abundant proteins after stress. blue: lower abundant proteins after stress. Important individual proteins are indicated in the oblique tiles. Connecting lines indicate the same metabolic pathways in different variants. However, these do not necessarily contain the same proteins. Glyoxalase in 'Kiebitz' NWD was only significantly changed in abundance in one experiment

in 'Kiebitz' suggesting that protein degradation was one of the key processes needed for plant survival under more severe stress. Upcoming studies need to be complemented by metabolic analyses related to the identified pathways (carbohydrate/energy and amino acid metabolism). The high abundance of a protease inhibitor in tolerant genotype 'Tomba' may be related to the overall better growth and less severe stress response of this genotype under NWD treatment. A possible explanation is that this genotype had already reduced proteolytic events at sampling. This hypothesis can be tested in follow-up studies applying a time-resolved sampling scheme. Collectively, our results suggest addressing the role of proteolytic events as a major focus in future functional studies.

The relatively low overlap of identified proteins when comparing the reaction to combined stress to responses to the single stresses rather drastically displays the need for test systems, which analyze double stressors on a broader scale for potatoes. This will be of grave importance in the future, when climate change, but also legal guidelines for fertilizer application, will lead to more challenging combinations of abiotic stresses.

Author contribution statement Material preparation, data collection, and analysis were performed by AMJ, KW, and CB. HPM, AS, SS, CB, and TW conceived and coordinated the project. The first draft of the manuscript was written by AMJ and KW. The manuscript was revised by CB, HPM, PM, AS, SS, and TW. All authors have read and approved the final document.

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Data availability The datasets generated during and analyzed during the current study are available in the IPK Gatersleben system e!DAL (Arend et al. 2014), available at: https://doi.org/10.5447/IPK/2023/4.

Declarations

Conflict of interest This study was financed by the Federal Ministry of Food and Agriculture (BMEL) through the Agency of Renewable Resources (FNR) (FKZ: 22001917 and 22007018). The authors have no competing interests to declare that are relevant to the content of this article. The authors declare that no human and/or animal material, data, or cell lines were used in this study.

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3. General discussion

3.1 Comparison of potato stress responses in an open greenhouse and in vitro

Due to the large amount of time and financial resources required for field studies and the rapid propagation and high sample throughput in the laboratory, in vitro studies are used for investigation of abiotic stress responses and narrowing down the test set of potato varieties for field trials in breeding processes (Schum et al. 2016; Gopal and Iwama 2007). Osmotic stress, as caused by e.g. sorbitol as osmoticum, is a part of drought stress response (Zhu et al. 1997). Therefore, an improved in vitro stress system based on Bündig et al. (2016a) was established in which the osmoticum could be added gradually and applied to rooted shoots (chapter 2.3). Similarities of the response to drought stress and to osmotic stress are e.g. growth reduction, increase of proline and alteration of specific proteins like linoleate 13s lipoxygenase 2 chloroplastic like (PGSC0003DMT400081909) (Bündig et al. 2016c). This lipoxygenase being the only protein found to be in common of the two test systems, in vitro and ex vitro, leads to the conclusion, that there are indeed differences between the responses in the two environments.

Besides the in vitro trials, two drought stress experiments were conducted in an open greenhouse, to gain insight into the early responses of potato to drought stress in the vegetative phase in terms of growth and expression of selected genes (chapter 2.2). In this chapter, the stress responses of plants in the open greenhouse to drought stress and plants in vitro to osmotic stress based on growth data and gene expression will be compared, to distinguish similarities and differences in the responses of the plant.

Growth data

Growth data from both, the open greenhouse experiments (2.2), and the in vitro experiments (2.3) showed morphological differences between control and stressed variants. Difference of water loss under stress (Stress/control in %; Table 1) show values between 27.5 % (‘Tomba’ experiment 1) and 57.1 % (‘Eurostarch’ experiment 1) in vitro after seven days, meaning that ‘Tomba’ showed a water loss 27.5 % lower under stress conditions than under control conditions. In the open greenhouse the decrease of water loss ranges from 14.0 % (‘Maxi’ experiment 1) to 64.6 % (‘Maxi’ experiment 2). After 14 days differences in water loss increased to up to 84.9 % (‘Eurobravo’ experiment 2 open greenhouse). In addition to reduced shoot length after drought/osmotic stress, a reduction of shoot dry mass was also observed in both scenarios. However, in the in vitro culture, the effect of reduced dry mass was exclusively significant after 14 days of stress in just one

experiment for all genotypes, while the differences in shoot DM in the open greenhouse were already significant after seven days of water withdrawal. A reason for the later increase of control shoot mass compared to stressed shoot mass in vitro could be the stress they are already experiencing by the in vitro environment. This creates a double stress for the plants, which has to be responded to simultaneously. However, due to the optimisation of the in vitro test system, we could prevent osmotic shock by letting the plants form roots before stress application. Root DM could not be statistically differentiated between control and stress condition in either environment after seven days. After 14 days root DM was decreased ex vitro in 'Eurobravo', 'Maxi', 'Ramses', and 'Tomba', while in vitro, root DM decreased only in 'Eurobravo'.

Table 1 Difference of water loss between stress and control variant of shoots in %. n=5. Percentages depict the difference between shoot water loss under drought or osmotic stress after 7 and 14 days. Ex1: exeriment 1, Ex2: experiment 2 (Stress/control).

		Open greenhouse		In vitro		
		Ex1	**Ex2**	**Ex1**	**Ex2**	**Ex3**
Decrease of water loss in shoots after 7 days of stress (%)	Eurobravo	40.0	52.0	35.6	59.2	51.8
	Eurostarch	57.1	46.1	36.8	54.2	50.1
	Maxi	49.0	43.2	14.0	64.6	54.7
	Tomba	27.5	41.5	22.7	50.8	27.1
Decrease of water loss in shoots after 14 days of stress (%)	Eurobravo	74.2	63.1	56.7	84.9	59.7
	Eurostarch	66.8	62.6	51.8	64.6	73.9
	Maxi	78.1	66.8	45.2	74.3	62.9
	Tomba	63.0	67.1	47.0	66.6	48.2

In the open greenhouse, the genotypes started the experiment with different masses. In vitro, this disparity in input weights can be avoided because the shoots used in the experiments were cut to the same length. However, the plants also showed differences in growth between genotypes in general. The growth quality of potato genotypes should be considered when assembling a test set for tolerance studies.

Biomarkers for osmotic or drought stress tolerance could not be determined in this study. This is because there were no genotypic differences in most of the growth data. In potato, there are suggestions for tolerant or sensitive potato genotypes (Sprenger et al. 2015; Meise et al. 2019).

In our study, we could not find comparable effects on those genotypes tested. Reason for that could be the test system. There are several differences between the test systems used in the studies (Table 2). Beginning with the environment, the open greenhouse, rainout shelter and field trials lead to differences in heat accumulation, water capacity in the soil and root space (compare to chapter 1.4). Furthermore, Bauer and Black (1992) showed, that water capacity is dependent on the texture of the soil. Therefore, responses can alter in different environments and soils. Also, the time of stress application, stress duration, and sampling comprises alterations in drought response. Next to acclimatisation, that can occur in genotypes until the samples are taken, potato plants respond with different strength based on the growth stage they were in, when stress occurrs (Obidiegwu et al. 2015). Other critical parameters, that can influence the outcome of stress tolerance classification are the choice of how to calculate a stress index. Meise et al. (2019) based their classification on the stress susceptibility index (SSI) (Fischer and Maurer 1978), whereas Sprenger et al. (2015) classified the tested genotype on the basis of deviation of relative starch yield from the experimental median (DRYM). Overall, these differences in the setups lead to different classification of the same genotypes (Table 2).

Moreover, in our study, in vitro trials and open greenhouse trials are not suitable to be compared directly due to the differences of the plants when they enter the experiments, the lab conditions (light, temperature, vessels etc.), and the mixotrophic growth in vitro. In the open greenhouse, reproducibility may be limited due to growth before the experiment, which can differ between the genotypes so that they start with different masses. Furthermore, other stressors might occure in addition to drought stress, depending on the weather, these might comprise heat stress or biotic factors that influence the results (Lamaoui et al. 2018; Intergovernmental Panel on Climate Change 2022).

The lack of differences in tolerance between genotypes through all growth parameters in the two environments we tested correlate with the comparison of the test systems of Meise et al. (2019) and Sprenger et al. (2015) to our study in the open greenhouse.To get to genotypic differences, that are required to derive biomarkers for drought sress tolerance, consistent growth data has to be

shown. The test set of genotypes should be changed to genotypes showing the highest contrast in shoot and root growth under drought stress conditions and the environment and experimental parameters should be considered carefully for interpretation of drought tolerance classifications. Therefore, it should be ensured that the drought stress conditions are appropriately applied, taking into account factors such as intensity, duration, and timing of stress imposition. Adjusting these parameters may help to create more distinct differences in growth responses among genotypes. Also, increasing the number of replicates or samples for each genotype to improve the statistical power of the analysis will help to ensure that any observed differences in growth parameters are reliable and significant. Besides shoot and root growth, evaluating other relevant growth parameters, such as leaf area, chlorophyll content, or stomatal conductance may be helpful.

Table 2 Comparison of drought stress studies.

Study	**Chapter 2.2**	**Meise et al. (2019)**	**Sprenger et al. (2015)**
Environment	Open greenhouse	Rainout shelter	Field at 2 sites Rainout shelter
Soil	pot substrate:sand (1:1 [v/v])	Peat:sand (95:5 [v/v])	Field: Soil with different quality Rainout shelter: Peat:sand (95:5 [v/v])
Sampling	Seven and 14 days after stress onset	After maturation	After maturation
Stress application	Water withdrawal until 15 % water holding capacity (WHC)	Water withdrawal	Water withdrawal And water withdrawal until 30 % WHC
Stress duration	14 days	12-13 days	Until maturation
Growth stage	Stolon initiation	Tuber initiation	Before and after flowering
Classification	-	Starch yield Tuber yield	Starch yield
‘Eurobravo’	-	Sensitive	Sensitive
‘Eurostarch’	-	In between	Sensitive

'Maxi'	-	Tolerant	Sensitive
'Tomba'	-	Tolerant	Tolerant

More consistent experimental conditions were expected to occur in in vitro experiments. In vitro culture is stress for the plants prior to the applied abiotic stress due to the environmental conditions. Parameters like temperature can be adapted, which is a huge advantage of the system. Single applied stresses like osmotic stress can be monitored better due to less external influences. Furthermore, it is important to notice, that plants grow mixotrophically in vitro and there are several differences in morphology and physiology, such as open stomata and rudimentary cuticle, that must be considered when interpreting and evaluating genotypes or stress response in general (Ševčíková et al. 2019). However, in vitro trials can be conducted to get a better insight into osmotic stress response of potato and processes like osmotic adjustment, as well as narrowing down the test set for breeders. For the osmotic adjustment response of the genotypes under osmotic stress changes in osmolyte accumulation in the stressed variant compared to control variant should be conducted. Also, relevant indicators like relative water content, electrolyte leakage, chlorophyll content, and stomatal conductance should be measured.

Gene expression

Gene expression assays were performed for the open greenhouse experiments and the in vitro experiments for the same GOIs based on proteins that were found differentially abundant under drought stress (chapter 2.1; Table 3). Since osmotic stress and drought stress have some responses in common, as osmotic stress is part of drought stress, the gene expression might also correlate between the systems. Due to different reactions, normalised gene expression could not be statistically evaluated combined for both open greenhouse experiments, but had to be evaluated seperately. Differences in the gene expression between the two experiments and the fact, that heat occurred in one experimental period, the expression of some genes was apparently strongly dependent on external influences such as temperature. In the in vitro experiments only the expression of *RPT5a*, *POD* and *SBT1.7* had to be statistically tested separately.

RPT5a (regulatory particle triple-A ATPase 5A) was downregulated in 'Eurobravo', 'Eurostarch' and 'Maxi' in the open greenhouse after seven days of drought stress. However, this only happened in the trial where high temperatures occurred before the drought stress phase. In vitro, the gene was

not regulated in the genotypes tested. This response may indicate that the gene is more responsive to combined stress or heat stress, and that the elevated temperature beforehand might be a trigger or a priming effect for the expression of *RPT5a*. *RPT5a* is a regulatory subunit of the 26S proteasome, that, when decreased, leads to heat shock sensitivity and reduced cell division. This leads to an increased 20S proteasome, which increases the oxidative stress tolerance by degrading oxidised proteins (Kurepa et al. 2009).

Table 3 Overview of fold changes (stress/control) of GOIs in the open greenhouse (chapter 2.2), in vitro experiments (chapter 2.3), and protein abundance (chapter 2.1). Significantly higher abundant proteins/upregulated genes are marked in orange, significantly lower abundant proteins/downregulated genes are marked in blue. Significance codes after Tukey's test or Kruskal-Wallis test between control and stress conditions in one genotype: *** = p < 0.001; ** = p < 0.01; * = p < 0.05. n=5 (open greenhouse), 4 (in vitro), 2x4 (Rainout shelter).

		Open greenhouse gene expression		In vitro gene expression		Rainout shelter protein abundance
		experiment 1	experiment 2	experiment 1	experiment 2	Wellpott et al. (2021)
Glyx	Eurobravo	0.66	1.83	0.68		
	Eurostarch	1.71	1.17	0.39***		0.63
	Maxi	1.54	0.63	0.78		
	Tomba	1.79	0.78	0.42**		0.63
RPT5a	Eurobravo	0.57***	0.91	1.15	0.85	
	Eurostarch	0.60***	1.12	1.11	1.14	1.62
	Maxi	0.75*	0.91	1.07	1.42	
	Tomba	1.11	0.9	1.01	0.72	2.53
ZBD	Eurobravo	0.74	0.87	0.56*		
	Eurostarch	1.03	1	0.6*		1.72
	Maxi	1.18	1.11	0.66*		
	Tomba	1.32*	0.89	0.54***		1.85
INH1	Eurobravo	4.18*	15.51***	1.41		
	Eurostarch	4.30*	10.37***	3.44***		2.21
	Maxi	3.77*	6.31***	1.12		
	Tomba	1.39	8.80***	2.42***		1.56
SHMT	Eurobravo	0.66	0.91	0.34***		
	Eurostarch	1.35	0.99	0.34***		1.52
	Maxi	1.59	0.47***	0.38***		
	Tomba	1.32	0.40***	0.51**		1.94
POD	Eurobravo	0.11**	0.08***	0.08**	0.03***	
	Eurostarch	0.13*	0.03***	0.09*	0.03**	0.64
	Maxi	0.29**	0.04***	0.3	0.18	
	Tomba	0.76	0.05***	0.16***	0.01***	0.63
SBT1.7	Eurobravo	0.33**	0.09***	0.22***	0.42	
	Eurostarch	0.63	0.08***	0.27***	0.21***	0.43
	Maxi	1.1	0.12***	0.32**	0.38	
	Tomba	1.25	0.21*	0.33*	0.09***	0.36
13-LOX	Eurobravo	0.71	0.44*	1.08		
	Eurostarch	1.2	0.16***	1.11		1.54
	Maxi	1.17	0.36*	0.42***		
	Tomba	1.98	1.11	0.61**		1.5

Glyx (lactoylglutathione lyase/glyoxalase I family protein) was generally higher expressed in the in vitro experiments. The protein is associated with the glyoxalase system and is responsible for detoxifying methylglyoxal (MG), which is a signal molecule for stress (Hoque et al. 2016). After seven days of osmotic stress, downregulation was observed in the 'Eurostarch' and 'Tomba' genotypes. In the open greenhouse, however, no regulation was observed after seven days of drought stress. As cultivation in vitro stresses the plant (Desjardins et al. 2009) the in vitro conditions and the mixotrophic growth could cause the observed regulation of the gene. The differences between genotypes could be explained by different mechanisms in dealing with osmotic stress. For example, 'Eurostarch' and 'Tomba' may be able to compensate the *Glyx* product through other enzymes like lactate dehydrogenase or glutathione reductase. This would provide an alternative route for detoxifying methylglyoxal (An et al. 2017).

ZBD is a gene for an allyl alcohol dehydrogenase and plays a role in plant growth, development, and stress adaptation. In the open greenhouse, the gene was not regulated (in genotype 'Tomba' it was upregulated in the experiment where high temperatures previously occurred). In vitro, the gene was downregulated in all genotypes after seven days of osmotic stress. In both environments, the gene was approximately at the same expression level at day 0 and in the control. Also, gene expression levels of the two environments were similar. The gene appears to be regulated under osmotic stress rather than under drought stress.

13-LOX is a lipoxygenase, linked to ABA synthesis and stomatal closure, oxylipin biosynthesis and therefore plant defense, and to tuberisatrion (Kolomiets et al. 2001; Liavonchanka and Feussner 2006). The downregulation in 'Eurobravo', 'Eurostarch' and 'Maxi' in one experiment ex vitro and in 'Maxi' and 'Tomba' in vitro may be due to overcompensation for a prior upregulation. A second hypothesis is, that *13-LOX* is downregulated to postpone tuber formation in order to save sugar and therefore energy, for the primary metabolism.

POD, a gene encoding a protein from the family of peroxidases, was downregulated in both environments, an exception being 'Maxi' after osmotic stress in vitro, where no regulation took place. POD detoxifies H_2O_2, which is produced in photorespiration when there is not enough CO_2 (Tourneux and Peltier 1995). O_2 will then be used to recover CO_2 for upkeeping function of the Calvin cycle. There is a higher level of photorespiration in vitro through the mixotrophic growth (Düring and Harst 1996). Thus, more H_2O_2 is produced, and more POD is needed. In the stress

variant, increased oxidative stress occurs. The downregulation could be a drop after a previous upregulation. To prove this hypothesis, gene expression studies at earlier time points are necessary. Genotype ‘Maxi’ showed no regulation in vitro. This may be due to a faster response of the gene to osmotic stress, leading to a return of gene expression to a steady state level.

SHMT is a gene linked to photorespiration, stomatal closure, and detoxification of ROS. In the open greenhouse it was downregulated after seven days of drought stress in ‘Maxi’ and ‘Tomba’ in one experiment. On the contrary, the response in vitro was much more consistent being downregulated in all genotypes after seven days of osmotic stress. Stomata do not close in vitro, because of the high relative humidity in the vessels (Santamaria et al. 1993). This might explain the overall lower expression in vitro of *SHMT*. In vitro, photorespiration and thus H_2O_2 production are higher (Collin 2019). The downregulation may be related to growth depression as Liu et al. (2019) found SHMT-mutants to be severly more sensitive to salt stress and thus growth deprived.

SBT1.7 is a gene for a protein of the subtilase family. It is involved in cell development and cell growth. In the open greenhouse and in vitro it was downregulated in all genotypes (with exception of ‘Eurobravo’ ex vitro in one experiment and ‘Eurobravo’ and ‘Maxi’ in vitro in one experiment). The rather small differences and the same trend in both environments leads to the conclusion of *SBT1.7* being a generally regulated gene in different environments under abiotic stress like drought and osmotic stress. When *SBT1.7* is downregulated, energy from cell development is saved for important stress responses. Moon et al. (2018) showed a subtilase to be downregulated in potato after six hours, and even stronger downregulated after 48 h of drought stress.

The gene *INH1* (cell wall/vacuolar inhibitor of fructosidase) was upregulated in all genotypes after seven days of drought stress in the open greenhouse. In vitro, the gene was also upregulated after osmotic stress, but only in ‘Eurostarch’ and ‘Tomba’. The gene was expressed at the same basic level in both environments at day 0 and in the controls, but it was upregulated to a five times higher level in the open greenhouse. Regulation here may depict the extent to which there is a difference between drought stress and osmotic stress. In general, the two stressors and thus the response are related, but it is more regulated under drought stress in the open greenhouse because it is more urgently needed under drought stress. This might be due to the mixotrophic growth in vitro. Whether the two genotypes in which the gene was not regulated in vitro, ‘Eurobravo’ and ‘Maxi’, are either more tolerant, might not need the regulation of *INH1* at the analysed time point, or they

are more sensitive to abiotic stress and cannot provide the regulation, remains unclear. An explanation for the lack of upregulation in vitro may be the supply of sugar as a carbon source in the in vitro culture. This may result in less pronounced upregulation of the expression of *INH1*, as the sugar is metabolized. Moreover, stomatal closure to which overexpression of *INH1* leads is not necessary in vitro (Wardle and Short 1983; Rodrigues et al. 2014).

An original goal of the gene expression analyses in the project VALPROKAR was to identify suitable biomarkers for drought tolerance. But since genotypic differences in the growth data could not be shown, the goal could not be reached with the genotypes tested. Nevertheless, since previous studies had classified them as more tolerant or more sensitive to drought and osmotic stress in different settings, this points to the stress response being very much depending on the experimental design and method used.

3.2 Regulation of drought stress responses on gene expression and protein levels

For the purpose of biomarker development, leaf samples of two rainout shelter experiments were used to identify proteins, that were differentially abundant under drought stress in two genotypes (‘Eurostarch’ and ‘Tomba’) which were postulated to be rather tolerant to drought stress (‘Tomba’) or combined drought stress and N deficiency (‘Eurostarch’)(Meise et al. 2019). From these identified proteins of interest primer pairs were derived based on their sequence and gene expression analyses in leaf material of two open greenhouse experiments were conducted. In this chapter the comparability of gene expression and protein abundance is discussed. It should, however, always be considered that the samples were derived from different experiments.

For protein biosynthesis translational and post-translational regulation as well as protein degradation regulation result in different protein abundances (Vogel and Marcotte 2012). Protein content is renewed through protein turnover and protein biosynthesis, which is also influenced by transcriptional and translational modifications (Nelson and Millar 2015). The turnover rate differs due to function, localisation or environmental factors of the proteins and can reach from several hours to several months (Li et al. 2017). Gene expression is regulated transcriptional and post-transcriptional. Also important to adress is alternative splicing, which is a way to modify genes leading to alternative isoforms of a protein (Farrell 2007). Petrillo et al. (2014) found light to regulate alternative splicing of proteins involved in RNA processing in *A. thaliana*.

For *INH1, POD,* and *SBT1.7* gene expression and protein abundance were comparable, at least regarding the direction of the observed changes (Table 3). Both the gene expression of *INH1* was upregulated (2.3) as well as a higher protein abundance was found (2.1) under drought stress ex vitro. *POD* and *SBT1.7* were both downregulated under drought stress in the open greenhouse experiments and the proteins were lower abundant after drought stress in a rainout shelter. From the GOI test set these three genes seem to indicate drought stress and osmotic stress in potato.

Glyx showed a lower protein abundance, but the gene expression level was stable under drought stress influence. This leads to the conclusion, that post transcriptional or translational regulation could have taken place. Furthermore, protein degradation could have occurred under drought stress. Protein degradation should be tested by azocasein assays and ^{14}C-methylated casein assays (Roy-Macauley et al. 1992; Peterson and Huffaker 1975), western blot, or pulse-chase analysis (Takahashi and Ono 2003).

RPT5a, SHMT, 13-LOX, and ZBD showed a higher protein abundance under drought stress, while the gene expression level was stable for *ZBD* and decreased for the other genes. For these genes earlier time points after stress need to be analysed to clarify whether the downregulation is an overcompensation effect after an upregulation within a rapid stress response. Further, it should be considered, that those proteins may have undergone post-transcriptional regulation and alternative splicing and therefore are not regulated equally.

The comparison of single genes and proteins is challenging, since various metabolic processes occur in plants under stress conditions. Moreover, more than one protein can derive from one single gene and low abundant proteins cannot be detected due to limitations. Sample preparation and even differences in the laboratories (such as methods or even handling) in which the experiments took place can be a problem for a comparison of gene expression and protein abundance (Greenbaum et al. 2002). Furthermore, plasticity of the plant metabolism and multiple genes involved in drought stress may make it more difficult to derive a biomarker for drought stress (Laitinen and Nikoloski 2019).

3.3 Choice of osmoticum for in vitro studies

Studies of osmotic stress reaction in vitro have now been conducted for many years. Mannitol was used for the induction of osmotic stress in culture media because of its non-toxic nature (Hanász et al. 2022). However, mannitol was shown to be taken up by plants like wheat, rape and potato and

transported to the shoot symplast (Fritz and Ehwald 2011; Sajid and Aftab 2022; Lipavská and Vreugdenhil 1996). PEG is a very large molecule and is also used for inducing osmotic stress in vitro. PEG with a high molecular weight (PEG8000) is not taken up by the plant, but it is limiting the oxygen movement due to its viscosity, leading to oxygen deficiency in potato (Sajid and Aftab 2022). This was also found for hydroponic cultures of maize (Verslues et al. 1998). It remains unclear whether the transferability of drought stress to osmotic stress works in vitro in terms of tolerance.

Sorbitol is the most common used osmoticum for osmotic stress studies in vitro. It has been used for various plants such as lupin (Legocka and Kluk 2005), macro algae (Gao et al. 2014), banana (Placide 2012), olive (Brito et al. 2003), cantaloupe (Mehmandar et al. 2023), but also for potato (Gopal and Iwama 2007; Albiski et al. 2012) because it is non-toxic for the plant. However, Bündig et al. (2016b) showed in an in vitro approach that sorbitol was indeed taken up at least in an in vitro system using solid medium and wounded plants without roots.

For that reason, the test system of Bündig et al. (2016b) was optimised as stated in chapter 2.4. Apparently, sorbitol was found in the shoots of plants that were allowed to form roots prior to the stress application. Whether sorbitol was taken up through roots, and transported to the shoots, or whether the shoots took up the osmoticum through the leaf area will be analysed by ^{14}C labeling. Bündig et al. (2016b) showed that the fructose concentration was reduced under stress, indicating that sorbitol was not converted to fructose by a sorbitol dehydrogenase. A sorbitol hydrogenase is exclusively predicted for potato (NCBI: LOC102595131, PGSC database: PGSC0003DMT400081907). For Rosaceae sorbitol is the main photosynthetic product and a sorbitol dehydrogenase was confirmed to occur in apple (NAD-SDH, NCBI: LOC103439704) (Wang et al. 2009; Li et al. 2012). Whether sorbitol is stored or even metabolised in potato is not known yet and must be veryfied by e.g. isotopic labeling in further studies.

3.4 Biomarkers for drought stress and drought stress tolerance in potato

Biomarkers are beneficial for breeders for several reasons. With biomarkers for drought stress, it is possible to detect drought stress prior to the appearance of visible symptoms. This allows prevention of yield loss. Further, with biomarkers for drought tolerance breeders can select varieties for further development faster than in conventional breeding methods without markers. Biomarkers may also help to develop varieties with specific desired traits. This can help to improve

sustainable agriculture, as yield losses could be prevented in areas with high impact of drought periods. Protein biomarkers are suitable for breeders next to molecular markers such as SNPs and microsatellites, because proteins are directly involved in the stress response of the plant. Moreover, there are proteins, that are highly specific towards the stress condition. This may lead to a more accurate and reliable choice of drought tolerant varieties (Barkla 2016).

A good biomarker should be specific, sensitive, and stable. High specificity to drought stress allows the variety selected with help of the biomarker to be specifically drought tolerant. Sensitivity allows an early detection of a tolerant or sensitive response of the plant to drought stress. Stability of the marker ensures consistent results under different circumstances (Ernst 1999; Brain and Cedergreen 2009). In potato, several biomarkers for drought stress and drought stress tolerance were suggested. Sprenger et al. (2018) suggested among others a lipoxygenase (PGSC0003DMT400082023), which we also showed to be genotypically differentially abundant in two rainout shelter experiments. Bündig et al. (2016c) suggested monohydroascorbate for osmotic stress tolerance, which is used to detoxify H_2O_2 or glycolate oxidase, which is assigned to photorespiration.

In this study, the stability could be identified for three GOIs: *INH1*, *POD*, and *SBT1.7*. These genes were similarly regulated in vitro and in the open greenhouse. Furthermore, gene expression and protein abundance displayed the same direction of regulation. However, a genotypic effect was found only in protein abundance (2.1). The overall predominantly downregulation of the selected GOIs (3.2) correlated with our findings in chapter 2.4. Differentially abundant proteins were mostly lower abundant under drought stress, and combined stress. Despite having found some similarities between those two stressors, it was striking, that combined stress showed a different palette of proteins that were differentially abundant. Because drought stress is not occuring individually in the field, and heat waves are expected just like drought periods, this leads to the conclusion, that analysing combinations of stressors is of great importance for deriving suitable biomarkers for stress tolerance.

3.5 Conclusions

For a deeper insight into responses to drought stress of potato, candidate proteins, that were identified in PROKAR and assigned to drought or osmotic stress were to be validated in material of two rainout shelter experiments. A protein, which showed up in both studies was 13-LOX. The

lipoxygenase was higher abundant under drought stress in rainout shelter material in those two genotypes, which were stated to be rather tolerant (‘Eurostarch’, and ‘Tomba’).

Further, new proteins indicating drought stress were identified by LC-MS (2.1). Eight candidate genes derived from the proteins identified could be assigned to drought stress responses. Three of them (*POD, SBT1.7*, and *INH1*) showed alterations of protein abundance in the same direction in all genotypes, assuming a general drought reaction. These proteins may be suitable candidates for further studies, such as metabolite analysis.

Based on the validation and identification of proteins, gene expression analyses took place to determine the level on which regulation takes place under drought stress in potato. These and drought stress tolerance in potato. These genes were also measured in two osmotic stress experiments in vitro. *INH1* showed similar upregulation in all genotypes and should be tested further for detection of early drought stress in potato. Genotypic differences and therefore a candidate gene for a biomarker for drought stress tolerance were not achieved. This may be solved with more diverging genotypes earlier sampling after inducting drought or osmotic stress, when tolerant and sensitive genotypes can be confirmed. Rapidly responding genes can then be detected and may show genotypic differences among the testset.

Also, the optismised in vitro test system for osmotic stress responses allowed to gradually apply osmotic stress after root formation. An increased content of sorbitol was detected in shoots under osmotic stress. It remains unclear, whether sorbitol is taken up through the roots, metabolised and/or stored. This has to be investigated in further studies with ^{14}C labelling.

3.6 Outlook

The outcomes of the protein and gene expression analyses lead to the conclusion, that seven days of water withdrawal are too long to identify the early drought stress responses of potato. Some genes like *13-LOX*, *POD*, or *SHMT* showed downregulation, while previous time points in different studies had shown upregulation.Therefore, earlier time points or better a time series from several hours to a few days after stress onset should be conducted for a better insight of early stress responses and genotypic differences.

Also, the presented promising candidate genes should be analysed with a diversified set of genotypes. This may result in genotypic differences in the stress response. For a deeper insight in the function of the genes as well as the stress mechanism of potato, genetic studies and mutants would be helpful. Metabolite studies would help to understand the link between gene expression, protein abundance, and drought stress tolerance. Further, the measurement of ABA levels could give a hint towards ABA-signaling, and ROS-detoxification, which is involved in drought stress and drought stress tolerance. For a functional study of the identified GOIs, genetic engineering to knock-out, or knock-down specific genes would be needed. CRISPR/Cas could comprise a useful tool for this purpose. However, genetically modified organisms (GMOs) are subject to European Union directives in Europe and to the GenTG in Germany (Eriksson et al. 2020). Furthermore, potato is tetraploid and therefore successful mutations of all alleles is difficult.

Since this study clearly revealed that the stress system is very important for the stress response, further comparative studies in different environments (in vitro, open greenhouse, climate chamber, field) should be conducted. It is important to carefully chose the best test system for the desired purpose. Maturation time and the season of the experiment should be considered when conducting drought stress experiments. Moreover, the substrate, temperature and light intensity should be considered relevant and implemented in the interpretation of the stress response of potato.

In the in vitro test system, the used osmoticum should be re-considered. Since sorbitol was probably taken up, it is important to include that fact into interpretation of data. In further studies a suitable osmoticum should be used as an alternative for sorbitol.

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5. Curriculum vitae

Name: Katharina Wellpott

Born: 19.06.1992 in Ochtrup

Education

Since 10/2018 **PhD**

Title: Response of starch potato (*Solanum tuberosum* L.) genotypes to osmotic stress in vitro and drought stress ex vitro

Leibniz University Hannover

Institute of Horticultural Production Systems

Research Team Woody Plant and Propagation Physiology

- **Expected degree: Dr. rer. nat.**

04/2016 – 10/2018 **M. Sc. Plant Biotechnology**

Leibniz University Hannover

Knock-out der Biphenylsythase in *Malus domestica* BORKH. Mittels CRISPR/Cas9

10/2015 – 03/2016 **Bachelor's Studies Biology**

Leibniz University Hannover

10/2012 – 09/2015 **B. Sc. Plant Biotechnology**

Leibniz University Hannover

08/2011 – 05/2012 **Professional Training**

Fachklinik für Kleintiere Dr. Uwe Romberger, Regensburg

Veterinary nurse

09/2002 – 06/2011 **Secondary School, Abitur**

Missionsgymnasium St. Antonius Bardel, Bad Bentheim

09/1998 – 08/2002 **Primary School**

Kath. Grundschule St. Johannes, Bad Bentheim

Professional Experience

10/2018 - 05/2022	**Research Assistant** Leibniz University Hannover Institute of Horticultural Production Systems Research Team Woody Plant and Propagation Physiology Implementation of a project funded by the BMEL and the FNR
11/2017 – 09/2018	**Student Assistant** Leibniz University Hannover Institute of Horticultural Production Systems Research Team Woody Plant and Propagation Physiology In vitro Work
04/2017 – 10/2017 + 04/2016 – 10/2016	**Student Assistant** Leibniz University Hannover Institute of Horticultural Production Systems Research Team Phytomedicine Field Trials Evaluation Work

Professional training

04/2022	**Statistics (RStudio)** Workshop of the WeGa-PhD Graduate Program
03/2022	**Business Management for Natural Scientists** Workshop of the WeGa-PhD Graduate Program
11/2021	**„Führungskompetenz – Was macht eine gute Führungskraft aus?“ (Leadership Competence)** Workshop of the WeGa-PhD Graduate Program
11/2020 – 01/2021	**Time Management** Workshop of the WeGa-PhD Graduate Program

11/2020	**„Unternehmerisches Denken und Handeln“ (Entrepreneurial Thinking and Acting)** „Erfolgsmodell DU – Traumjobs werden häufiger geschaffen als gefunden“ Workshop from the Graduate School of Natural Sciences
01/2020	**Writing Scientific Articles** Workshop of the WeGa-PhD Graduate Program
11/2019	**Good Scientific Practice – Protecting Scientific Integrity** Workshop from the Graduate School of Natural Sciences
10/2016 – 03/2017	**Gene Technology, Biosafety and Biosecurity** Educational Course according to § 15 para. 2 GenTSV

6. List of publications

6.1 Peer reviewed publications

Wellpott K., Jozefowicz A.M., Mock H.P., Meise P., Schum A., Winkelmann T., Bündig C. (2021). Identification of candidate proteins in drought stress tolerant and sensitive starch potato genotypes (*Solanum tuberosum* L.) for biomarker development. (Conference Paper for the Annual Conference DGG and BHGL, At: Stuttgart (online)) DOI: 10.5288/dgg-pr-10-04-kw-2021

Wellpott K., Jozefowicz A.M., Meise P., Schum A., Seddig S., Mock H.P., Winkelmann T., Bündig C. (2023). Combined nitrogen and drought stress leads to overlapping and unique proteomic responses in potato. Planta 257:58. DOI: 10.1007/s00425-023-04085-4

6.2 Submitted/ under review

Wellpott K., Straube J., Winkelmann T., Bündig C. (submitted) Expression analysis of candidate genes as indicators for commencing drought stress in starch potatoes. J. Agron. Crop Sci.

6.3 Publications in preparation

Wellpott K., Herde M., Winkelmann T., Bündig C. (in preparation) Liquid culture system allows rooting of *Solanum tuberosum* and gradual application of osmotic stress through sorbitol in vitro.

6.4 Poster presentations

Wellpott K., Winkelmann T., Bündig C. (2018) Untersuchung von Trockenstress an Kartoffeln. Die Nacht, die Wissen schafft 10.11.18. Posterbeitrag

Wellpott K., Winkelmann T., Bündig C. (2019) Entwicklung von Schnelltests für die Züchtung von stick-stoffeffizienten und trockentoleranten Stärkekartoffeln auf Basis von Proteomdaten. DGG & BHGL Jahrestagung Berlin, 6.-9.3.19. Posterbeitrag.

Wellpott K., Bündig C., Winkelmann T. (2019) Untersuchung der Reaktion von Stärkekartoffeln (*Solanum tuberosum* L.) auf osmotischen Stress in vitro sowie Trockenstress in vivo. WeGa Doktorandentag 10.-11.10.19. Posterbeitrag.

Breil J.M., Wellpott K., Winkelmann T., Bündig C. (2021) Response of roots of *Solanum tuberosum* L. to drought stress. Symposium on Horticulture in Europe (DGG: SHE) Stuttgart (online), 8.-12.3.21.

Struzina S., Wellpott K., Winkelmann T., Büündig C. (2023) Reaktion von *Solanum tuberosum* L auf osmotischen Stress in einem Flüssigkultursystem in vitro. 55. Gartenbauwissenschaftliche Jahrestagung der DGG und des BHGL Osnabrück, 01.-04.03.23.

6.5 Oral presentations

Wellpott K., Jozefowicz A., Mock H.P., Winkelmann T., Bündig C. (2021) Identification of candidate proteins in drought stress tolerant and sensitive starch potato genotypes (*Solanum tuberosum* L.) for biomarker development. Symposium on Horticulture in Europe (DGG: SHE) Stuttgart (online), 8.-12.3.21.