Responses of starch potatoes (*Solanum tuberosum* L.) to osmotic stress *in vitro* regarding growth, proteome and metabolite profile

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades
Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation
von

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2016

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Tag der Promotion:	27.05.2016

Abstract

Starch potatoes are important for industrial applications such as the production of ethanol, adhesives and gelling agents. Through the foretold climate change drought spells will be prolonged and arise more often. As potato plants are relatively susceptible to drought it is of utmost interest to gain new insights into stress responses upon water shortage. Especially starch potatoes suffer from water shortage, as a mobilisation of sugars from starch reduces the market value and increases the risk of acrylamide production during processing. However, while field trials are labour and cost intense and other stresses can influence the results, an alternative to analyse stress responses was proposed using the *in vitro* technique. Stress was applied to 18 genotypes of *Solanum tuberosum* L. and two wild type species (*S. tarijense* and *S. chacoense*) through the addition of 0.2 M sorbitol, which reduces the medium's osmotic potential. As drought stress comprises osmotic stress, analysing these responses in contrasting genotypes can be expected to give insight into pathways specific for the reaction to osmotic stress. In this thesis, special emphasis was put on the growth, proteomic and metabolite responses of starch potatoes upon osmotic stress *in vitro*. Plants were successfully stressed in the *in vitro* experiments with special regard to growth reduction, and significant differences in the extent of growth reduction were observed for the genotypes tested. Dry mass reduction after 21 days was between 43.8 % and 12.2 %. A correlation to tuber yield in pot trials, in which drought stress was applied, was however not detected. The most divergently reacting genotypes *in vitro* were then analysed for specific responses on the proteomic and metabolite level. On day 11 after the onset of the osmotic stress, samples were taken and analysed. By 2D-IEF PAGE (two dimensional- isoelectric focussing polyacrylamide gel electrophoresis) and subsequent identification of the proteins, differentially abundant proteins especially in the sensitive genotype were detected. A lower number of differentially abundant proteins was detected in the tolerant compared to the sensitive genotype (20 in tolerant; 118 in sensitive). The analysis of specific metabolites through GC/MS (gas chromatography/ mass spectrometry) also displayed that the sensitive genotype comprised stronger responses compared to the tolerant genotype in terms of the number of altered metabolites. For most analysed metabolites a decrease was observed. Major differences between the genotypes were identified for fumaric acid, glycine, sucrose and phenylalanine. Taken together, indications for the following osmotic stress responses were obtained: an increased photorespiration for both genotypes, but a different response to oxidative stress and an elevated proteolysis in the sensitive genotype. For the first time, uptake of sorbitol was shown for both genotypes. As a consequence, this leads to a discussion of future improvements of *in vitro* osmotic stress test systems.

Keywords: *Solanum tuberosum*, *in vitro*, osmotic stress, proteomic, metabolite

Zusammenfassung

Stärkekartoffeln (*Solanum tuberosum* L.) sind bedeutend für industrielle Anwendungen wie z. B. die Produktion von Ethanol, Klebemitteln und Geliermitteln. Durch den prognostizierten Klimawandel wurde eine erhöhte Anzahl und längere Dauer von Trockenperioden vorausgesagt. Da Kartoffelpflanzen als relativ anfällig gegenüber Trockenheit gelten, ist es von höchstem Interesse neue Einblicke in Trockenstressreaktionen zu bekommen. Stärkekartoffeln werden besonders von Trockenheit beeinflusst, da Zucker aus Stärke mobilisiert werden und dies den Marktwert verringert. Zudem ist dadurch die Gefahr der Bildung von Acrylamid während der Verarbeitung erhöht. Da Feldversuche kosten- und arbeitsintensiv sind und andere Stressoren nicht ausgeschlossen werden können, wurde die *In-vitro*-Technik als Alternative vorgeschlagen um Stressantworten zu untersuchen. In dieser Arbeit wurden 18 *S. tuberosum* Genotypen und zwei Wildarten (*S. tarijense* und *S. chacoense*) durch die Zugabe von 0.2 M Sorbitol, welches das osmotische Potential des Mediums verringert, gestresst. Da Trockenstress osmotischen Stress beinhaltet, können durch die Untersuchungen dieser Stressantworten neue spezifische Stoffwechselwege, die an der Reaktion auf osmotischen Stress beteiligt sind, gefunden werden. Der spezielle Schwerpunkt dieser Arbeit lag auf Antworten auf osmotischen Stress von Stärkekartoffeln *in vitro* in Hinblick auf Wachstum, Proteom und Metabolite. Im *In-vitro*-Versuch wurde erfolgreich Stress aufgebaut, wie durch die Reduktion des Wachstums belegt wurde. Signifikante Unterschiede in der Wachstumsreduktion wurden zwischen den getesteten Genotypen beobachtet. Die Reduktion in der Trockenmasse nach 21 Tagen betrug zwischen 43.8 % und 12.2 %. Eine Korrelation zum Knollenertrag unter Trockenstress in Topfversuchen konnte jedoch nicht gezeigt werden. Die beiden *in vitro* am stärksten kontrastierenden Genotypen wurden dann für weitere Analysen ihrer spezifischen Antwort auf Proteom und Metabolit Ebene untersucht. An Tag 11 nach Beginn des osmotischen Stresses, wurden Proben genommen und analysiert. Mittels der 2D-IEF PAGE (zweidimensionale-Isoelektrische Fokussierung Polyacrylamid Gelelektrophorese) und anschließender Identifikation der Proteine wurden differentiell abundante Proteine besonders im sensitiven Genotyp nachgewiesen. Es zeigte sich, dass der tolerante Genotyp weniger differenziell abundante Proteine aufwies als der sensitive Genotyp (20 im toleranten, 118 im sensitiven). Die Analyse von spezifischen Metabolite mittels GC/MS (Gaschromatographie/ Massenspektrometrie) zeigte ebenfalls eine stärkere Reaktion des sensitiven Genotyps in Bezug auf die Anzahl der veränderten Metaboliten. Für die meisten Metaboliten konnte eine Abnahme beobachtete werden. Erhebliche Unterschied konnte für Fumarsäure, Glycin, Saccharose und Phenylalanine zwischen den Genotypen nachgewiesen werden. Zusammengefasst wurden Indizien für folgende Antworten auf osmotischen Stress *in vitro* gefunden: eine erhöhte Photorespiration für beide Genotypen, aber eine unterschiedliche Antwort auf oxidativen Stress und eine erhöhte Proteolyse für den sensitiven Genotyp. Zum ersten Mal konnte gezeigt werden, dass beide Genotypen Sorbitol aufnehmen. Als Konsequenz ergibt sich eine Diskussion über zukünftige Verbesserungen für osmotische Stresstestsysteme *in vitro*.

Schlagworte: *Solanum tuberosum*, *in vitro*, osmotischer Stress, Proteomik, Metabolite

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Abbreviations

2D	two dimensional
ABA	abscisic acid
ATP	adenosine triphosphate
C	carbon
Ca^{2+}	calcium
CAT	catalase
CLD	compact letter display
CO_2	carbon dioxide
cv.	cultivar
DM	dry mass
FM	fresh mass
GABA	*γ*-aminobutyric acid
GC	gas chromatography
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HSPs	heat shock proteins
IEF	isoelectric focussing
IPG	immobilized pH gradient
K^+	potassium
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MS medium	Murashige and Skoog (1962) medium
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonium-lyase
PCA	principal component analysis
PEG	polyethylene glycol

pI	isoelectric point
PSII (PS2)	photosystem II
Q-TOF	quadrupole-time-of-flight
ROS	reactive oxygen species
RTI	retention time index
RubisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SSI	stress susceptibility index
TCA	tricarboxylic acid
TFs	transcription factors
TOF	time of flight

1 Chapter 1 - General Introduction

Plants as sessile organisms have developed a number of mechanisms to deal with inadequate environmental conditions. Abiotic stresses, such as drought and salt stress are of major importance as they challenge the plants response system to lead to molecular, biochemical and physiological changes in the plant. This thesis is devoted to analyse answers of genotypes of *Solanum tuberosum* L to osmotic stress *in vitro* and their specific response on the proteome and metabolite level.

1.1 *Solanum tuberosum* L., an important crop

Solanum tuberosum L., commonly known as potato, belongs to the family of Solanaceae and the genus Solanum. With 1000 - 2000 species it is the largest genus in the family of Solanaceae (family estimates between 3000 - 4000 specimens) (PBI Solanum Project 2015). The Latin word *tuberosum* describes the fact that the plant is producing tubers at underground stem parts, which are called stolons. In 1753 Carl Linnaeus described the genus and the species *S. tuberosum* for the first time (Fig.1.1).

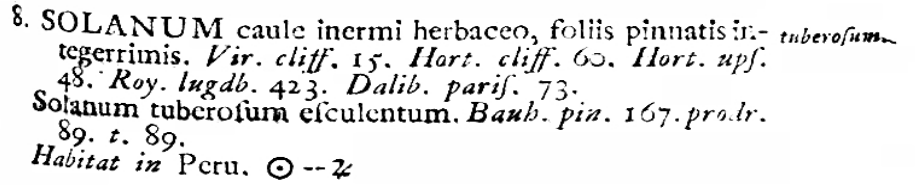
8. SOLANUM caule inermi herbaceo, foliis pinnatis in- *tuberoſum.*
tegerrimis. *Vir. cliff.* 15. *Hort. cliff.* 60. *Hort. upſ.*
48. *Roy. lugdb.* 423. *Dalib. pariſ.* 73.
Solanum tuberoſum eſculentum. *Bauh. pin.* 167. *prodr.*
89. *t.* 89.
Habitat in Peru. ⊙ -- ♃

Figure 1.1: First description of *S. tuberosum* by Linnaeus in Species Plantarum (adjusted after Botanicus.com)

Linnaeus depicted the species as an annual shrub with herbaceous stems and pinnate leaves. The original habitat lays in Peru and nowadays also Chile and Argentina are acknowledged as a native habitat. The genotypes cultured today are mostly of the species *S. tuberosum*. Potatoes were first introduced to the "old world" (Europe) in the 16th century by Spanish conquerors, but it was not until the 17th century that this crop was widely used as a food resource (Salaman and Burton 1985). It quickly became an important crop and due to its inexpensiveness and wide distribution it became the solemnly affordable food for numerous people.

Today potato is one of the most important crops worldwide. In 2013 the potato production was estimated to be around 368 million tonnes grown on ca 19 million ha (FAO 2015). This crop is grown throughout the world in over 125 countries (Mullins et al. 2006). Potato tubers are very nourishing, because they are rich in starch, proteins, antioxidants and vitamins (Burlingame et al. 2009) (Fig. 1.2). Due to this fact they get more and more important in developing countries to meet the demands of poor and undernourished people (Thiele et al. 2010).

Nowadays modern potato cultivars are either categorised as table potatoes or starch/ industrial potatoes. Starch potatoes usually contain a higher amount of starch then the table potatoes and are also grown for

other reasons than human diet. Universal applications for potato starch include the production of adhesives, bioethanol, biogas, biopolymers, but it is also used as a gelling agent in the food industry and in the pharmaceutical industry (Röper 2002).

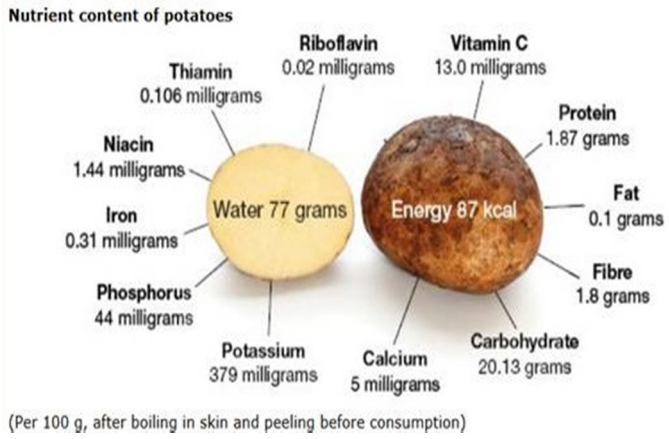

Figure 1.2: Nutrient content of potato tubers after boiling.

Source: FAO 2008.

In August 2015 the BVS (Bundesverband der Deutschen Stärkekartoffelerzeuger e.V.) proposed that the German starch potato production for 2014 was around 2.3 million tonnes of starch potatoes produced on 53288 ha of land (Bundesverband der Deutschen Stärkekartoffelerzeuger 2015). When unfavourable conditions such as drought arise, a reduction in starch and a mobilisation of sugars can be observed (Geigenberger et al. 1997 and references therein). A reduction in starch and an accumulation in reducing sugars (such as glucose and fructose) are unwanted as this reduces the tuber quality. Reducing sugars together with asparagine can produce acrylamide during processing of potatoes (Mottram et al. 2002). As industrial potatoes are produced for their starch content a reduction in starch would lower the total yield and therefore the value.

1.2 Potato, a drought sensitive crop

Because of its shallow root system, *S. tuberosum* is widely known to be drought sensitive (Iwama and Yamaguchi 2006). Especially in its vegetative growing period, in spring and early summer before the onset of tuber formation (Fig 1.3), drought has a major effect on tuber setting. If drought occurs during this early stage fewer tubers per stem are produced, but after the tuber initiation water shortage has few effects on tuber number (de Lis et al. 1964, MacKerron and Jefferies 1986). Haverkort et al. (1990) reported that after stolon formation the impact of drought on tuber yield was very low.

Drought not only affects the tuber number, but also the tuber quality. Tubers might be misshaped due to secondary growth, if drought occurs together with high temperature (Bodlaender et al. 1964). This might lead to knobby, bell shaped or bottlenecked tubers and reduces the market value.

In potato it was also shown that high ozone values increased the vitamin C concentration in tubers and decreased sugars leading to higher value tubers (Piikki et al. 2003). Reducing sugars such as glucose are unwanted in potato tubers, because they tend to go through the Maillard browning reaction with amino acids to produce acrylamide during potato chip production (Mottram et al. 2002).

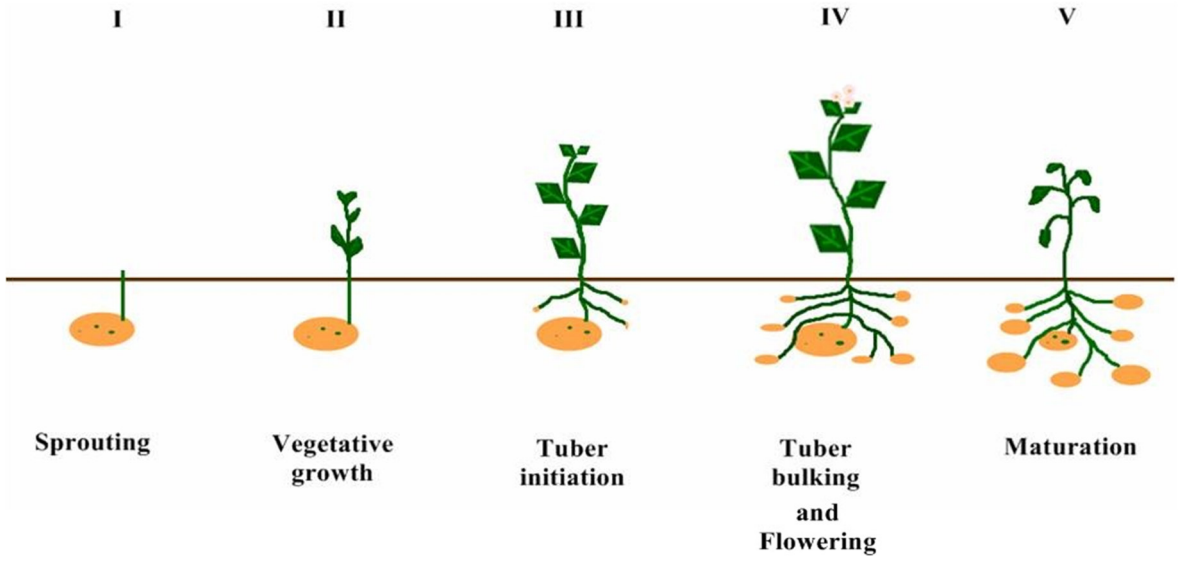

Figure 1.3 : Growth stages of potatoes after Johnson (2008).

Stage I: Sprouting of the foliage; Stage II: Vegetative growth of the foliage; Stage 3: Tuber initialisation as the tip of the stolons swell; Stage 4: tuber bulking at the tips of stolons, flowering; Stage 5: Maturation of the tubers (Adjusted after Johnson 2008).

Drought spells arise on a regular basis and usually fall into the growing period before tuber initiation when the plant is most susceptible. To assure sufficient yield, farmers irrigate the land with artificial systems. To reduce the need for irrigation and the amount of water needed, it is important to breed new varieties, which tolerate water deficiency better without compromising yield. For this purpose, physiological parameters leading to drought tolerance have to be identified.

1.3 Potato improvement through breeding

Modern potato breeding is mainly performed by clone breeding (BDP 2016). This breeding technique comprises crossing potato plants sexually, which have desirable traits such as resistance against pests or higher drought tolerance. A schematic overview is given in Figure 1.4 (adjusted after Becker 2011). After crossing of the parent plants, the F1 generation is further cultivated. Seedlings are then evaluated for the desired trait and termed A clones, because each plantlet derived from these cuttings belongs to a clone, a genetically identical plant. Specific A clones are then further selected to narrow the number of clones (derived from individual plants) and increase the number of individual plants (of one clone). These clones are then further selected and the number of individual clones is further reduced. After the D clone selection, the number of individual clones is small, but each clone has been propagated to several cuttings. These cuttings are usually stored in different places and tested individually for the desired traits

at different places. This happens from the C clone level onwards. After the last selection of clones with desirable traits only a few clones hold the potential to become a new cultivar. The *in vitro* technique is widely used, as it holds the potential to produce disease and virus-free material. The selection of suitable clones and their propagation is performed *in vitro*. After acclimatisation the material can then be cultivated in the greenhouse to produce mini-tubers, which can further be propagated in the field (Becker 2011).

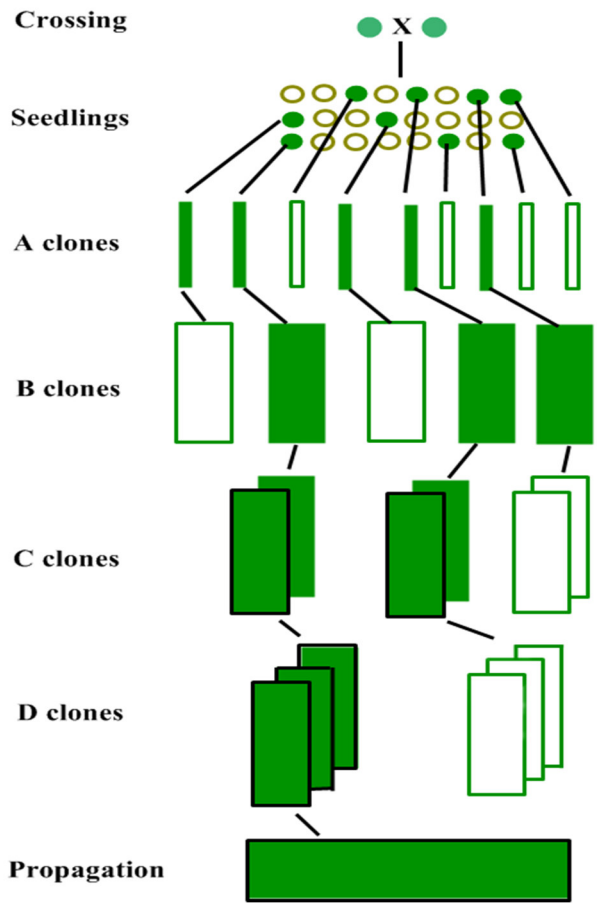

Figure 1.4: Schematic overview of clone breeding strategy of potato after Becker 2011.

Potato plants are crossed. Seedlings with desired traits of the F1 generation are then selected for further use. Further reduction of the clones is performed on A to D clone level. The propagation number of the selected clones is however increased. From the C clone level onwards the clones are stored and tested in different places. After the last selection only the D clones with desired traits are used for the propagation to high number. (Picture was adjusted after Becker 2011).

Main goals in potato breeding generally comprise resistance against pests such as *Phytophthora infestans*, high nutrient use efficiency, increased yield, increased nutritional value and improved tolerance to abiotic stresses such as drought and high temperature. As drought is recognised as one of the major yield reducing abiotic stresses, in the last years increased efforts have focused on distinguishing specific responses upon drought (Deikman et al. 2012). Specific goals for breeding starch potatoes include the increase of starch yield, especially amylopectin and an increase in drought tolerance. Starch is made up of amylopectin (70-80 %) and amylose (20-30 %) in potato tubers. As amylopectin is the part of starch with interesting properties for the industry such as its high water binding capacity, it is separated from amylose enzymatically or chemically for industrial uses. The high demand for amylopectin resulted in a genetically modified potato variety, which was released in 2010 and was called 'Amflora'. This potato does not produce amylose (BASF 2016). Another important breeding aspect of starch potatoes is to increase the drought tolerance. As starch is remobilised during drought and used as sugars, this would decrease the market value of the potato tubers.

Cultivated potatoes are autotetraploid (2n = 4x = 48 chromosomes) and heterozygous (Carputo and Frusciante 2011, Becker 2011). It was proposed that crossing of polyploid plants would lead to reduction of fertility and disorders during meiosis (Becker 2011). This is the reason why part of the breeding strategy is based on diploid level under usage of haploid techniques such as anther culture. A reduction of the ploidy level in potatoes can also be reached through crossing tetraploid clones with selected clones of *S. phureja*, which occasionally generates diploid offspring (Butler et al. 1998). However, as cultivated potatoes in Europe have a limited genetic background as they originated mainly from *Solanum tuberosum* ssp. *tuberosum* (Potato Genome Sequencing Consortium 2011), the around 200 wild relatives of *S. tuberosum* came into focus (Brandeen and Haynes 2011). These wild relatives such as *S. tarijense* and *S. chacoense* might display interesting new features such as improved drought tolerance or pest resistance, but are usually diploid.

1.4 Drought, an abiotic stress for plants

Drought is one of the major yield limiting abiotic stresses worldwide. This abiotic stress occurs on a regular basis and is foretold to get even more severe in the future in terms of frequency and intensity. In the tempered region there will be more rainfall during winter time and less during summer (Haverkort and Verhagen 2008). Together with salt stress and low temperature it comprises the major problem for agriculture because these abiotic stresses prevent plants from using their full genetic potential (Zhu 2002). Different definitions of drought exist. There are definitions for meteorological drought, where precipitation is lower than usual for the time period, hydrological drought, where the water flow and the reservoirs are depleting or agricultural drought, where water supply does not meet the relative demands of the crops planted (Changnon 1987). The word drought will further be used as defined under agricultural drought.

Different soil types can hold different amounts of water depending on their texture and structure. This is termed the field capacity. The lower limit of water capacity is called the permanent wilting point at which plants permanently wilt. This is dependent on the soil type, but is generally defined as the water content at -1.5 MPa. The available water for plant production lies between the field capacity and the permanent wilting point (Dalla Costa and MacKerron 2006).

It is still difficult to accurately predict drought periods and their time span. Because of this problem, droughts are usually only recognized while they last (Wilhite and Glantz 1985). Especially in the fields it is difficult to simulate and analyse the effects of drought on plants, because other factors such as light and temperature influence the plants response to the stress. Combinations of stress might alter the response of the plant, because signalling pathways may interact or counteract with another or share parts of a common pathway (Mittler 2006). Therefore, pot trials with limited soil space and *in vitro* experiments with artificial medium in climate chambers are frequently conducted to evaluate drought stress responses. This allows scientist only to test for a certain stress on the plant in an otherwise stable environment.

1.5 Responses to drought

Because plans are sessile organisms, they developed mechanisms to overcome stress. Drought tolerance is the generic term used to cover a range of mechanisms to withstand drought (Jones et al. 1981). Levitt (1972) depicted stress as a force and a counter force as was described by Newton´s laws for motion. Because plants are living organisms, he proposed two major differences between Newton´s laws for motion and biological stress. First, plants are able to produce barriers between the living matter and the surrounding environment, therefore stress should not be measured in units of force, but in units of energy and second, biological stress always holds the possibility of injury. He therefore defined biological stress as any part of the environment able to induce serious damage upon the plant.

Now two general categories of stress tolerance can be determined. First plants can avoid or adapt to stress. Plants, which avoid or escape stress, would finish their life cycle before stress occurs. Others might have morphologically adapted to the stress. These would be e.g. plants in summer dry lands completing their live cycle before drought arises or plants which have adapted to very arid environments e.g. succulent plants. Through alteration of their morphology or metabolism in a long term perspective, plants can live and grow in unfavourable environments (Araus et al. 2002).

As a second mechanism, plants can in general tolerate a certain stress. This means that the plant will suffer injuries through stress later than other plants if the stress is persistent. Tolerant genotypes can keep growing where others might already be in a stage of growth arrest and therefore produce greater yield than sensitive genotypes. Nowadays it is well known that plants tolerate stress through alteration of gene expression, metabolic and protein adjustments, but it is not yet understood how exactly this is

regulated. These alterations might comprise accumulation of transcription factors, chaperones or osmolytes upon stress.

It was proposed that plants respond differently to mild or severe stress situations. For *A. thaliana* it was shown that during early stress sensing other genes are regulated than in later phases of stress (Cheong et al. 2002). This might indicate that plants sense the stress much earlier than certain responses appear. Plants can also be primed for a stress answers. When plants are primed, they encounter stress at a low level and accumulate certain amplifiers. This leads to a sort of tolerance where the plants can respond to a certain stress faster, because the stress response pathway is accumulating low levels of dormant signal amplifiers, which are activated during the next stress phase (Bruce et al. 2007, for review see Hilker et al. 2015). The priming of cellular responses can also be performed in the laboratory by externally supplying stress as was shown for *A. thaliana* with externally applied BABA (beta-aminobutyric acid) leading to enhanced salt and drought resistance (Jakab et al. 2005).

1.6 Drought sensing and signalling in plants

In the following sections major stress responses will further be introduced, which are thought of to be the main responses leading to acclimatisation to a certain stress.

To accurately respond to water shortage and stress in general, plants have to sense and mediate stress precisely. Every response consumes energy and therefore an adequate reaction must be found to keep the cost/efficiency factor to a minimum without jeopardising survival. Through a complex regulatory network, plants can cope with unfavourable conditions by signal perception and transduction.

The drought stress signal transduction consists of many different parts, such as ionic adjustments and signalling, detoxification responses e.g. for reactive oxygen species (ROS) and growth regulation (Zhu 2002). Environmental stresses lead to changes in transcriptome, metabolome and proteome.

The drought stress and osmotic stress signal perception in plants is not well understood. Most of the knowledge on osmosensors is transferred from the insights gained in yeast and bacteria (Shinozaki and Yamaguchi-Shinozaki 1997). The “two-component system” is a sensor widely distributed across bacteria and is known to act in diverse stress transduction pathways. In yeast a “two –component system” which is comprised from the three gene products Sln1p, Ypd1p, and Ssk1p was identified. Because a *SLN1* homologous of *A. thaliana* namely *ATHK1* (*Arabidopsis thaliana* histidine kinase 1), was able to complement yeast *Sln* mutants it was proposed that *ATHK1* could also function as an osmosensor in plants (Urao et al. 1999). It was shown through Northern blot that the *ATHK1* transcript is highly abundant in roots (Urao et al. 1999). Because an over-expression of *ATHK1* leads to more tolerant plants, it was suggested that sensing of the osmotic stress could be a novel way to improve stress tolerance of the plant instead of genetically altering osmolyte accumulation and transcript levels of protective proteins (Wohlbach et al. 2008).

Signal perception might be the point where specificity of the stress response occurs. However, plant transduction pathways overlap and a reason could be that plants might not be able to distinguish between certain stresses such as drought and cold (Knight and Knight 2001). For example, most abiotic stresses involve the rise of free cytosolic Ca^{2+} levels and involvement of protein kinases and phosphatases (Knight and Knight 2001). It was proposed that the elevated Ca^{2+} level in the cytosol might give rise to a specific stress response, depending on the calcium signature. The calcium signature is comprised of kinetics, magnitude and cellular source of the stimulus-induced cytosolic calcium and it was shown that particular calcium signals specifically regulate certain gene expressions (Hardingham et al. 1997). Specific gene regulation might also be the result of ABA (abscisic acid) accumulation upon osmotic stress, as it was shown that ABA-dependent and ABA-independent signal transduction pathways exist (Shinozaki and Yamaguchi-Shinozaki 2000).

The plant hormone ABA is synthesized *de novo* when plants encounter stressful situations and confers stress tolerance. ABA was shown to be synthesized via the 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme. In *A. thaliana* it was shown that the expression of the AtNCDE3 (*A. thaliana* 9-*cis*-epoxycarotenoid dioxygenase) was induced by drought stress and that this is the major rate limiting step controlling endogenous ABA levels (Iuchi et al. 2001).

Proteins under the influence of hormones are an important part of the stress response in plants. ABA is the major drought responsive hormone and to date two ABA-dependent and two ABA-independent pathways are known for stress responses upon drought, salt and cold stress. An overview is given in Figure 1.5. Transcription factors (TFs) such as MYC/MYB (myelocytomatosis / myeloblastosis) and bZIP (basic Leucine-zipper protein) are regulated via the ABA-dependent pathway. These TFs bind to the MYB recognition sequence (PyAACPyPu), the MYC recognition sequence (CANNTG) or the ABA-responsive element (PyACGTGGC) (Shinozaki and Yamaguchi-Shinozaki 1997). These recognition sequences are part of stress responsive promoter elements in specific genes and binding of the TFs leads to increased stress-responsive gene expression through the recruitment of polymerases (Yamaguchi-Shinozaki and Shinozaki 2006).

Before the accumulation of ABA, it was observed that ERD1 (EARLY RESPONSE TO DEHYDRATION STRESS 1) was accumulating in response to dehydration and salt, suggesting the presence of an ABA-independent pathway (Nakashima et al. 1997). Nowadays it is documented that ABA-independent pathways comprise the transcription factors DREB (Drought responsive element binding) and NAC/ ZF-HD (NAC/ zinc-finger homeodomain). NAC is comprised of three abbreviations namely NAM (no apical meristem), ATAF (*Arabidopsis thaliana* activating factor) and CUC (cup shaped cotyledon) and binds to a CATGTG motif (Tran et al. 2004). DREB binds to the dehydration-responsive element (TACCGACAT) (Yamaguchi-Shinozaki and Shinozaki 1994) and can also be induced via the ABA-dependent pathway but to a lesser extent. These transcription factors are extensively studied (Shinozaki and Yamaguchi-Shinozaki 2000). The downstream genes code for

different proteins, which give rise to tolerance of the plant to the specific stress (Shinozaki and Yamaguchi-Shinozaki 2006).

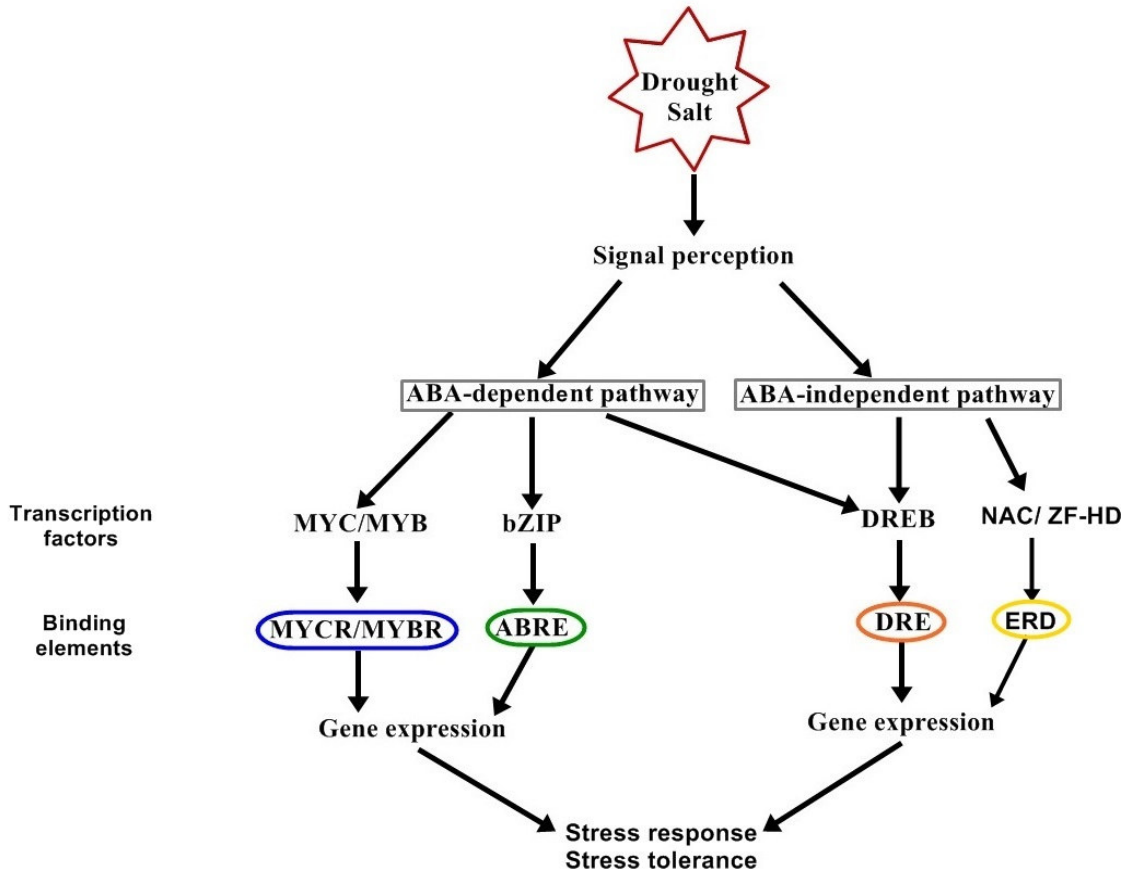

Figure 1.5 Schematic overview over the ABA-dependent and ABA-independent pathways.

Drought stress is sensed via a signal perception. This signal is either mediated via the ABA-dependent or ABA-independent pathway. Through the transcription factors MYC/MYB and bZIP in the ABA-dependant and DREB and NAC/ZF-HD in the ABA-independent pathway stress responsive elements are targeted. The binding of the TFs to their specific target sequence gives rise to altered gene expression leading to stress responses and subsequently to stress tolerance. (**ABA** = abscisic acid; **MYC/MYB** = myelocytomatosis / myeloblastosis; **bZIP** = basic Leucine-zipper protein; **DREB** = drought responsive element; **NAC/ZF-HD** = no apical meristem-, *Arabidopsis thaliana* activating factor-, cup shaped cotyledon/ zinc-finger homeodomain; **ABRE** = ABA-responsive element; **ERD** = EARLY RESPONSE TO DEHYDRATION STRESS).

Adjusted after Saibo et al. (2008), Yamaguchi-Shinozaki and Shinozaki (2006) and Shinozaki and Yamaguchi-Shinozaki (2006).

Gene expression is altered depending on the stress intensity and a reprogramming of the whole plant to reach a new steady-state level (Chaves et al. 2009). It was also demonstrated that plants respond differently to mild and severe drought stress. Responses of plants are affected by the intensity, duration

and by the progression of the stress. These factors regulate if a stress response will take place and if there will be acclimatisation to the occurring stress (Chaves et al. 2009). Mild stress is usually mediated via an altered water balance leading to cell wall-plasma membrane interaction, which is resulting in the activation of receptor-like kinases and is assisted by an increase in ABA. On the other hand, severe or suddenly appearing stresses are associated with membrane destabilisation, which triggers phospholipid signalling (for review see Kacperska 2004). Phospholipid signalling leads to phospholipid peroxidation, an increase in ROS and increased synthesis of injury related hormones especially jasmonates and ethylene (Creelman and Mullet 1997, Kacperska 2004).

An increase in calcium together with an increased phosphorylation status of the cell lead to inactivation of a number of protein targets, changes gene expression and subsequently leads to an appropriate response upon the environmental stress (Munnik and Testerink 2009).

1.7 Growth reduction and stomata function as important stress response mechanisms

A reduction in growth is a response to abiotic stress observed in plants. Growth arrest was proposed to be an important mechanism as it is regulated by numerous factors including hormones. Under stress conditions an arrest in growth through the inhibition of the activity of CDKs (cyclin-dependant protein kinases) blocks the transition from G1 to S phase of the cell cycle, therefore arresting the cell cycle until stress is relieved or the cell has adapted (Ogawa et al. 2011). ABA, a hormone which is known to be up-regulated under osmotic stress conditions, is known to inhibit the transition from the S phase to the G2 phase of the cell cycle by promoting ICK (inhibitor of cyclin-dependant protein kinase), therefore keeping the cell in a transition state (Stals and Inzé 2001). While a growth arrest in plants is a common response to drought, root and shoot growth are not affected equally. After a short period of total growth arrest, it was shown that during drought stress root growth in many plants is promoted compared to the shoot growth in stressed plants (Sharp et al. 1988, Jefferies 1993, Zeid and Shedeed 2006). In *A. thaliana* it was shown that ROS production in roots is necessary for root growth, because ROS stimulates Ca^{2+} influx during root hair formation and root cell elongation (Foreman et al. 2003). A reduction in shoot mass and an increase in root mass is a common feature documented in several species. This is beneficial for the plants as it leads to a reduction in water loss through the canopy while on the other hand more water can be taken up through a larger root system.

Another very important aspect of drought tolerance under *in vivo* conditions consists of the function to open and close stomata (guard cells) of leaves. Under water deficit stomata are closed to avoid water loss. This results in a shortage of carbon dioxide (CO_2), which alters the electron transport and biochemical pathways, leading to a reduction in photosynthesis (Boyer 1976). Stomata movement regulation is associated with ABA, ion channels and the diurnal cycle (Daszkowska-Golec and Szarejko 2013). During drought stress an efflux of K^+ and H_2O over K^+-channels and aquaporins and an influx of Ca^{2+} leads to stomata closure. This is mediated through a depolarisation of the plasma membrane as the H^+-ATPases are inhibited (Daszkowska-Golec and Szarejko 2013).

1.8 Drought and osmotic stress test systems

Test systems such as screening under field, shelter, greenhouse or *in vitro* conditions for drought and osmotic stress tolerance are commonly used to identify mechanisms related to drought tolerance in genotypes. Each screening system has its advantages and drawbacks.

Screening under field conditions

Studying drought stress under field conditions is very labour, time and cost intense especially when the root system is analysed (Iwama and Yamaguchi 2006). In fields genotypes can be tested for drought tolerance under naturally occurring conditions. Because every field is different in terms of soil composition and water availability within the soil, one test side is usually not enough to test genotypes for their performance. Drawbacks of this system are especially those arising from the open environment, because a control of water input is difficult and moreover, light intensity, temperature, ozone and other stresses cannot be excluded from the field trial. Therefore, experiments have to be conducted and analysed over several years to get a good overview over the different factors (Haverkort et al. 1990, Deblonde and Ledent 2001).

Shelter trails

A shelter is a roof which holds off the rain from the experimental plot. This plot can either be on the ground in a field or simulated by pots filled with soil. Analysing the stress performance of different genotypes in shelter trials is becoming more popular (Haverkort et al. 1990, Steyn et al. 1998, Sprenger et al. 2015). Both types of shelter have their advantages and drawbacks. The shelter over a field where the plants are grown in the soil usually display the same disadvantages as plants in field trials comprising other stresses such as wind and pests, but also the labour intense analysis of the root system. Water control can only be gained for the applied water, but if ground water levels rise, the controlled water feeding is useless. Advantages of this system are the space and plant-soil interaction as under field conditions. Performing experiments under a shelter in pots filled with soil is another system which is used to screen plants for their stress performance (Sprenger et al. 2015). The advantages of this system with pots are that the soil type can be analysed and known amounts of fertilisers can be added. The water capacity of these soils is usually known and a weighing of the pot can be performed to reach a certain water regime. A major drawback of this system in pots is the limited space of the pots for rooting. Both systems display drawbacks in different light conditions depending on the roof fabric used, which might not be comparable between experiments.

Greenhouse or climate chambers

Screening plants under greenhouse or climate chamber conditions are probably the most often used screening systems to analyse plant responses upon a certain stress (Nemali and van Iersel 2006, Bowne et al. 2012, Witt et al. 2012). Under greenhouse and climate chamber conditions plants can be screened

under a controlled environment. Water can be applied in a certain amount like in the shelter system with pots but usually without other stresses such as high temperature and wind influencing the results. On the other hand, drawbacks are very limited pot size, cost intensity through climate regulation (heating/ cooling, ...) and light intensity depending on the lights used. Specific drawbacks during greenhouse experiments are that an increase in temperature might occur, especially in summer when no sufficient cooling takes place, while climate chambers have a fully controlled environment, but are usually very narrow in size. Advantages especially when screening for drought tolerance are that soil with a known water holding potential can be used. When water is withheld, the pots can be weighted and a certain amount of water can be filled up e.g. 60 % of the water holding capacity of the soil.

***In vitro* growth**

Breeders in general use the *in vitro* system as a technique to keep important potato plant material disease-free in a controlled environment. *In vitro* grown plants display unique features in their growth. Because sugars are added directly to the medium as a carbon source, the photosystem is less active and plants grow C-mixotrophically. This is necessary because an alternative C source has to be provided, due to low CO_2 concentrations and low light intensity in the vessels which are the reason for a low performance of the photosystem.

Because of high air humidity in the vessels most stomata are constitutively open. This feature of *in vitro* plants was shown not to be the cause of biochemical impairment of inflation and deflation of guard cells (Wardle and Short 1983). Together with a decrease in cuticular waxes this results in poor water loss control and marks a problem for acclimatising *in vitro* plants to *ex vitro* conditions (Grout 1975, Ghashghaie et al. 1992, for review see Hazarika 2006). However, it was shown for potato that transpiration still takes place under *in vitro* conditions and is increasing with the growth time (Tanaka et al. 1992). Therefore, when reducing the osmotic potential in the culture medium to simulate water deficiency a loss of water is still taking place from the shoot.

It was shown by Gopal and Iwama (2007) that a reduction of the osmotic potential of the medium used *in vitro* could best be achieved through the addition of 0.2 M sorbitol or 0.003 M PEG 8000. As the medium supplemented with PEG was viscose, they proposed that sorbitol would be the best choice to screen potatoes for their variability to osmotic stress tolerance.

The *in vitro* technique is a fast and relatively easy technique to maintain plant material in a disease-free environment. Plants which are amenable to the *in vitro* culture like potatoes can be multiplied in a short period of time. However, drawbacks of this technique are the high humidity in the vessels, which alters the morphology of the plant e.g. in terms of stomata function and cuticular waxes. It was also reported that the artificial medium conveys stress on the plant especially during long term maintenance *in vitro* (Cassells 2000). However, the *in vitro* technique and stress application through the addition of sorbitol is a promising approach to unravel specific osmotic stress responses in potato (Gopal and Iwama 2007).

1.9 Strategies in identifying novel responses in potato

Changes upon stress can occur on different levels in the plant. Besides gene expression these include changes in protein abundance and metabolite concentration. Analysing the changes in the proteome and the metabolome are of utmost interest, as they represent the actual physiological and biochemical state of the cell at a given time point.

The separation of the whole proteome by gel-based methods has become the most popular technique for separation and quantification of proteins in biological samples. This usually includes separation by 2D-IEF PAGE (two dimensional isoelectric focusing polyacrylamide gel electrophoresis), analysing the spot volume, picking of differentially abundant proteins and the identification of proteins by mass spectrometry, which were previously separated according to their pI (Isoelectric point) and their MW (molecular weight) on the gels. The 2D-PAGE method was independently introduced by O`Farrell (1975) and by Klose (1975). This way of protein profiling through gel-based methods and mass spectrometry possesses a high resolving power and enables the detection of hundreds to thousands of proteins on a single gel. The use of IEF strips, ampholyte-based buffers, sensitive dyes and precise gel imaging software have lowered the variability between gels to a minimum. The variation between gels can therefore be pin pointed to the loss of proteins during extraction (Magdeldin et al. 2014 and references herein).

Major drawbacks of this method despite the potential protein loss during extraction include that low abundant proteins, very acidic or basic, very small or large, hydrophobic and membrane bound proteins cannot be analysed as the extraction and the separation are not suitable for these proteins (Santoni et al. 1999, 2000). Also high abundant proteins might mask low abundant proteins. The detection limit of proteins through staining varies with the dye used. The detection limit for Coomassie stained gels was proposed to be around 100 ng, where the colloidal Coomassie staining increased the sensitivity to 10 ng protein. Silver staining might increase the detection sensitivity to 1 ng of protein, however it is not compatible with further analysis through mass spectrometry (Magdeldin et al. 2014). Nevertheless, due to the labour intensiveness of this method only low throughput can be achieved and few sample numbers can be processed. During the last years non gel-based techniques have been developed, where proteins are digested before the peptides are separated by LC-MS/MS (liquid chromatography- tandem mass spectrometry). However, even though significant advantages have been made in non gel-based methods (also termed shotgun proteomics) the gel-based methods will remain popular to perform proteomic studies as they allow a rather simple visualisation of the proteome.

Identification of stress responses of potatoes on metabolite level has come into focus over the last years. Nowadays gas chromatography (GC) coupled to mass spectrometry (MS) is a commonly used technique for the identification and quantification of metabolites in a complex compound mixture. GC was established by Martin and James (1952). The metabolome of tissues at a certain time point can be separated by GC/MS (gas chromatograph/ mass spectrometry). With the GC, compounds can be

separated and through the coupling to MS can further be identified based on their fragment pattern. Advantages of this method include the identification of several compounds in a complex mixture and the fast throughput of samples. However, major drawbacks include that the metabolites which should be analysed must be volatile or can readily convert into the volatile phase upon heating, the instability of metabolites, which might lead to a number of by-products and the co-elution of metabolites (Steinhauser and Kopka 2007). The co-elution of metabolites is however only a major problem, if both compounds are identified by the same fragment masses. The loss of metabolites during extraction and derivatisation is another limitation encountered. However, this can be overcome through the addition of internal standards. On the other hand, internal standards have to be chosen carefully so they do not overlay compounds of interest. As compounds are identified by their mass fingerprint of fragments through the search against a metabolite library of standards, it might be difficult to analyse rare compounds or determine metabolites with very similar structures. However, as a big number of metabolites has already been analysed, many compounds can be identified in complex samples (Koek et al. 2011). Metabolite profiling by GC/MS to analyse specific responses in potato is a promising technique, as major metabolites can be compared between different samples.

Together these two techniques of proteomic and metabolite analysis, comprise powerful tools to unravel specific responses in potatoes upon osmotic stress *in vitro*.

1.10 Protein alteration takes place during stress acclimatisation

The term proteome was first defined by Wilkins et al. (1996) as "the total protein complement of a genome". Later it was more specifically defined that the proteom is "the quantitative analysis of proteins present in an organism at a certain time and under certain conditions" (Lottspeich 1999). This definition describes more precisely that a proteome is highly dynamic and results from protein biosynthesis, degradation and modification such as phosphorylation at a given time point. These proteins present at a given time point either in a cell, tissue or whole plant can further be modified and form complexes with other proteins. To depict the actual physiological state of a cell the proteomic approach is the most promising to study biological changes arising from environmental unfavourable conditions.

Alterations in the proteome of stressed plants are of major importance and contribute to acclimatisation to the stress. Detoxification enzymes for instance are a group of proteins which are an important part of the stress response. These proteins scavenge ROS (reactive oxygen species), which are produced as a side product of aerobic metabolism (Apel and Hirt 2004). After the onset of stress several ROS scavenging enzymes such as SOD (superoxide dismutase) or catalase (CAT) are more abundant in numerous plants to reduce the amount of ROS. Under normal conditions ROS can work as signalling molecules and are produced during normal growth. Under stress plants tend to produce more ROS and an active detoxification machinery helps to protect plants from harmful effects including oxidative damage on DNA and membranes. ROS production under osmotic stress is usually a side effect of lower water availability and a decrease in photosynthesis. The excessive excitation energy in the photosystem

II (PSII) together with a decrease in photosynthetic function leads to the accumulation of ROS in the chloroplast (Aranjuelo et al. 2011). Through the Asada-Halliwell cycle a photoreduction of dioxygen to water at PSI takes place with the electrons generated at the PSII (Asada 1999). A reduction in the detoxification rate and an accumulation of high ROS concentrations would result in oxidative stress and subsequent photoinhibition. An increased photorespiration also helps to eradicate excessive energy by the consumption of NADPH and ATP, while at the same time generating CO_2 as an electron acceptor in the chloroplast (Asada 1999).

Other important proteins which are known to be altered in their abundance upon stress are heat shock proteins, which are known to function as molecular chaperons and help in folding, assembly and translocation of proteins under control conditions. However, they can also stabilize proteins and membranes and help in refolding under environmental unfavourable conditions (Wang et al. 2004). Heat shock proteins (HSPs) were first identified to be higher abundant under heat stress conditions. Nevertheless, these proteins are also essential for the maintenance of normal growth and development (Kotak et al. 2007). Five major families of HSPs/chaperones exist. These comprise the HSP70 (DnaK) family, the chaperonins (GroEL and HSP60), the HSP90 family, the HSP100 (Clp) family and the small HSP (sHSP) family. Other proteins might also function as chaperons such as calnexin/ calreticulin which function in the endoplasmatic reticulum (Wang et al. 2004). In cucumber plants it was recently shown, that hydrogen peroxide mediates abscisic acid-induced HSP70 accumulation and confers heat tolerance (Li et al. 2014). This result displays the importance of the interplay between protective protein accumulation and ROS and ABA (abscisic acid) signalling.

1.11 Metabolite alteration as part of the stress response

Not only protein composition but also metabolite composition within the plant is altered when unfavourable conditions arise. This has been demonstrated in grapevine (Hochberg et al. 2013), in tomato for secondary metabolites (Sánchez-Rodríguez et al. 2011) and also for potato tubers (Roessner et al. 2000).

A general focus lies on osmotic active compounds such as special amino acids (e.g proline, GABA (γ-Aminobutyric acid)) and sugars (e.g. sucrose) (Hare et al. 1998). These compounds are nontoxic for the plant even in higher concentrations and are of major importance for plants to actively protect hydration shells of proteins and reach a positive water balance. Proline for example is the most studied osmolyte. It has generally been recognised as important for plants to reach homeostasis within cells under unfavourable conditions, but proline concentrations could not be correlated to yield or to tolerance in potatoes (Schafleiter et al. 2007). Under field conditions GABA is another very important non-protein incorporated amino acid which is a common component of stress responses. After stress application GABA accumulates rapidly in plant tissue (Roberts 2007). Beyond its role as an N source it was proposed to act as a signalling molecule for N uptake in *Brassica napus* (Beuve et al. 2004). These osmolytes were shown to accumulate during environmental unfavourable conditions. However, the only

report of a positive correlation of osmolyte accumulation and stress tolerance were identified under very severe drought responses, where survival of the plants is usually the only benefit and yield still decreases (for review see Serraj and Sinclair 2002).

Many secondary metabolites are derived from the phenylpropanoid pathway leading from the shikimate pathway over phenylalanine converted by PAL (phenylalanine ammonium-lyase). This pathway leads to many metabolites functioning in the interaction between the plant and its environment. Secondary metabolites such as lignins and anthocyanes derived from this pathway are also reported to be increased after stress application in plants. Plant secondary metabolites are of economic value as they are a unique source for flavours, pharmaceuticals and biochemicals for the industry (Ramakrishna and Ravishankar 2011). Also for the production of secondary metabolites the intensity and frequency of the stress is important. Sperdouli and Moustakas (2012) displayed that under mild drought stress *A. thaliana* does not increase its anthocyanin concentration, but under moderate drought a significant increase was detected, which is sufficient enough to shield against excessive light energy. In *A. thaliana* it was demonstrated that anthocyanins also accumulated to a low extent when plants were grown in the presence of sucrose (Mita et al. 1997). Anthocyanins may shield against excessive light energy. This might also be important for plants grown c-mixotrophically *in vitro*. A low photosynthetic rate was reported for roses (Capellades et al. 1991), strawberry plants (Hdider and Desjardins 1994) and for potatoes (Kubota and Kozai 1992) *in vitro*.

1.12 Collaboration project PROKAR

The studies in this thesis were conducted as part of the collaboration project „Characterization of the proteom under nitrogen and water deficiency as a basis for the development through breeding of nitrogen efficient and drought tolerant starch potatoes" (Charakterisierung des Proteoms unter Stickstoff- und Wassermangelstress als Grundlage für die züchterische Entwicklung stickstoffeffizienter und trockentoleranter Stärkekartoffeln (PROKAR)), which was financed by the Federal Agency for Renewable Resources (Fachagentur Nachwachsende Rohstoffe, FNR) e.V. which is supported by the German Federal Ministry for Food and Agriculture [Grant number Leibniz Universität Hannover 22023511].

The aim of this project was to identify proteins which are differently abundant after stress application, which could be used as biological markers for the early nitrogen and water deficiency responses. Therefore, *in vitro* experiments and pot trials were conducted and the proteom was analysed.

Sub-project 1: Investigation of the proteom of starch potatoes under nitrogen deficiency (JKI Groß Lüsewitz)

This sub-project was processed in Groß Lüsewitz/ Sanitz at the Julius Kühn-Institute, Federal Research Centre for Cultivated Plants (JKI) – Institute for Resistance Research and Stress Tolerance; coordinator Dr. Annegret Schum and processed by Philipp Meise. For this sub-project an *in vitro* screening of

different potato genotypes as well as pot trials under a rain out shelter were performed to analyse the response of the starch potatoes upon nitrogen deficiency. Drought responses and combined stress (nitrogen deficiency and drought) were also analysed under the rain out shelter in two years. From the *in vitro* nitrogen deficiency experiments samples were taken and the proteom of the divergent reacting genotypes was dissected.

Sub-project 2: Investigation of the phosphoproteome and the plasmamembrane (IPK Gatersleben)

The sub-project 2 was performed in Gatersleben and Groß Lüsewitz/ Sanitz at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) – Department Physiology and Cell Biology – working group Applied Biochemistry and at the IPK Potato collections at Groß Lüsewitz (GLKS), coordinators Dr. Hans-Peter Mock and Dr. Klaus Dehmer, processed by Anna Jozefowicz and Katja Löschner, respectively.

In Gatersleben (IPK) the roots of *in vitro* grown potato plants under nitrogen deficiency stress were analysed. Here the quick changes of the root proteom and especially the phosphoproteome were in focus.

At the Potato Genebank in Groß Lüsewitz (GLKS) wild species were established in *in vitro* culture and some of these were analysed for their response upon osmotic stress and nitrogen deficiency *in vitro*.

Sub-project 3: Investigation of the proteome under water deficiency (LUH Hannover)

Water deficiency was simulated *in vitro* at the Leibniz Universität Hannover in the Institute for Horticultural Production Systems, coordinator Prof. Dr. Traud Winkelmann, processed by Christin Bündig.

This thesis was written based on the results of this part of the collaboration project. Potato plants were subjected to osmotic stress *in vitro* and the morphological responses, changes in the proteome and the metabolome were analysed. The time point of 11 days which was used for proteomic and metabolite analysis was decided based on previous work done at the JKI. This time point marked the point in time at which first responses to nitrogen deficiency were noted. Roots were not analysed, as they were extensively studied at the IPK in Gatersleben.

1.13 Thesis objectives

The demand for more tolerant genotypes to drought will increase in the coming years as a result of the changing climate and the associated prolonged drought periods. Today new genotypes can be produced through breeding and virus-free plants are generated and stored *in vitro* by breeders throughout the world. Screening a set of potatoes *in vitro* for osmotic stress tolerance comparing genotypes and analysing the proteome and metabolome after stress application might give insight into specific responses of potatoes. As osmotic stress is part of drought stress, selection of specific proteins

differentially abundant after stress application might also comprise good candidates to further develop protein markers. These could help to identify genotypes faster which are more tolerant to the applied stress.

The three main objectives of this thesis were:

1. To investigate if a screening *in vitro* would be an efficient way to identify genotypes which are more tolerant to the applied stress by
    - Applying osmotic stress *in vitro* to 18 genotypes and 2 wild species and evaluating the morphological reactions
    - Determine sensitive and tolerant genotypes in comparison to the set analysed
    - Comparison of osmotic stress responses to drought response data obtained under a rain out shelter in pots
2. Dissecting the proteome of *S. tuberosum* L. after stress application and identifying differentially abundant proteins after osmotic stress *in vitro*, which might function as a biochemical marker through
    - Proteome separation by 2D-IEF/SDS-PAGE and sub sequential detection of differential abundant protein spots
    - Identification of proteins in these spots with mass spectrometry techniques and categorisation into functional groups and pathways
3. Analysing major metabolites to gain an insight into the metabolism of *S. tuberosum* L. to understand responses in a sensitive and a tolerant genotype by
    - Stressing plants *in vitro* and analysing two genotypes (one sensitive and one tolerant) for a specific set of metabolites (targeted approach)
    - Comparing the genotypes to predict if the altered metabolites might contribute to the stress tolerance or susceptibility
    - Identifying if sorbitol, the osmoticum of choice, is taken up by the plants *in vitro*

The manuscripts presented in the next chapter are organised according to the three main objectives of this thesis. A general introduction and an overall discussion with concluding remarks complete the picture and can be found at the beginning and the end of this thesis, respectively.

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2 Chapter 2 - Manuscripts

Manuscript 1

2.1 Variability in osmotic stress tolerance of starch potato genotypes (*S. tuberosum* L.) as revealed by an *in vitro* screening: Role of proline, osmotic adjustment and drought response in pot trials

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Type of authorship:	First author
Type of article:	Research article
Contribution to the publication:	Planned and performed *in vitro* experiments, analysed data, prepared all figures and wrote the manuscript
Co-authors contribution	T. H. Vu: Performed proline experiments, manuscript correction P. Meise: Performed pot trial experiment under a rain out shelter in Groß Lüsewitz, manuscript correction S. Seddig: Planned and performed pot trial experiments, manuscript correction A. Schum: Wrote parts of the material and methods (pot trial), planned the pot trial experiment, manuscript correction T. Winkelmann: Planned *in vitro* experiment, correction of the manuscript
Status	Under Revision; Journal of Agronomy and Crop Science

Variability in osmotic stress tolerance of starch potato genotypes (*S. tuberosum* L.) as revealed by an *in vitro* screening: Role of proline, osmotic adjustment and drought response in pot trials

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Abstract

S. tuberosum (potato) as a drought sensitive plant is also one of the most promising plants to meet the demands for food and starch of a growing population. Distinguishing genotypes into tolerant and susceptible is therefore of utmost interest. We subjected eighteen potato genotypes and two wild species, *S. tarijense* and *S. chacoense*, to osmotic stress applied *in vitro* by addition of 0.2 M sorbitol to a solid medium. Here we report that a ratio of root:shoot dry mass (DM) together with the SSI (stress susceptibility index, equivalent to drought susceptibility index by Fischer and Maurer 1978) of shoot DM were found to be relevant parameters to characterize genotypes *in vitro* for their osmotic stress tolerance. Drought stress data from pot trials in a rain out shelter (2013 and 2015) correlated poorly with the data obtained in *in vitro* experiments. However, the most tolerant and most sensitive genotypes *in vitro* were also categorised to be more tolerant or sensitive than the average to drought stress *in vivo*. Both, under *in vitro* and *in vivo* conditions, proline displayed an increase under osmotic stress conditions in nearly all potatoes tested, but no direct correlations were found to stress tolerance. However, a genotype classified as tolerant displayed earlier proline accumulation. Proline is thought of as one factor for plants to withstand stressful conditions, but cannot be used to distinguish potato genotypes for their stress tolerance to osmotic stress *in vitro*. Analysis of the osmotic potential of *in vitro* and *in vivo* stressed plants displayed a general increase compared to the control.

Key words: *in vitro*, osmotic stress, *Solanum tuberosum*.

Introduction

Drought is one of the major yield limiting stresses worldwide. This abiotic stress occurs on a regular basis and is foretold to get even more severe in terms of frequency and intensity. In the temperate regions, there will be more rainfall during winter time and less during summer (Haverkort and Verhagen 2008). The extreme drought in 2003 (Schär and Jendritzky 2004) and recently in2015, are examples of extreme weather conditions in Central Europe. Due to this prognosis, it is of utmost importance to select and breed genotypes with increased stress tolerance.

Solanum tuberosum (*S. tuberosum*) is sensitive to drought stress due to its shallow root system (Iwama and Yamaguchi 2006). Especially in early growth stages water shortage is critical. When drought occurs in spring and early summer, quantity and quality of the tubers might be drastically reduced (MacKerron and Jefferies 1986; de Lis et al. 1964). Because a selection for drought tolerant genotypes in the field is time consuming, cost intensive and difficult to reproduce, it was proposed that screening genotypes for their response to stress conditions *in vitro* might be an alternative to efficiently check material for its reaction to osmotic stress (Gopal and Iwama 2007). Previous studies by Gopal and Iwama (2007) and Gopal et al. (2008) simulated drought stress condition *in vitro* by the addition of PEG (polyethylene glycol) or sorbitol to solid MS medium. This resulted in the identification of 0.2 M sorbitol to give the best response of the potato genotypes to distinguish more tolerant ones among the test set. Osmotic stress was chosen as it is one of the factors contributing to drought stress.

Because plants are sessile organisms, they developed mechanisms to overcome stress. These include morphological, physiological and biochemical adaptations (Bartels and Sunkar 2005). Osmotic adjustment is one of the major mechanisms that lead to tolerance against drought and salt stress. Compatible solutes or osmolytes do not interfere with normal biochemical reactions, but act as osmoprotective compounds during stress (Yoshiba et al. 1997). The amino acid proline is one of the major compatible solutes, which is known to be up-regulated under stress conditions (Delauney and Verma 1993). It plays a major role in the stabilization of membranes and prevents degradation of proteins and enzymes under stress (Farooq et al. 2009). It was shown that proline accumulates under numerous stresses in various species (reviews by Hare and Cress 1997; Verbruggen and Hermans 2008)

such as potato (Schafleitner et al. 2007), wild relatives of *Solanum lycopersicum* (Tal et al. 1979) and *Arabidopsis thaliana* (Chiang and Dandekar 1995).

The goal of our study was to analyse morphological and specific physiological responses of potatoes *in vitro* subjected to osmotic stress by determining biomass production, proline accumulation, osmotic adjustment and to compare their response to drought stress in pot trials in terms of yield. For this purpose sixteen starch potatoes as well as two table potato cultivars and two wild species were subjected to osmotic stress through the addition of 0.2 M sorbitol *in vitro*. The osmotic potential and proline levels of specific genotypes were analysed, to determine an optimal parameter for screening potatoes under osmotic stress conditions. These genotypes were selected based on their performance upon osmotic stress with help of the calculated SSI. In addition, potato genotypes were subjected to drought stress in pot trials and among the determined parameters the stability of tuber yield was used for the comparison to results of the *in vitro* screening.

Material and methods

Plant material

Eighteen genotypes of *S. tuberosum* and two wild species (*S. chacoense* and *S. tarijense*) were used in this study (Supplementary Table 1). *In vitro* plant material of the eighteen genotypes was kindly provided by the respective breeders and plants of the wild species were retrieved from Dr. Klaus Dehmer Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) (Groß Lüsewitz Potato Collections, Germany). Wild species were chosen because of their natural habitat with recurrent drought periods. The plant material was grown *in vitro* on solid MS medium (3 % sucrose; 7.5 g plant agar/l (Duchefa Biochemie B.V., Haarlem, The Netherlands)) (Murashige and Skoog 1962) at constant 18 °C in a 16 h light/ 8 h dark cycle with a light intensity of approx. 35 µmol m^{-2} s^{-1}. Nodal cuttings were used for propagation.

In vitro screening

Plants were grown on MS medium for 2 weeks before they were transferred to the experimental media. Only shoot tips were transferred to solid MS medium without and with 0.2 M sorbitol as control and treatment, respectively. After three weeks of culture ten vessels per genotype and treatment with five

shoots each were analysed for each repetition of the experiment. For this purpose, plants were separated into roots and shoots, weighed (fresh mass, FM) and dried until constant weight in an oven at constant 70 °C for 48 hours (dry mass, DM). Data was collected from two independent experiments.

Proline analysis

Proline was extracted and analyzed according to Bates et al. (1973). Shoot tips were microwaved for 5 sec. before they were dried. To determine the free proline content, 25 mg of shoot dry mass obtained after three weeks of culture on control or treatment (0.2 M sorbitol) medium was homogenized to a fine powder with stainless steel beads (7 mm diameter) in a ball mill MM 400 (Retsch, VERDER Group, Netherlands). After the addition of 1.8 ml 3% (w/v) sulfosalicylic acid the mixture was incubated for 30 min on ice and centrifuged at 16162xg (Sigma 1-14, Sartorius AG - Göttingen, Germany) for 15 min. 150 µl of the supernatant was then diluted with 90 µl glacial acetic acid (90%) and 90 µl 25% (w/v) ninhydrin reagent (838 ml/l 60 % acetic acid, 162 ml/l 85 % phosphoric acid and 25 g/l ninhydrin). Afterwards the mixture was placed in a boiling water bath at 100 °C for 45 min. The reaction was cooled down by placing the test tubes in cold water for 2-3 min. The samples were mixed with 1.5 ml toluene. 200 µl of the supernatant were transferred into a well of a microtiter plate, and absorption was measured at a wavelength of 520 nm. For each sample, five biological and three technical replications were measured. Proline analysis was repeated two times with samples from the two individual repetitions of the experiment. Toluene was used as a blank.

For the time course experiment shoot tip samples were collected at different time points (0 d, 1 d, 3 d, 7 d, 11 d and 21 d) after the transfer of the shoot tips to the experimental media. The procedure for proline analysis was as described above. Five samples per genotype and treatment were measured at each time point.

Measurement of osmotic potential

Osmotic potential was calculated based on osmotic measurements with a vapor pressure osmometer (Vapro® Vapor Pressure Osmometer Modell 5520, Wescor ELI Tech Group, St. Ingbert, Germany). Plant material was ground with stainless steel beads (7.00 mm diameter) in a ball mill MM 400 (Retsch,

VERDER Group, Netherlands) for 2 * 1 min at 23 Hz. Samples were briefly centrifuged and 10 µl of supernatant was pipetted on a sample disk (SS-033, Wescor ELI Tech Group, St. Ingbert, Germany). Measurements were performed against osmolality standards (Opti-Mole^{TM} 1000mmol/kg, 290 mmol/kg and 100 mmol/kg; Wescor ELI Tech Group, St. Ingbert, Germany). Three biological with two technical replications each were measured.

Osmolality was converted to osmotic potential

$$Osmotic\ potential\ [MPa] = (-c * R * T) * 10^{-6}$$

c = concentration measured [mmol/kg]

R = gas constant in J/mol*K

T = temperature in Kelvin

Pot trial under rain out shelter

In vitro propagated plants were acclimatized and planted 2013 and 2015, respectively, into a peat substrate with 5 % sand in 5 l pots and cultivated in a rain out shelter starting at the beginning of May. The pots were weighed daily and the individual loss of water was balanced to a substrate moisture capacity of 60 %. For the exertion of drought stress, watering was completely stopped for 14 days during the tuber induction phase, which was empirically determined. During this period the moisture capacity of the substrate rapidly dropped down to 15 % resulting in severe wilting of plants. Samples of the youngest fully developed leaves were taken at day 5 and 13 of the drought period for the determination of proline contents and osmolality. Plants were cultivated until natural senescence. After removal of the haulm, tubers rested for two weeks in the substrate before yields were determined. The experiment was conducted in a block design with a total of 8 replicates per genotype and variant.

Data analysis and statistics

Data analysis was performed with R software (R version 3.1.2; The R Project for Statistical Computing, Lyon, France (www.r-project.org)). Significant differences were calculated based on multiple comparisons of means (Hothorn et al. 2008) or Tukey´s test. Compact letter display (CLD) was calculated based on log transformed data. The stress susceptibility index (SSI) (Fischer and Maurer

1978) was calculated for the identification of more sensitive or more tolerant genotypes based on the following formula:

$$SSI = \frac{\left(1 - \frac{Ps}{Pc}\right)}{\left(1 - \frac{mean\ Ps}{mean\ Pc}\right)}$$

Ps = parameter determined under stress conditions (0.2 M sorbitol)

Pc = parameter determined under control conditions

meanPs = mean of all genotypes under stress conditions

meanPc = mean of all genotypes under control conditions

Results

Sorbitol affects the growth of potato plants *in vitro*

To identify osmotic stress conditions which best distinguish different genotypes a test with various concentrations of sorbitol (0.1 M, 0.2 M, 0.3 M and 0.4 M) and PEG 8000 (0 %, 4.8 % and 9.6 %) was performed (data not shown). The results were similar to those obtained before by Gopal and Iwama (2007) and indicated that with 0.2 M sorbitol the best differentiation of the genotypes among a test set could be achieved. In Figure 1 the effect of sorbitol on selected potato cultivars after 21 days of culture on control and treatment medium containing 0.2 M sorbitol is displayed. Growth reduction was observed for all genotypes after stressing the potato plantlets with 0.2 M sorbitol, however to a different degree.

A reduction in shoot and root fresh and dry mass was observed for each genotype (Table 1 and 2). Well growing genotypes like cv. Euroflora (29.0 % reduction DM shoot) or the wild species *S. chacoense* (30.1 % reduction DM shoot) were not necessarily among those with the highest reduction in dry mass. The highest reduction after treatment with 0.2 M sorbitol was found in cv. Ramses, cv. Eurobravo, and the wild species *S. tarijense* with a reduction in shoot DM of 43.8 %, 41.2 %, and 40.6 %, respectively. The least reduction in shoot DM was observed in cv. Maxi and cv. Verdi with 12.2 % and 21.1 %, respectively (Table 1).

The highest reduction in root FM was observed in cv. Eurobravo, cv. Ramses and cv. Tomba (83.3 %, 71.9 % and 66.7 %, respectively) (Table 2). The least was recorded in cv. Maxi, cv. Eurostarch and cv. Euroflora with 25.0 %, 26.5 % and 28.9 %, respectively. The decrease in root DM was the highest in

cv. Eurobravo, cv. Ramses and *S. tarijense* (68.4 %, 55.6 % and 50.0 %, respectively). Even a slight increase in root DM was observed for cv. Maxi (+7.7 %) and for cv. Eurostarch (+6.7 %), whereas the cv. Euroflora did not display any loss or gain (+/- 0 %).

To group the genotypes into more sensitive and more tolerant genotypes, the SSI of the shoot and root DM was calculated (Figure 2). According to the SSI for both parameters, cv. Maxi was found to be the most tolerant genotype among the test set whereas cv. Eurobravo was among the most sensitive. Due to the weak performance after acclimatisation of cv. Ramses, which could not be subjected to drought in the pot experiment, cv. Eurobravo was chosen for further analysis. Generally, the SSI based on the root DM allowed a clearer differentiation of the genotypes in response to osmotic stress than that calculated for the shoot DM (Figure 2a and b).

A shift from shoot to root growth was observed in more tolerant genotypes to osmotic stress *in vitro* (Figure 3). In Figure 3, genotypes are ordered according to their mean calculated SSI (Mean of SSIs of the shoot DM and root DM), where the genotypes on the left are more tolerant to the applied stress and the ones on the right are more sensitive within the set tested. It is interesting to note that the two most tolerant cv. Maxi and cv. Eurostarch show a significant shift towards root growth upon stress conditions, whereas the two more sensitive cv. Eurobravo and cv. Ramses are characterized by a reduced root:shoot ratio based on the DM (Figure 3).

In order to assess if the data of osmotic stress tolerance determined under *in vitro* conditions of the selected genotypes would be comparable to the data of drought responses in a pot trial under a rain out shelter, the SSI for the total DM *in vitro* was compared to the SSI for yield under drought (data not shown). Data for tuber yield was obtained at the experimental station of the JKI in Groß Lüsewitz in 2013 and 2015. Not only between the repetitions of the *in vitro* experiment, but also in pot experiments a variation between the repetitions was observed. The overall view of the mean SSIs determined for DM production under osmotic stress *in vitro* did not coincide with the mean SSIs of the tuber yield calculated from the drought stress pot experiment. However, the most tolerant (cv. Maxi) and the most sensitive (cv. Eurobravo) genotypes were identified consistently under both conditions.

Osmotic potential in starch potatoes subjected to osmotic stress

To elucidate if the effect of stress tolerance is only due to the differential adjustment of osmotic potential in different genotypes, shoot tips of the sensitive cv. Eurobravo, the tolerant cv. Maxi and the two intermediate cv. Eurostarch and cv. Tomba were analysed for the osmotic potential of the cell sap. Control plants from the most tolerant cv. Maxi did not differ in their osmotic potential compared to the other genotypes (Figure 4). Cultivation on medium with 0.2 M sorbitol resulted in a significant increase of the osmotic potential in each of the tested genotypes but was most prominent in cv. Maxi. There was no effect on the osmotic potential of the experimental medium under control or treatment conditions, when plants were grown on these media (Supplementary data Table 2). In the pot experiments the osmotic potential of the leaves after 5 days and 13 days of drought also changed significantly (Figure 5).

Tolerance is not (only) an effect of proline accumulation after 21 d

Shoot tips were analysed for their proline content after 21 days on medium with and without 0.2 M sorbitol. It was observed, that control plants of several genotypes as cv. Kiebitz and cv. Tomba contained comparatively high levels of proline (Figure 6). These accumulated around 30.0 µmol proline/g DM in case or cv. Kiebitz and around 22.5 µmol proline/ g DM in cv. Tomba. Treatment with 0.2 M sorbitol resulted in a substantial and significant increase in proline concentration in all genotypes with the exception of *S. chacoense* (Figure 6). In all other genotypes proline increased at least 2-fold after stress application. The highest increase in proline concentration, as compared to the respective controls, was found in cv. Kolibri, cv. Topas and cv. Lambada with an up to 7-fold increase. Because all genotypes and one wild species displayed a similar response, it was not possible to distinguish the genotypes for their tolerance to osmotic stress. Therefore, a correlation analysis between further parameters was performed. Neither the increase in proline, nor proline in control plants nor proline concentration in treated plants could be correlated to the SSI for shoot DM (Table 3).

As we did not find any correlation between proline levels and the determined SSIs of the shoot DM, the dynamic change of proline over time was analysed in the tolerant cv. Maxi and the sensitive cv. Eurobravo (Figure 7). In both genotypes the proline concentration in plants grown on control medium increased until day 7, dropped to the initial levels at day 11 and increased slightly again until day 21.

Stressed plants of cv. Maxi displayed a steady and rapid increase in proline concentration until at the end of the experiment a total of around 100 µmol proline/g DM was reached. In contrast, the increase in proline concentration in plants stressed with 0.2 M sorbitol was distinctly slower in the more sensitive cv. Eurobravo until after 21 days a total of around 65 µmol/ g DM was reached. In both genotypes significant differences in proline contents of plantlets grown on media with and without 0.2 M sorbitol were determined starting with day 11.

The proline concentration in leaves of drought stressed plants under the rain out shelter in the pot experiment was analysed after 5 and 13 days (Figure 8). For both analysed genotypes the control plants had around 1 µmol proline/g DM at day 5 and day 13. The drought stressed plants of cv. Eurobravo displayed 17 µmol proline/ g DM after 5 days and around 130 µmol proline/ g DM after 13 days. The tolerant cv. Maxi showed a concentration of 25 µmol proline/ g DM at day 5 and around 140 µmol proline/ g DM at day 13. At the time points analysed both genotypes displayed similar proline concentrations.

Discussion

Growth reduction after the addition of sorbitol to *in vitro* plants was reported before for sweet potato (Jarret and Gawel 1991), African violet (Sawwan et al. 2000), potatoes preselected for root morphology (Gopal and Iwama 2007) and for olive trees (Brito et al. 2002). A growth inhibition is a common response to drought and osmotic stress in plants (Hsiao and Acevedo 1974, Skirycz and Inzé 2010). Gopal and Iwama (2007) analysed three genotypes, which were selected before based upon their root mass production under field conditions. They found, that the overall effect of water deficit on *in vitro* plant growth was similar to that under field conditions. We report here the feasibility of identifying the most sensitive and tolerant genotype to osmotic stress *in vitro* for a larger test set ofstarch and table potatoes, which were not pre-selected for root characteristics.

A common response of plants to drought and osmotic stress is a shift towards higher root growth to improve water uptake and survival *ex vitro* (Lloret et al. 1999, Gedroc et al. 1996, Jefferies 1995). This effect was also observed in our investigation in genotypes that were more tolerant to osmotic stress *in vitro* (Figure 3). It can be assumed that also under *in vitro* conditions a shift towards root growth is

beneficial for the plants and displays a genotype specific tolerance mechanism. A higher root to shoot ratio was also observed under control and drought stress conditions for grafted plants on a tolerant root stock by Jefferies (1993). Higher root DM compared to shoot DM was also observed for alfalfa by Zeid and Shedeed (2006), who stressed their plants with PEG 4000 with subsequent application of putrescine and by Sharp et al. (1988) for maize after stress application with different regimes of $CaCl_2$ in vermiculite.

Variability between the two repetitions of the *in vitro* experiment (Supplementary data Table 3) was most likely due to variation in growth which is often observed in tissue cultures. The *in vitro* plants of the genotypes ranked in the middle of the SSI scale were very similar in growth morphology and strength under drought stress conditions. Therefore, minor differences might have a big impact on the SSI ranking. Variation in the ranking order between years was also found for the SSIs determined for tuber yields in pot experiments (data not shown). Under greenhouse conditions variation was also present between different years of potato screening experiments (Lahlou et al. 2003).

It was reported before by Lahlou and Ledent (2005) that among different parameters, tuber yield was significantly, but weakly, correlated with root dry mass in the field. A highly significant correlation was found between the tuber fresh weight of potatoes grown in a high salinity field and the root fresh weight of *in vitro* plantlets under salt stress (Morpurgo 1991). Higher root DM was stated to postpone leaf senescence under field conditions and therefore to prolong the tuber bulking phase (Iwama et al. 1982). However, root DM was very low after a three week culture under osmotic stress *in vitro*, and therefore might not be a suitable parameter on its own for determining stress tolerance. Therefore, we compared the SSI of the total plant DM produced *in vitro* to the SSI of the tuber yield in pot trials. This comparison was chosen, because plants in pot experiments were allowed to grow to maturity, therefore FM or DM values of the foliage were not available to directly compare the parameters. Considerable variation was noted between time-independent repetitions in both experimental systems. However, the most tolerant genotype identified among the test set under *in vitro* conditions was also among the higher yielding genotypes after drought stress in pots. Difficulties in correlating *in vitro* plant performance to tuber yield in pots might be due to differences in plant physiology as plants e.g. grow C-mixotrophically and display

low transpiration rates under *in vitro* conditions. Other differences to plants grown *in vivo* include low to no responsiveness of stomata and a low amount of cuticular wax deposition. As these comprise major factors contributing to the drought stress response under natural conditions, a correlation to *in vitro* osmotic stress responses might be difficult. In addition, in field or pot trials drought stress is a slowly increasing process, whereas we subjected potato shoot tips directly to an osmotic shock. The shoots had to regenerate roots and to adjust to the osmotic stress at the same time. In contrast, in field grown potatoes drought usually occurs after sprouting and subsequent rooting. These facts most likely explain, that no overall correlation was found between the SSI ranking of *in vitro* plant DM and the SSI ranking of tuber yields. Further studies will be conducted to improve the *in vitro* test system with a gradually increasing osmotic stress. In this study responses of juvenile *in vitro* plantlets to osmotic stress were compared with tuber yields of plants after their natural senescence. It is possible that other parameters, which characterize the biological tolerance to drought stress, are more suited for a correlation rather than the agronomic tolerance.

However, for the identification of the most tolerant and most sensitive genotypes to osmotic stress among a set of potatoes *in vitro*, the consideration of the SSI together with the ratio of root to shoot DM appears to be a practicable approach. Therefore, it is concluded, that *in vitro* screening of osmotic stress responses will allow a check for the variability of the tolerance level of the genotypes within a test set, but cannot be considered as a sole test system to replace field trials and screen for drought tolerance.

The osmotic potential of the medium (Supplementary data Table 2, liquid medium) was comparable with values obtained by Gopal and Iwama (2007) and Albiski et al. (2012), if the contribution of the agar was subtracted. The effect of agar on the osmotic potential of MS medium was analysed before (Gopal et al. 2008). The osmotic potential of the shoot tips in control plants were found to be relative similar among the genotypes (Figure 4). Also the genotypes in the pot trial displayed similar responses after drought (Figure 5). However, osmotic potential of the shoot tips of the plants *in vitro* did show a significant increase when cultivated on medium with of 0.2 M sorbitol, but did not vary over time in the different genotypes (Figure 4). Only the most tolerant cv. Maxi showed a higher osmotic potential during the first 11 days of culture compared to the others tested. A similar response was also observed in respect

to the proline accumulation which was considerably faster in cv. Maxi during the first 11 days of culture compared to the sensitive genotype 2 (Figure 7). Our data suggests that the more tolerant genotype accumulates proline faster and has a higher overall osmotic potential in shoot tips under osmotic stress. This might lead to a faster acclimatisation and a subsequently increased growth. The osmotic potential of leaves from drought stressed plants in pots also varied between control and stressed plants (Figure 5). Within the set of 17 potato cultivars in pot trials ranging in size under control conditions between 50.0 and 77.4 cm at the time point of first sampling, cv. Eurobravo and cv. Maxi were highly comparable in vegetative development with a height of 53.1 and 54.1 cm, respectively. The osmotic potential displays an increase after 5 and 13 days of drought in the stressed plants and after 13 days the tolerant genotype cv. Maxi displays a slightly higher osmotic potential than the sensitive cv. Eurobravo. If this is a common feature and holds true of more tolerant genotypes in general has to be analysed further.

The accumulation of osmolytes is a common feature of many plant species to withstand drought. Proline as one of the major compatible solutes was analysed in shoot tips of selected genotypes and the wild species. Kavi Kishor et al. (1995) showed that transgenic tobacco plants overexpressing P5CS (Δ^1-pyrroline-5-carboxylate synthetase) have an increased proline production which was shown to be related to drought and salt stress tolerance. Due to the medium which contains sugar and agar, a notable proline concentration was already detected in the control plants (Figure 6). Both substances increase the osmotic potential as Gopal et al. (2008) showed in case of agar and Michel (1972) in case of sucrose. Likewise, gene expression studies showed that plants suffer stress from *in vitro* conditions (Desjardins et al. 2009). Badr et al. (2015) demonstrated that *in vitro* plants accumulated high levels of proline, which decreased rapidly after acclimatisation in the greenhouse. They explained the effect with stress responses under *in vitro* conditions due to the artificial culture medium containing sucrose. This might also account for stress reactions of control plants on medium without sorbitol and might differ between genotypes. On the other hand, *in vitro* grown potatoes also need proline for root and shoot elongation under control conditions (Mattioli et al. 2009) and therefore require a basic amount. The increase until day 7under control conditions might be a stress reaction to the wounding by cutting the nodal segments and subsequent transfer to fresh medium (Figure 6). From day 7 onwards all plants displayed rooting after which an increase in proline would display the particular response to the osmotic stress. An increase

over time was also observed in the pot experiment with a highly significant increase after 13 days of drought stress (Figure 8). The more tolerant genotype also displayed a slightly faster increase in proline concentration over time compared to the sensitive cv. Eurobravo as was the case *in vitro*. In order to find out if this is a general feature in more tolerant potato genotypes further tests should be performed with genotypes known to be tolerant and sensitive.

Our findings are in agreement with Teixeira and Pereira (2006) who demonstrated that proline accumulated under greenhouse conditions significantly after drought and salinity stress in all organs of potato. The cv. Désirée displayed an increase of around 1.6- to 2-fold in different organs (Teixeira and Pereira 2006), whereas the genotype Marfuna accumulated around 4-times more proline under drought stress in the greenhouse (Farhad et al. 2011). Plants of the clone Sullu, which is thought to be more tolerant to drought stress, increased in proline concentration around 4-fold after drought in field trials (Evers et al. 2010). Schafleitner et al. (2007) indicated that proline accumulation in potato can be considered as a sign of plant stress, but should not be used as a tool for prediction of drought stress mechanisms. In our own pot and *in vitro* experiments accumulation of proline under drought stress differed significantly between genotypes but could not be linked directly to tolerance. The comparison of the proline concentration at day 21 of the time course experiment (Figure 7) and the initial assay (Figure 6) revealed considerable differences in the absolute values. Moreover, it cannot be excluded that proline would further accumulate during prolonged culture time. Therefore, proline accumulation should rather be regarded as a general plant response to osmotic stress, but not as a biochemical marker which allows the discrimination between tolerant and sensitive genotypes.

The two wild species *S. tarijense* and *S. chacoense* were introduced to the test set because these were promising candidates for stress tolerance based upon their natural habitat. Because the genotypes displayed very similar morphological reactions when subjected to osmotic stress by 0.2 M sorbitol, it was assumed that the addition of promising candidates for stress tolerance would allow an equalisation of the SSI middle range for the test set. The wild species *S. chacoense* ranged in the middle ranks for the SSIs and did not display a significant increase in proline in contrast to all other genotypes tested

(Figure 6). *S. tarijense* showed a stress response similar to those of other genotypes and was among the three most sensitive for the SSI of the shoot DM (Figure 3a).

In conclusion, neither early proline accumulation nor the increase of the osmotic potential in shoot tips could be correlated directly to osmotic stress tolerance in a set of 18 potato genotypes under *in vitro* conditions. Nevertheless, it is noticeable that the most tolerant genotype cv. Maxi was outstanding in both features. As the osmolality was only investigated in four selected genotypes, the analysis of further genotypes should be performed in order to test if higher osmotic potentials are characteristic for more tolerant genotypes and could therefore be used as a selection criterion in potato. After treatment with 0.2 M sorbitol, all genotypes decreased in FM and DM production. Most notably, a shift towards root development was observed under osmotic stress and the root to shoot DM ratio significantly increased in the more tolerant genotypes. The computed SSI (Fischer and Maurer 1978) for the DM of the plants together with the ratio between DM of the root and the shoot are considered suitable parameters to rank genotypes within a test set according to their tolerance. A comparison between the SSI for tuber yield in pots and the SSI for total plant DM displayed, that no close correlation was noticeable for these parameters. Differences in the ranking order between years and experiments arise from the sometimes small differences between genotypes with, however, a huge impact on the SSI. In addition, a considerable variation between repetitions was observed in pot trials and *in vitro* experiments. Further repeats of the experiments would help to address the variability of the data and to gain a better insight. However, in this study the most sensitive and the most tolerant genotype tested *in vitro* were also identified in the same response categories under rain-out-shelter conditions. In conclusion, a pre-screening of a test set for its variation of osmotic stress tolerance between the genotypes can be performed under *in vitro* conditions. However, it cannot replace field screenings of drought stress response. Until now the identification of an outstanding and reproducible screening system for the parameter drought tolerance in potato remains difficult.

Acknowledgements: The study was conducted with funding provided by the Federal Ministry of Food and Agriculture (BMEL) through the Agency of Renewable Resources (FNR), funding no.: 22023511 (LUH) and 22023311 (JKI). The authors would like to thank Dr Klaus Dehmer for *in vitro* material of *S. chacoense* and *S. tarijense*.

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Figures and Tables

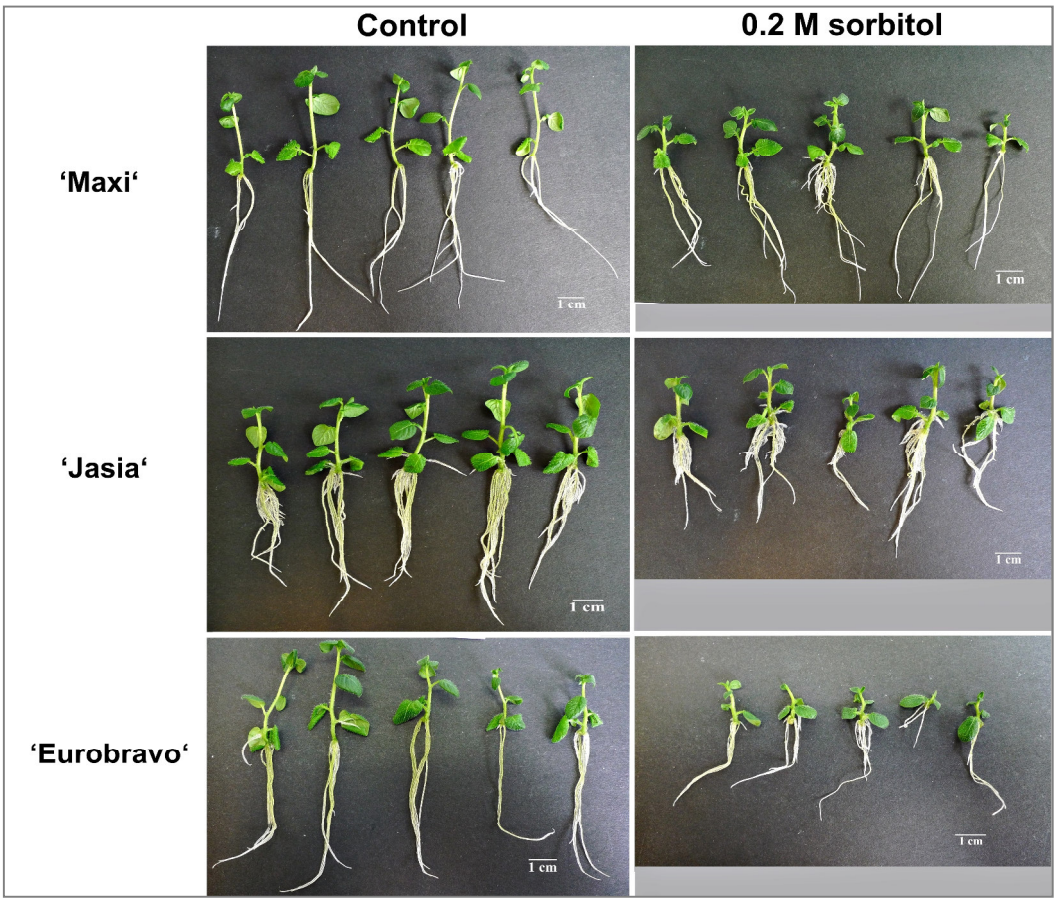

Fig.1 Plants of three contrasting genotypes after 21 days of culture on control medium or medium supplemented with 0.2 M sorbitol *in vitro*. Bars indicate 1 cm. Cv. Maxi = tolerant; cv. Jasia = middle range; cv. Eurobravo = sensitive.

Table 1 Fresh and dry mass of shoots after three weeks of culture on control medium and medium with 0.2 M sorbitol *in vitro*. n=20; CLD = Compact letter display; different letters between genotypes indicate significant differences on a global significance level of 5 %, CLD was calculated based on log transformed data. % red. = % reduction after treatment with 0.2 M sorbitol compared to control.

Genotype	FM shoot Control [mg]	Stdw +/-	CLD	FM shoot 0.2 M sorbitol [mg]	Stdw +/-	CLD	% red.	DM shoot Control [mg]	Stdw +/-	CLD	DM shoot 0.2 M sorbitol [mg]	Stdw +/-	CLD	% red.
cv. Eurobravo	0.370	0.082	fghi	0.131	0.030	fh	64.6	0.034	0.007	cdf	0.020	0.004	cfg	41.2
cv. Euroflora	0.756	0.106	a	0.320	0.055	a	57.7	0.062	0.008	ab	0.044	0.008	ab	29.0
cv. Euronova	0.531	0.105	bcde	0.187	0.039	bcde	64.8	0.039	0.011	cdef	0.024	0.005	cdefg	38.5
cv. Euroresa	0.333	0.086	fg	0.146	0.040	bfgh	56.2	0.028	0.007	cg	0.019	0.005	cfh	32.1
cv. Eurostarch	0.491	0.121	bcdh	0.220	0.073	cdij	55.2	0.038	0.007	cdef	0.028	0.009	deij	26.3
cv. Heidrun	0.492	0.075	bcd	0.229	0.043	dij	53.5	0.042	0.006	deh	0.031	0.005	dij	26.2
cv. Jasia	0.547	0.126	bde	0.259	0.044	aij	52.7	0.048	0.010	aeh	0.034	0.006	dij	29.2
cv. Kiebitz	0.425	0.057	bcfhi	0.189	0.025	cde	55.5	0.038	0.005	def	0.027	0.003	deij	28.9
cv. Kolibri	0.419	0.045	bcfhi	0.176	0.024	bcde	58.0	0.035	0.004	cdef	0.025	0.004	defg	28.6
cv. Kormoran	0.454	0.050	bcdhi	0.209	0.036	cdeij	54.0	0.037	0.003	cdef	0.027	0.005	deij	27.0
cv. Lambada	0.584	0.120	ade	0.262	0.053	aij	55.1	0.039	0.006	def	0.030	0.006	dij	23.1
cv. Maxi	0.519	0.145	bcd	0.277	0.070	ai	46.6	0.041	0.010	defh	0.036	0.008	aij	12.2
cv. Priamos	0.680	0.128	ae	0.263	0.043	ai	61.3	0.054	0.009	ah	0.036	0.006	ai	33.3
cv. Ramses	0.361	0.039	fghi	0.131	0.017	fgh	63.7	0.032	0.003	cdf	0.018	0.002	ch	43.8
cv. Sibu	0.408	0.100	cfhi	0.179	0.054	bcdeg	56.1	0.036	0.008	cdef	0.024	0.006	cdefg	33.3
cv. Tomba	0.363	0.099	fgi	0.161	0.047	befg	55.6	0.030	0.007	cfg	0.022	0.008	cefg	26.7
cv. Topas	0.288	0.080	g	0.111	0.030	h	61.5	0.023	0.005	g	0.014	0.003	h	39.1
cv. Verdi	0.412	0.077	bcfhi	0.197	0.039	cdij	52.2	0.033	0.006	cdf	0.026	0.006	degj	21.2
S. chacoense	1.287	0.218	j	0.511	0.111	k	60.3	0.083	0.013	b	0.058	0.013	b	30.1
S. tarijense	0.439	0.108	bcfhi	0.163	0.035	bcefg	62.9	0.032	0.008	cdfg	0.019	0.004	cfgh	40.6

Table 2 Fresh and dry mass of roots after three weeks of culture on control medium and medium with 0.2 M sorbitol *in vitro*. n=20; CLD = Compact letter display; different letters between genotypes indicate significant differences on a global significance level of 5 %, CLD was calculated based on log transformed data. % red. = % reduction after treatment with 0.2 M sorbitol compared to control.

Genotype	FM root Control [mg]	Stdw +/-	CLD	FM root 0.2 M Sorbitol [mg]	Stdw +/-	CLD	% red.	DM root Control [mg]	Stdw +/-	CLD	DM root 0.2 M Sorbitol [mg]	Stdw +/-	CLD	% red.
cv. Eurobravo	0.264	0.062	ach	0.044	0.024	ach	83.3	0.019	0.003	abef	0.006	0.003	fg	68.4
cv. Euroflora	0.315	0.060	ab	0.224	0.044	ab	28.9	0.023	0.004	abc	0.023	0.004	ab	0.0
cv. Euronova	0.196	0.037	cdef	0.111	0.025	cdef	43.4	0.015	0.003	defg	0.012	0.003	cd	20.0
cv. Euroresa	0.146	0.045	dg	0.059	0.024	dg	59.6	0.011	0.003	d	0.006	0.002	efg	45.5
cv. Eurostarch	0.212	0.044	cefh	0.156	0.044	cefh	26.4	0.015	0.003	defg	0.016	0.004	chi	-6.7
cv. Heidrun	0.297	0.047	abh	0.152	0.032	abh	48.8	0.021	0.003	abe	0.016	0.003	chi	23.8
cv. Jasia	0.469	0.121	i	0.237	0.048	i	49.5	0.033	0.007	c	0.024	0.004	a	27.3
cv. Kiebitz	0.263	0.043	ach	0.103	0.014	sch	60.8	0.019	0.003	abe	0.011	0.002	cd	42.1
cv. Kolibri	0.318	0.058	abi	0.196	0.032	abi	38.4	0.022	0.003	abc	0.021	0.003	abh	4.5
cv. Kormoran	0.293	0.040	abh	0.155	0.043	abh	47.1	0.020	0.005	abef	0.017	0.005	bchi	15.0
cv. Lambada	0.353	0.098	abi	0.178	0.060	abi	49.6	0.025	0.005	abc	0.018	0.005	abhi	28.0
cv. Maxi	0.176	0.026	defg	0.132	0.021	defg	25.0	0.013	0.002	dfg	0.014	0.002	ci	-7.7
cv. Priamos	0.407	0.074	bi	0.167	0.044	bi	59.0	0.027	0.005	bc	0.017	0.004	abhi	37.0
cv. Ramses	0.256	0.070	aceh	0.072	0.017	aceh	71.9	0.018	0.004	aefg	0.008	0.002	dej	55.6
cv. Sibu	0.195	0.048	cdef	0.085	0.030	cdef	56.4	0.015	0.003	defg	0.009	0.004	defj	40.0
cv. Tomba	0.156	0.055	dfg	0.052	0.028	dfg	66.7	0.011	0.003	d	0.006	0.003	g	45.5
cv. Topas	0.176	0.058	dfg	0.082	0.034	dfg	53.4	0.012	0.003	dg	0.008	0.003	efj	33.3
cv. Verdi	0.130	0.025	g	0.080	0.021	g	38.5	0.012	0.002	d	0.009	0.002	dj	25.0
S. chacoense	0.299	0.050	abh	0.134	0.036	abh	55.2	0.023	0.003	abc	0.016	0.004	chi	30.4
S. tarijense	0.061	0.015	j	0.029	0.009	j	52.5	0.006	0.001	h	0.003	0.001	k	50.0

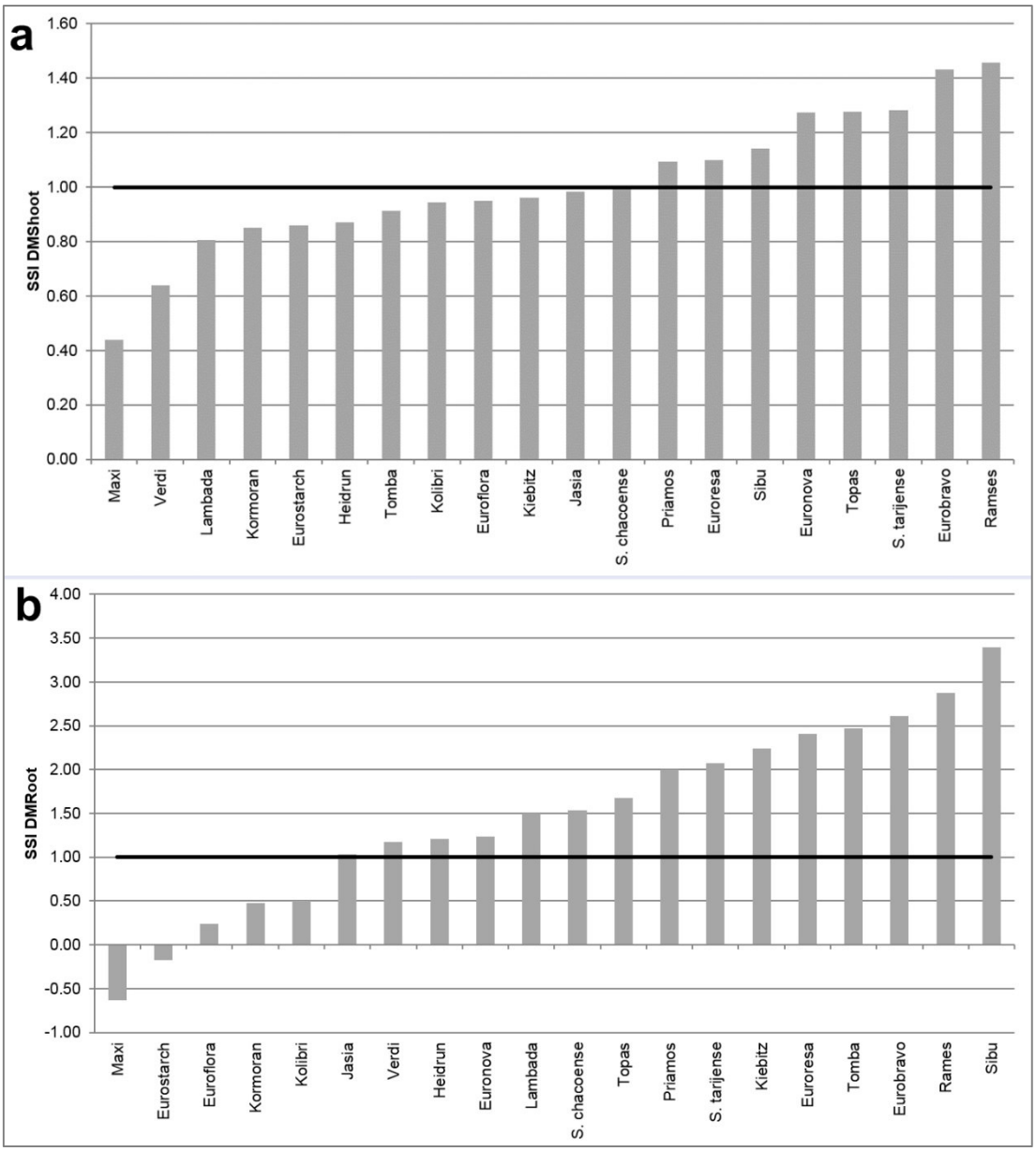

Fig.2 Stress susceptibility index (SSI) based on the shoot dry mass or the root dry mass after 21 days as an average of two repetitions of the *in vitro* experiment. a SSI for shoot dry mass after 21 days of culture, **b** SSI for root dry mass after 21 days of culture. Black line indicates 1. Genotypes with an SSI above 1 are more sensitive to the stress applied and below 1 more tolerant compared to the test set.

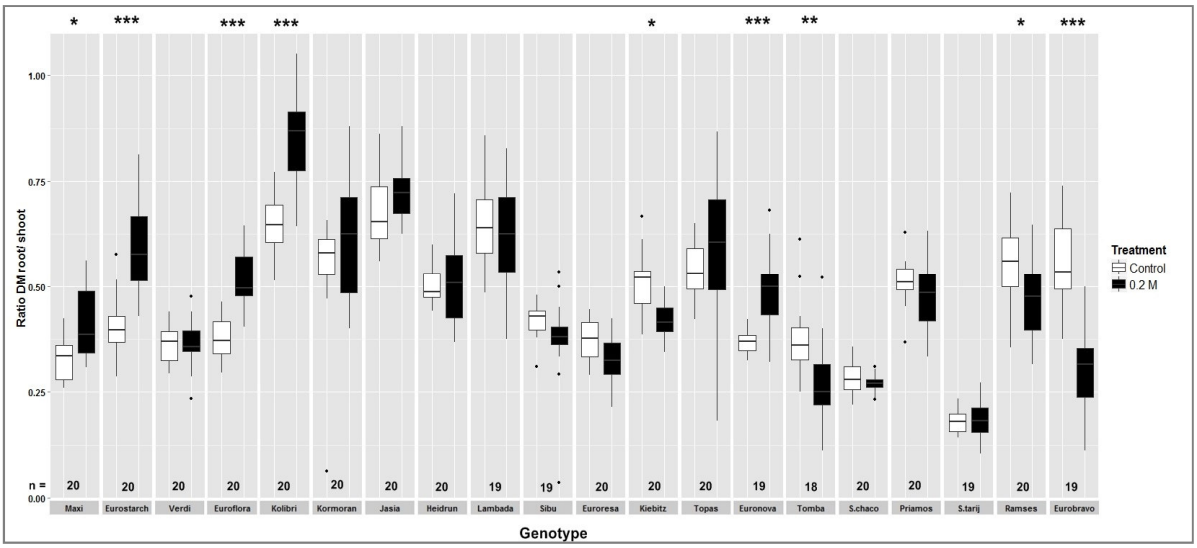

Fig. 3 Ratio of root to shoot dry mass of plants after 21 days of culture on control medium or medium with 0.2 M sorbitol *in vitro*. S.chaco = *S. chacoense*, S.tari = *S. tarijense.* *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Multiple comparison of means, after Hothorn et al. (2008). Genotypes were ordered according their SSI over four parameters. Most tolerant are on the left side, the most sensitive on the right.

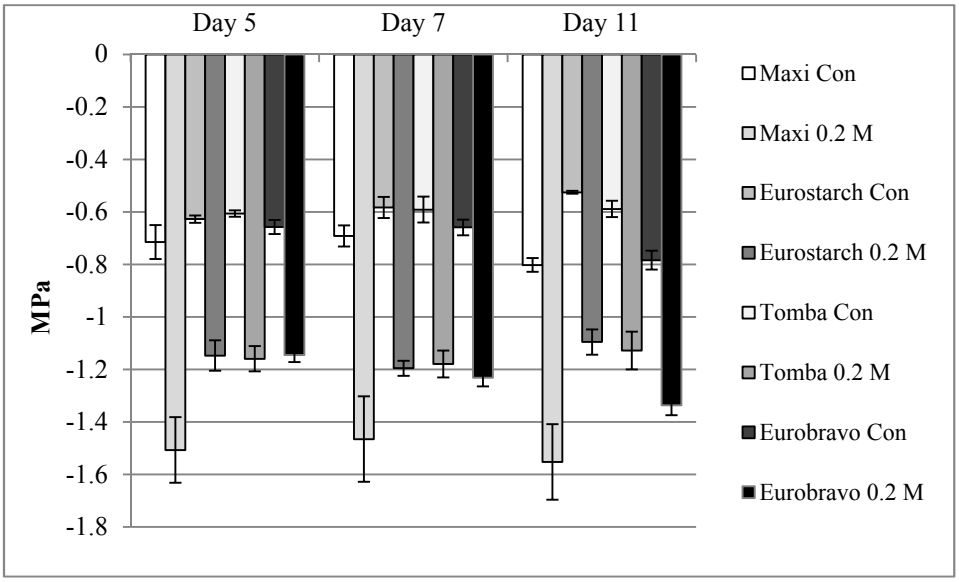

Fig.4 Osmotic potential of shoot tips after 5, 7 and 11 days on liquid control medium or medium with 0.2 M sorbitol *in vitro*. All genotypes display highly significant differences at each day between control and plants stressed with 0.2 M sorbitol ($p<0.001$; ***). n = 6 from two repetitions.

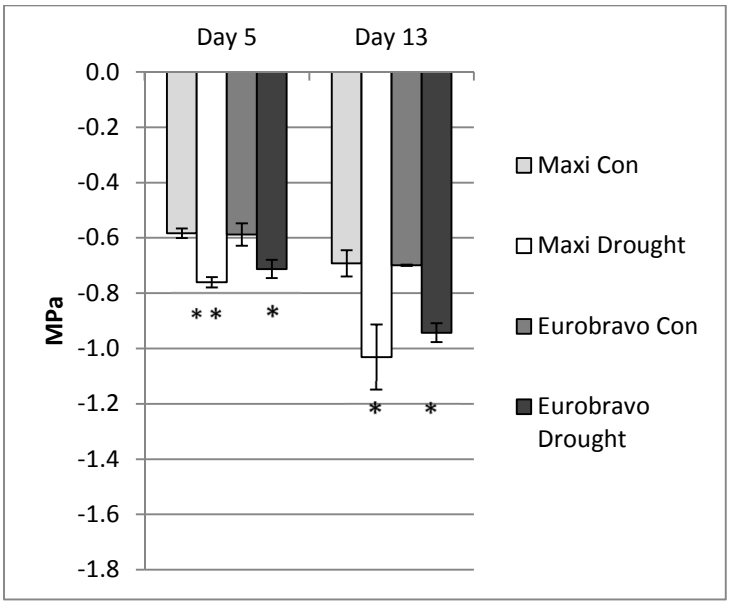

Fig.5 Osmotic potential of leaves after 5 and 13 days of drought under a rain out shelter in pots. Two mixed samples were analyzed using two plants each from which the pieces of the two youngest fully developed leaves were pooled. Significant difference were calculated with Tukey´s test, (** = $p<0.01$; * = $p< 0.05$).

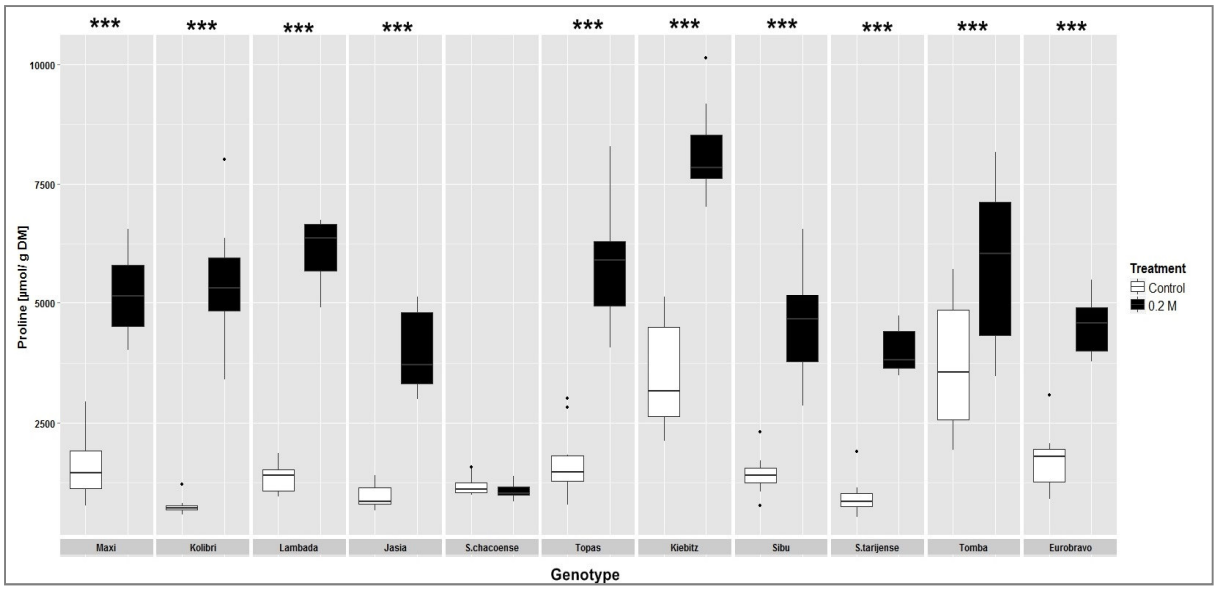

Fig. 6 Proline concentrations of shoot tips after 21 days on control medium or medium with 0.2 M sorbitol *in vitro*. n = 10, cv. Jasia n = 9. *** = p <0.001. Multiple comparison of means, after Hothorn et al. (2008).

Table 3 Correlation of proline concentration in shoots of control plants, treated plants or the ratio of both against the calculated SSI for the shoot dry mass (DM) *in vitro*.

	R^2	p	Pearson-R
Proline concentration in control vs SSI DM shoot	0.0125	0.74	-0.11
Proline concentration after 0.2 M sorbitol vs SSI DM shoot	0.033	0.59	-0.18
Proline.ratio (0.2 M sorbitol/ control) vs SSI DM shoot	0.0033	0.87	-0.057

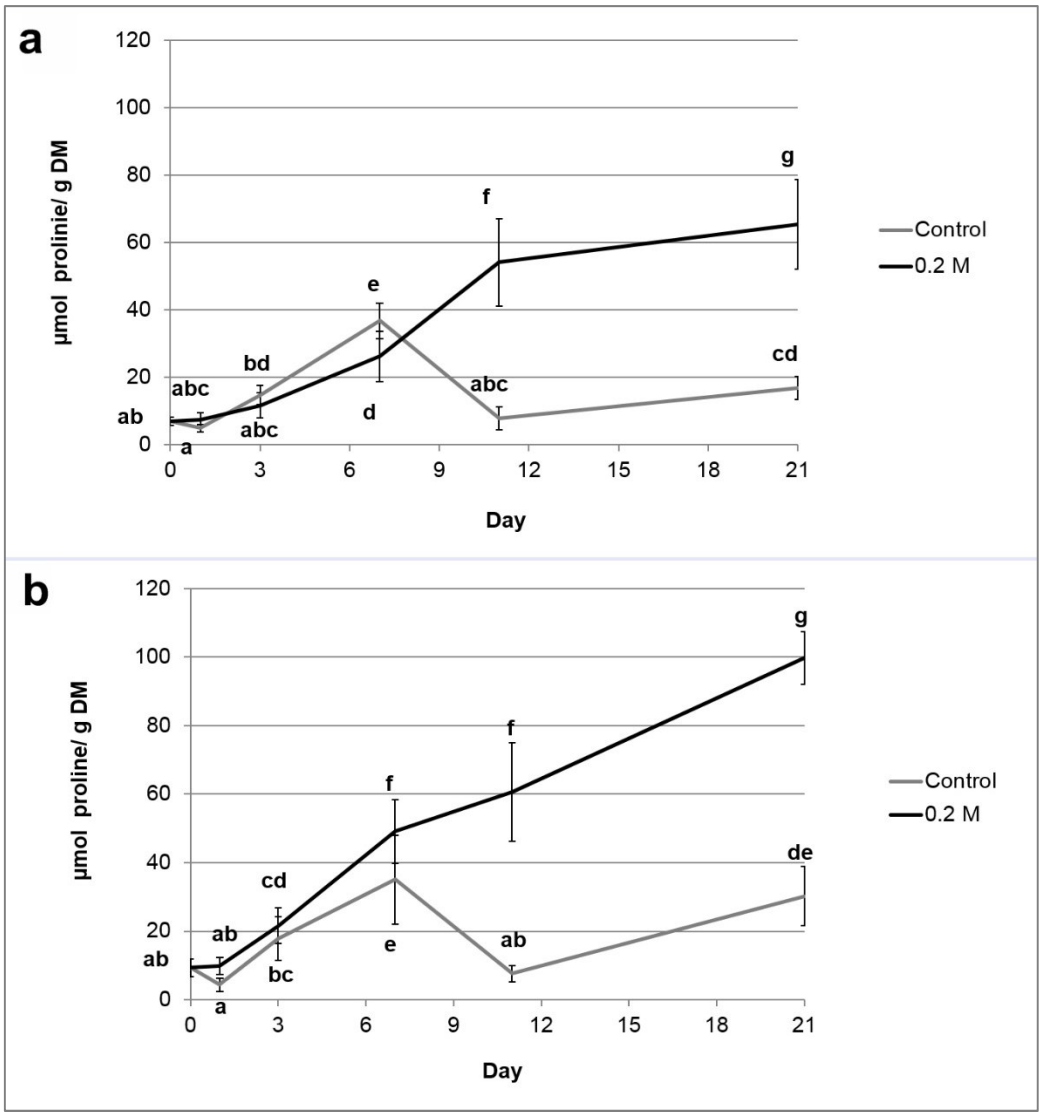

Fig.7 Changes of proline concentration in shoot tips of two contrasting genotypes *in vitro* over time. a cv. Eurobravo, more sensitive to the applied stress, **b** cv. Maxi, tolerant to the applied stress. Different letters show significant differences based on Tukey's test at $p \leq 0.05$, mean ±standard deviations, n=10 vessels with five explants each.

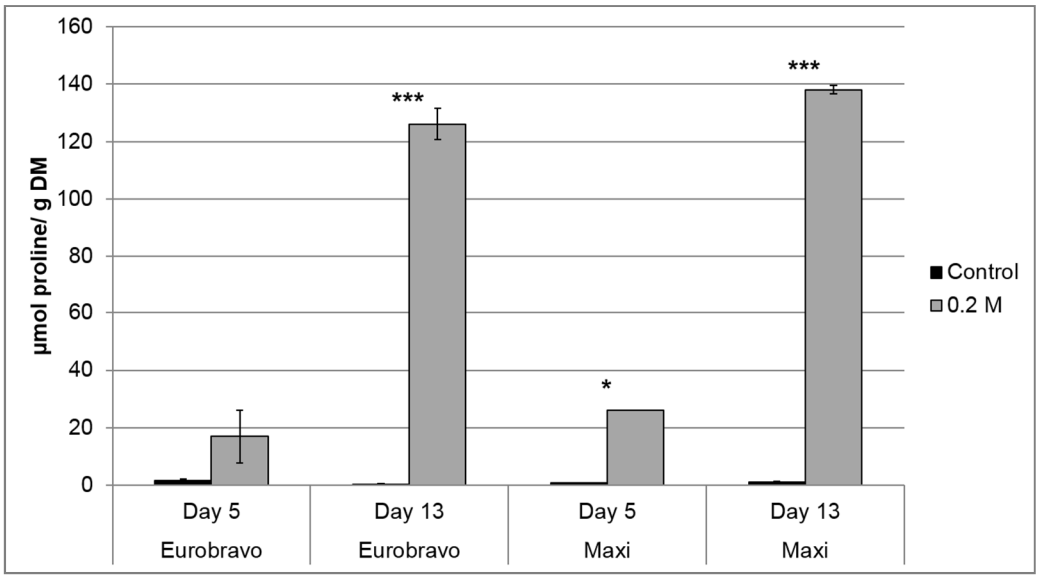

Fig.8 Proline concentration in young leaves after 5 and 13 days of drought stress in the pot experiment under rain out shelter conditions. 2 mix samples were analyzed using 2 plants each from which the two youngest fully developed leaves were pooled. Significant differences between control and drought were calculated. *** = $p<0.001$; * = $p<0.05$.

Supplementary data

Supplementary Table 1: Overview over the potato genotypes tested in this study. Information as described online.

Genotype	year of registration	Maturation time	Tuber yield	Starch yield	Varity	Breeder
cv. Eurobravo	2006	mid late- late	high	high	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Euroflora		late	high	medium	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Euronova		mid late	very high	high	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Euroresa	2010	very early	very high	high	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Eurostarch	2005	mid late	high	high	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Heidrun	1987	late	high		Starch	Saatzucht Fritz Lange; Bad Schwartau
cv. Jasia		mid late	high	very high	Starch	Dr. K. H. Niehoff, Bütow
cv. Kiebitz	2009	mid early-early		medium	Starch	NORIKA.Kartoffelzucht GmbH, Sanitz
cv. Kolibri	1998	early	medium	high	Starch	NORIKA.Kartoffelzucht GmbH, Sanitz
cv. Kormoran	2003	mid late-late	high	high	Starch	NORIKA.Kartoffelzucht GmbH, Sanitz
cv. Lambada	2003	mid early	high		Table	NORIKA.Kartoffelzucht GmbH, Sanitz
cv. Maxi	2004	mid late-late	high	high to very high	Starch	Bayerische Pflanzenzuchtgesellschaft eG &Co KG, Freising
cv. Priamos	2002	mid early	high	high	Starch	SaKa Pflanzenzucht GmbH & Co KG; Hamburg
cv. Ramses	2004	mid early	medium to high	very high	Starch	SaKa Pflanzenzucht GmbH & Co KG; Hamburg
cv. Sibu	1993	mid late-late	high	high to very high	Starch	SaKa Pflanzenzucht GmbH & Co KG; Hamburg
cv. Tomba	1995	late	medium	high	Starch	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Topas	2005	medium	high		Table	Europlant Pflanzenzucht GmbH; Lüneburg
cv. Verdi	2003	medium	medium	high	Starch	SaKa Pflanzenzucht GmbH & Co KG; Hamburg

Supplementary Table 2: Osmotic potential of liquid medium at the end of the experiment after 21 days for three selected genotypes. n= 3 (one repetition).

Genotype	Control [MPa]	Stdw +/-	0.2 M sorbitol [MPa]	Stdw +/-
cv. Kolibri	-0.479	0.008	-1.003	0.017
cv. Tomba	-0.488	0.012	-0.968	0.012
cv. Eurobravo	-0.484	0.007	-0.991	0.001

Supplementary Table 3: Genotype ranking by their respective SSI calculated for the fresh mass or the dry mass of the shoot and root after the first or second repetition of the *in vitro* experiment.

	1st repetition		2nd repetition			1st repetition		2 nd repetition	
Genotype	**SSI FMShoot**	**SSI DMShoot**	**SSI FMShoot**	**SSI DMShoot**	**Genotype**	**SSI FMRoot**	**SSI DMRoot**	**SSI FMRoot**	**SSI DMRoot**
cv. Eurobravo	19	18	20	16	**cv. Eurobravo**	20	12	20	20
cv. Euroflora	13	14	6	2	**cv. Euroflora**	4	4	2	2
cv. Euronova	20	20	12	11	**cv. Euronova**	5	10	7	6
cv. Euroresa	10	7	7	15	**cv. Euroresa**	18	18	15	14
cv. Eurostarch	12	15	5	5	**cv. Eurostarch**	3	3	1	1
cv. Heidrun	8	16	2	6	**cv. Heidrun**	7	7	10	8
cv. Jasia	2	3	14	19	**cv. Jasia**	9	5	11	11
cv. Kiebitz	6	9	11	10	**cv. Kiebitz**	16	17	16	15
cv. Kolibri	14	11	8	4	**cv. Kolibri**	8	6	4	4
cv. Kormoran	3	10	9	7	**cv. Kormoran**	6	2	13	12
cv. Lambada	5	4	10	15	**cv. Lambada**	10	11	8	7
cv. Maxi	1	1	1	3	**cv. Maxi**	1	1	3	3
cv. Priamos	17	12	16	8	**cv. Priamos**	14	14	19	10
cv. Ramses	18	19	18	18	**cv. Ramses**	19	19	14	19
S. chacoense	11	6	17	20	***S. chacoense***	12	8	17	17
S. tarijense	16	13	19	17	***S. tarijense***	12	15	9	13
cv. Sibu	15	17	3	12	**cv. Sibu**	15	20	6	16
cv. Tomba	7	2	13	13	**cv. Tomba**	17	16	18	18
cv. Topas	9	8	15	9	**cv. Topas**	11	12	12	9
cv. Verdi	4	5	4	1	**cv. Verdi**	2	9	5	5

1= lowest SSI (most tolerant); 20 = highest SSI (most sensitive); Blue colour indicates large difference between repetitions (> 7 SSI places).

Manuscript 2

2.2 Proteomic analysis of two divergently responding potato genotypes (*Solanum tuberosum* L.) following osmotic stress application *in vitro*

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Type of authorship:	First author
Type of article:	Research article
Contribution to the publication:	Planned and performed all experiments, analysed data, prepared all figures and wrote the paper
Co-authors contribution	A. M. Jozefowicz: Helped with data collection, manuscript correction H.-P. Mock: Helped in experimental design, manuscript correction T. Winkelmann: Planned the experiment, corrected the manuscript
Submitted to	Journal of Proteomics
Status	in press
DOI:	doi:10.1016/j.jprot.2016.04.048

ARTICLE IN PRESS

JPROT-02537; No of Pages 15

Journal of Proteomics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomic analysis of two divergently responding potato genotypes (Solanum tuberosum L.) following osmotic stress treatment in vitro

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article info

Article history:
Received 3 March 2016
Received in revised form 20 April 2016
Accepted 28 April 2016
Available online xxxx

Keywords:
Solanum tuberosum
Osmotic stress
In vitro
Proteomics
Mass spectrometry
Stress response

abstract

Starch potatoes (Solanum tuberosum L.) are of interest for production of starch, ethanol, and biopolymers. Due to the predicted increase in drought periods, the breeding of starch potatoes for drought tolerance is essential. This study aims to elucidate the physiological mechanisms that give rise to drought tolerance. Two genotypes contrasting in drought tolerance were compared. We applied osmotic stress which is a known component of drought stress under in vitro conditions. Shoot tips were harvested after 11 days of culture on control medium and medium supplied with 0.2 M sorbitol. Their proteomes were analyzed using two-dimensional isoelectric focussing sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D-IEF/SDS-PAGE). Of a total of 679 distinct protein spots, 118 and 20 spots with differential abundance were found in the sensitive and the tolerant genotype, respectively, after the application of stress. Using mass spectrometry, the proteins in 100 differentially abundant spots were identified; a majority of these proteins were from the chloroplast. For the sensitive genotype, an increase in the abundance of proteinase inhibitors and their precursors, changes in stress responsive proteins and an altered RNA/DNA-binding response were observed. The differentially abundant spots of the tolerant genotype comprised one chaperone and one hydrogen peroxide detoxifying protein.
Biological significance: Our findings reveal that the two genotypes have different responses to osmotic stress in terms of protein degradation and reactive oxygen species (ROS) scavenging and production. Our data suggest that the tolerant genotype might adjust to the applied stress more quickly. A comparative temporal analysis might provide further insights into these rapid changes and assist in the development of biomarkers.

1. Introduction

Plants are often subjected to unfavourable conditions, such as drought, cold temperatures and high salinity. These conditions result in reduced growth and yield losses in crop plants; if the stress is too severe or lasts for an extended period, plant death will occur [1]. Drought is one of the most severe abiotic stress conditions and has accelerated famines in the past [2]. In the context of climate change, periods of drought are predicted to occur more frequently in many regions of the world [3]. Such conditions will result in reductions in useable agricultural land or the need for irrigation.

Solanum tuberosum L. (potato) is an important source of human nutrition and starch. The potato plant is sensitive to drought stress due to its shallow root system [4]. Therefore, potatoes will be negatively affected by climate change; rainfall is expected to increase in the winter and decrease in the summer in temperate regions [5]. In the early growth stages, which occur in spring and early summer, water shortages drastically reduce tuber quantity and quality [6,7]. Potatoes have been screened for drought stress tolerance using in vitro systems with 0.2 M sorbitol, which to reduces the osmotic potential of Murashige and Skoog (MS) medium [8]. Such assays can distinguish genotypes based on stress tolerance and are less costly and time consuming than field trials; in vitro assays are also easier to reproduce [9].

Even though potatoes are known to be drought sensitive, some genotypes perform better than others under diverse water regimes. Proteomic studies have increased in precision and provide powerful tools used to compare the physiological states of plant organs under specific stress conditions [10,11]. The potato genome was fully sequenced by the Potato Genome Consortium in 2011 [12]. Proteomic studies are an interesting approach to analysing changes in divergently responding genotypes during abiotic stress. As gel-based proteomic techniques allow for excellent visualization of the actual physiological status of a tissue at a given time, they are commonly used for comparative studies. We are aware of the limitations of these techniques and will apply LC-based approaches in a complementary manner for whole proteome analyses or to analyse subcellular fractions such as plastids. Proteomic studies have been performed previously [13] using cold treated and dehydrated potato plants to analyse cryopreservation stress; proteomic techniques have also been applied for the study of salt treated potato plants [14] and plants under drought and salt stress [15]. A number of

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http://dx.doi.org/10.1016/j.jprot.2016.04.048
1874-3919/

Please cite this article as: C. Bündig, et al., Proteomic analysis of two divergently responding potato genotypes (Solanum tuberosum L.) following osmotic stress treatment in vitro..., J Prot (2016), http://dx.doi.org/10.1016/j.jprot.2016.04.048

proteins specific to stress responses were found to increase in abundance, including heat shock proteins (HSPs). Other proteins decreased in abundance, such as proteins related to photosynthesis (e.g., rubisco). Stress responses were also analysed at the protein level in other plant species, including tomato seedlings subjected to salt stress [16], rice leaf sheaths under drought stress [17] and oak leaves under drought stress [18].

In this study, we analysed two starch potato genotypes. These genotypes were identified in a larger set of 18 potato genotypes to react tolerant and sensitive to both, osmotic stress in vitro and drought stress in a pot trial (Bündig et al., submitted manuscript). The tolerance or sensitivity of these genotypes was determined based on their dry mass performance in vitro and their tuber yield in pot trials under stress conditions. These two genotypes were compared using 2D IEF/SDS-PAGE. Our aims were as follows: (i) elucidate the stress responses of potato by examining changes in plant physiology and metabolism at the protein level and (ii) highlight specific proteins that might give rise to stress tolerance.

2. Material and methods

2.1. Plant material

For this study, two genotypes of S. tuberosum L. were selected from a set consisting of 18 genotypes and 2 wild species. The selected genotypes were the most tolerant (genotype 13, cv. Maxi, Bayerische Pflanzenzuchtgesellschaft e.G. & Co KG) and the most sensitive (genotype 2, cv. Eurobravo, EUROPLANT Pflanzenzucht GmbH). Plants were kindly provided as in vitro cultures by Dr. A. Schum (Julius Kühn-Institut, Groß Lüsewitz, Germany). The plant material was propagated in vitro on solid MS (Murashige and Skoog, 1962) medium (3% sucrose; 7.5 g Plant agar/l (Duchefa Biochemie B.V., Haarlem, The Netherlands)) [8] at a constant temperature of 18 °C with a 16 h light cycle and a photon flux density of about 35 µmol m^{-2} s^{-1}.

2.2. Stress application and harvest

Plants were grown on MS medium as described above for 2 weeks before use in the experiment. Shoot tips 0.5 cm in length were transferred to solid MS medium [8] without or with 0.2 M sorbitol as the control or the treatment, respectively. After three weeks of culturing, ten vessels for each genotype and treatment containing five shoot tips each were analyzed; two repetitions of the experiment were performed. To document plant development, fresh mass (FM) and dry mass (DM) were compared after 21 days. Data analysis was performed using R software (R version 3.1.2; The R Project for Statistical Computing, Lyon, France (www.r-project.org)). Significant differences were identified based on a multiple comparison of means [19].

For the proteomic analyses, two samples from each repetition of the experiment (=four biological replicates) were harvested after 11 days of culturing; early responses were analyzed. At this time point, shoots of both genotypes had formed roots under stress conditions. Approximately 100 mg of shoot tip material from five shoot tips (the first 0.5–1.0 cm) cultured together in one vessel was instantly frozen in liquid nitrogen (LN) to serve as one biological replicate. The samples were then ground using a ball mill (MM 400, Retsch, VERDER Group, Netherlands) with stainless steel beads (7 mm diameter) in a reaction tube and either stored at −80 °C or used immediately for protein extraction.

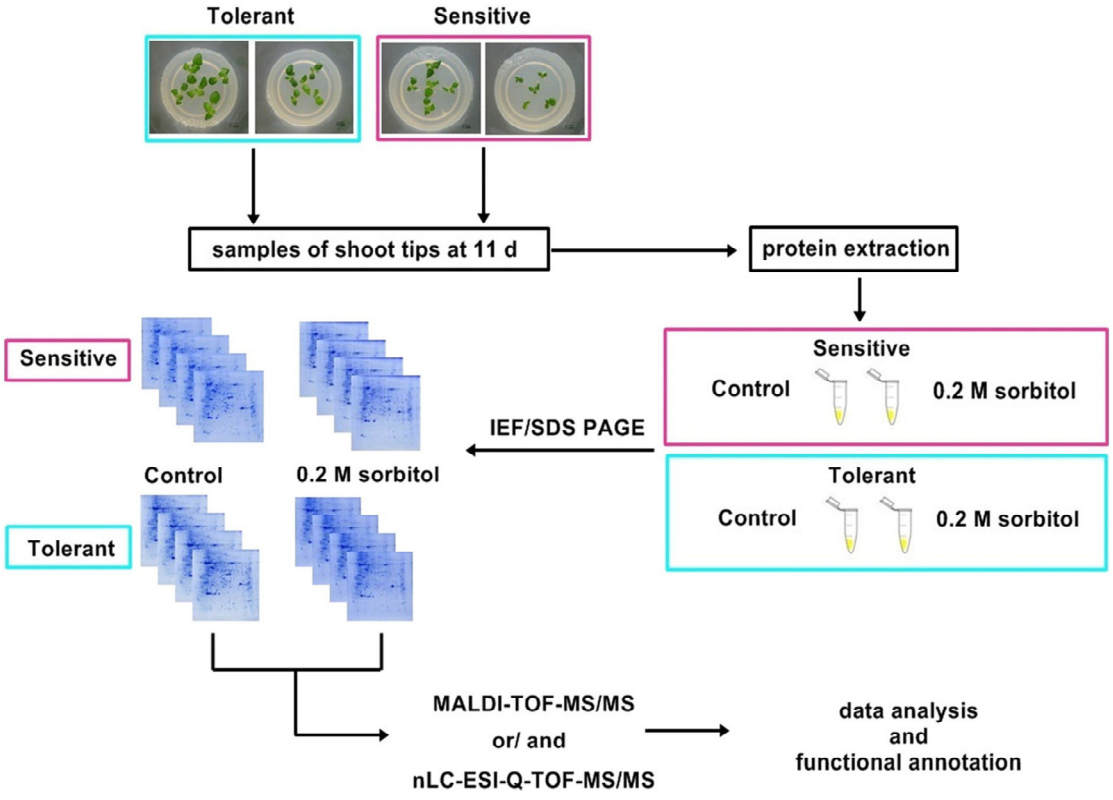

Fig. 1. Experimental design for the proteomic characterization of stress response upon osmotic stress in vitro. Plants were stressed through MS medium with 0.2 M sorbitol. After 11 days of culture shoot tips were harvested. Proteins were extracted by TCA and acetone followed by 2D IEF/SDS gel electrophoresis (four replications for each treatment; two from each repetition of the experiment). Spot quantification was performed with Delta2D and proteins differing in abundance were identified by MS analysis. Functional annotation was performed to assign functional categories to the identified proteins.

Please cite this article as: C. Bündig, et al., Proteomic analysis of two divergently responding potato genotypes (Solanum tuberosum L.) following osmotic stress treatment in vitro..., J Prot (2016), http://dx.doi.org/10.1016/j.jprot.2016.04.048

Table 1
List of spots with altered volumes and identified proteins within the comparisons. Each row displays the number of spots found to be higher abundant between the respective comparison. Numbers in brackets represent the number of proteins found in the respective spots.

Comparison	Samples to be compared		Differential abundant protein spots	Spots identified via MALDI (proteins)	Spots identified via QTOF (proteins)	Total number of identified spots (proteins)
1	Genotype 13 Treatment		5	–	3 (6)	3 (6)
		vs Genotype 13 Control	15	3 (3)	3 (4)	6 (7)
2	Genotype 2 Treatment		49	6 (7)	16 (30)	23 (38)
		vs Genotype 2 Control	69	7 (7)	23 (25)	30 (32)
3	Genotype 2 Treatment		62	12 (13)	6 (16)	18 (29)
		vs Genotype 13 Treatment	72	11 (12)	–	11 (12)
4	Genotype 2 Control		54	13 (14)	3 (3)	16 (17)
		vs Genotype 13 Control	68	13 (14)	2 (3)	15 (17)
Total			394	65	56	122

2.3. TCA protein extraction

Protein extraction was performed for each of the four biological replicates as described previously [20] with modifications. In total, 1 ml of cold TCA solution (10% (w/v) TCA, 20 mM DTT in acetone) was added to the shoot tip powder, and the samples were incubated at −20 °C for 45 min. The samples were then centrifuged for 15 min at 35,000 × g at 4 °C. The pellet was then resuspended in washing solution (20 mM DTT, 1 mM PMSF in acetone), and the samples were incubated at −20 °C for 1 h before centrifugation at 35,000 × g for 15 min. The samples were again resuspended in washing solution and centrifuged for 15 min at 35,000 × g. The pellet was then dried for 20 min under a fume hood. The protein pellet was weighed and then frozen at −80 °C until further use.

2.4. Protein quantification

The protein pellet was resuspended in 50 µl rehydration buffer (8 M urea, 2 M thiourea, 2% (v/v) Triton-X, a small amount of bromophenol blue, 100 mM DTT, 12 µl/ml DeStreak-reagent (GE Healthcare, Freiburg, Germany), and 0.5% (v/v) IPG-buffer pH 3–11 NL (GE Healthcare)) per mg of protein. Protein quantification was performed using the 2D Quant Kit (GE Healthcare) against BSA as a standard as described by the manufacturer's instructions.

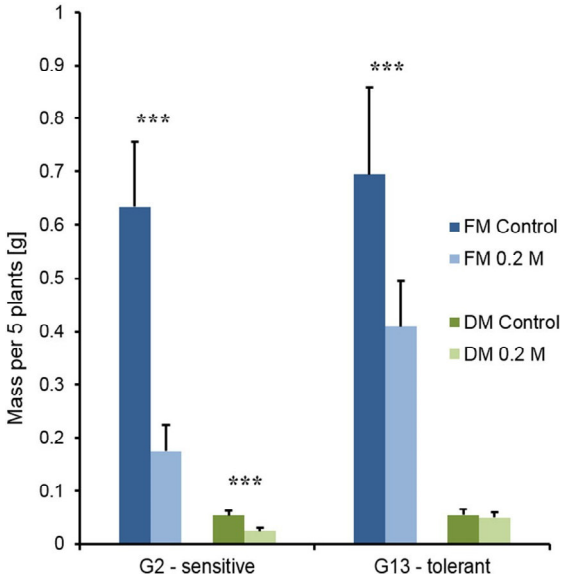

Fig. 2. Comparison of fresh and dry mass per vessel of the two genotypes after 21 days of culture. n = 20, genotype 2 0.2 M. n = 19. ***p b 0.001. Multiple comparison of means, after [16].

2.5. 2D IEF/SDS-PAGE

Approximately 500 µg of protein suspended in 350 µl rehydration buffer was then separated using 2D gel electrophoresis. The samples were transferred to IEF strips (18 cm, pH 3–11 NL, GE Healthcare). Isoelectric focussing was performed as described previously [21]. A polyacrylamide gel (13.5 ml 49.5T/3C acrylamide and 15 ml tricine gel buffer (3 M Tris, 0.3% (w/v) SDS, 6 ml 87% glycerine, 10.5 ml bidest H_2O, 150 µl 10% ammonium persulfate (APS) and 15 µl TEMED)) was poured between two glass plates 20 × 20 cm in size. The gel thickness was 2 mm. The IEF strips were equilibrated for 15 min in 40 ml equilibration solution (50 mM Tris-Cl (pH 8.8), 6 M Urea, 30% (v/v) glycerin, 2% (w/v) SDS, and a small amount of bromophenol blue) containing 0.4 g DTT. A subsequent equilibration step in 40 ml equilibration solution containing 1 g iodoacetamide (IAA) without DTT for 15 min was performed and followed by a wash step in tricine gel buffer. The IEF strips were then placed on top of the acrylamide gel and run for 18 h at a maximum of 500 V and 30 mA per gel.

2.6. Gel staining procedure

Protein spots were fixed in the gel for 2 h (15% ethanol and 10% acetic acid) and stained over night with Coomassie blue CBB G-250 (Merck, Darmstadt, Germany) in a solution containing 1% (w/v) ortho-phosphoric acid (85%) and 10% (w/v) ammonium sulphate).

2.7. Quantitative gel analysis of the proteomes of two genotypes differentially responding to osmotic stress

Scanned images of the Coomassie-stained gels were analyzed as described previously [22] using Delta2D software 4.4 (Decodon, Greifswald, Germany). Four replicate gels per genotype and treatment were analyzed, and spots were automatically detected. Minor corrections of gel disturbances were performed manually. To determine significant differences in spot patterns between the treatment and the control within a genotype, as well as control versus control and treatment versus treatment between the genotypes, a Student's t-test based on the normalized relative spot volume was performed (p-value ≤ 0.05). Only spots with a fold change greater than 1.5 were taken into consideration. Four individual comparisons were made between the different groups (treatment and control samples of the tolerant genotype, treatment and control samples of the sensitive genotype, treatment samples between genotypes, and control samples between genotypes).

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Table 2
Identification of proteins from spots with changed abundance in comparison 1 between the treatment with 0.2 M sorbitol of the tolerant genotype and the control of the tolerant genotype. A Student's t-test was performed (p-value ≤ 0.05) to determine significant changes in spot volume on the basis of normalised relative spot volume. Only alterations of at least 1.5-fold in spot volume were considered to represent true alterations in protein level.

ID[A]	Reg[B]	Protein name	PGSC numbers	KEGG[C]	Score[D]	PI Calc[E]	MW Calc[F]	Pep[G]	Method[H]	Size[I]	Normalized spot volume[J]
G13C 431	0.39	chaperone protein chloroplastic-like	PGSC0003DMT400003652	Environmental information processing ++	127	6.17	110252	13	MALDI	m	
G13C 421	0.46	probable polyribonucleotide nucleotidyltransferasechloroplastic like	PGSC0003DMT400019741	Nucleotide metabolism	321.55	5.79	98592	12	QTOF	m	
G13C 349	0.50	plastocyanin precursor	PGSC0003DMT400092049	Energy metabolism	366	5.04	16970	3	MALDI	m	
G13C 466	0.56	peptidyl prolyl cis trans isomerase fkbp62 like	PGSC0003DMT400051809	Genetic information processing++	2086.31	5.05	63456	16	QTOF	m	
G13C 466	0.56	protein executer chloroplastic like	PGSC0003DMT400069690	Environmental information processing ++	210.29	5.03	71445	6	QTOF	m	
G13C 608	0.57	1-aminocyclopropane-1-carboxylate oxidase	PGSC0003DMT400055332	Amino acid metabolism	102	5.21	36363	4	MALDI	m	
G13C 491	0.66	far upstream element binding protein 2 like	PGSC0003DMT400031096	Genetic information processing++	1569.80	4.72	82586	9	QTOF	l	
G13T 483	2.32	v type proton atpase catalytic subunit a like	PGSC0003DMT400074197	Energy metabolism	2407.39	5.08	68594	15	QTOF	m	
G13T 483	2.32	nad binding rossmann fold superfamily protein isoform 1	PGSC0003DMT400073075	Genetic information processing	821.94	7.94	65202	16	QTOF	m	
G13T 483	2.32	ru large subunit-binding protein subunit chloroplastic	PGSC0003DMT400061700	Genetic information processing	632.02	5.57	62995	12	QTOF	m	
G13T 483	2.32	t complex protein 1 subunit epsilon like	PGSC0003DMT400027968	Genetic information processing	627.21	5.50	24624	9	QTOF	l	
G13T 208	2.18	peptidyl prolyl cis trans isomerase fkbp15 1 like	PGSC0003DMT400022512	Environmental information processing ++	11148.51	8.21	16019	5	QTOF	m	
G13T 243	2.10	Monodehydroascorbate reductase	PGSC0003DMT400032099	Carbohydrate metabolism	4017.67	8.06	53133	13	QTOF	s	

A = ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labelled with the same ID. G13 stands for the genotype 13 and C or T are standing for control or treatment with 0.2 M sorbitol, respectively.

B = Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (stress/ control).

C = Functional classification according to the KEGG Pathway Database (++ = if no classification was automatically annotated, the proteins were manually classified).

D = The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database, which was based upon the sequences from Solanum tuberosumgroup Phureja DM1-3, which was completely sequenced by the [12].

E = Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

F = Calculated MW obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

G = Number of peptides matched to the protein through the database search.

H = Method with which the protein was identified. MALDI = MALDI-TOF-MS/MS and QTOF = nLC-ESI-Q-TOF-MS/MS.

I = Molecular weight (MW) in gel as compared to the theoretically expected MW.

m: MW in gel corresponding to the theoretically expected MW ± 15 kDa

s: MW in gel lower than theoretically expected

l: MW in gel larger than theoretically expected

J = Mean relative spot volume obtained in the four gels of control or plants treated with 0.2 M sorbitol at day 11 illustrated by graphs. The first bar (purple) represents the mean normalized spot volume in the gel of the control plants of the tolerant genotype, the second bar (green) represents the mean normalized spot volume in the gel of the treated plants of the tolerant genotype, the third bar (light purple) stands for the mean normalized spot volume in the gels of the control plants of the sensitive genotypeand the fourth bar (light green) represents the mean normalized spot volume in the gel of the treated plants of the sensitive genotype.No graph is given if more than one protein per spot was identified.

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Table 3
Identification of proteins from spots with changed abundance in comparison 2 between the treatment with 0.2 M sorbitol of the sensitive genotype and the control of the sensitive genotype. A Student's t-test was performed (p-value ≤ 0.05) to determine significant changes in spot volume on the basis of normalised relative spot volume. Only alterations of at least 1.5-fold in spot volume were considered to represent true alterations in protein level.

ID[A]	Reg[B]	Protein name	PGSC numbers	KEGG[C]	Score[D]	PI Calc[E]	MW Calc[F]	Pep[G]	Method[H]	Size[I]	Normalized spot volume[J]
G2C 470	0.18	atp dependent zinc metalloprotease ftsh chloroplastic like	PGSC0003DMT400044601	Environmental information processing	596.85	6.10	74369	14	QTOF	m	
G2C 557	0.20	utp alpha d glucose 1 phosphate uridylyltransferase	PGSC0003DMT400034699	Carbohydrate metabolism	6067.07	5.60	51774	22	QTOF	m	
G2C 431	0.21	linoleate 13s lipoxygenase 2 chloroplastic like	PGSC0003DMT400081909	Lipid metabolism	850.61	6.14	101911	20	QTOF	m	
G2C 422	0.22	structural maintenance of chromosomes protein 4 like	PGSC0003DMT400003351	Cellular Processes	21.66	6.14	141514	9	QTOF	s	
G2C 474	0.23	ru large subunit binding protein subunit chloroplastic like	PGSC0003DMT400006501	Genetic Information Processing	1174.02	5.36	64272	7	QTOF	m	
G2C 471	0.26	atp-dependent zinc metalloprotease ftsh chloroplastic-like	PGSC0003DMT400044601	Environmental information processing	62	6.14	74370	6	MALDI	m	
G2C 510	0.35	heat shock protein sti like	PGSC0003DMT400012208	Environmental information processing ++	1153.72	5.82	65090	21	QTOF	m	
G2C 565	0.39	methionine synthase	PGSC0003DMT400072279	Amino acid metabolism	1653.58	6.17	84544	23	QTOF	m	
G2C 78	0.43	ribulose--bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	46	7.23	40242	2	MALDI	s	
G2C 476	0.49	ru large subunit-binding protein subunit chloroplastic	PGSC0003DMT400061700	Genetic Information Processing	3413.16	5.57	62995	23	QTOF	m	
G2C 476	0.49	glucose 6 phosphate isomerase like	PGSC0003DMT400033620	Carbohydrate metabolism	775.11	5.32	67598	12	QTOF	m	
G2C 619	0.50	tubulin alpha chain like	PGSC0003DMT400022574	Cellular Processes	2522.22	4.73	49576	7	QTOF	s	
G2C 386	0.51	2476393 glutamate 1 semialdehyde aminomutase	PGSC0003DMT400082447	Metabolism of cofactors and vitamins	8356.27	6.57	51401	13	QTOF	m	
G2C 418	0.53	elongation factor ts isoform 2	PGSC0003DMT400008133	Genetic Information Processing ++	72	4.72	114201	8	MALDI	s	
G2C 473	0.54	ru large subunit-binding protein subunit chloroplastic	PGSC0003DMT400061700	Genetic Information Processing	7607.11	5.57	62995	29	QTOF	m	
G2C 507	0.54	glycine trna ligase mitochondrial like	PGSC0003DMT400036931	Genetic Information Processing	887.92	5.60	70141	16	QTOF	m	
G2C 60	0.55	29 kda ribonucleoprotein chloroplastic like	PGSC0003DMT400055527	Genetic Information Processing ++	11810.17	4.45	31305	13	QTOF	m	
G2C 477	0.56	threonine dehydratase chloroplastic like	PGSC0003DMT400033801	Amino acid metabolism	1265.12	5.04	64723	12	QTOF	m	
G2C 568	0.56	methionine synthase	PGSC0003DMT400072279	Amino acid metabolism	282.28	6.17	84544	12	QTOF	m	
G2C 658	0.56	oxygen evolving enhancer protein chloroplasticlike isoform x1	PGSC0003DMT400002421	Energy metabolism	3381.04	6.38	30373	10	QTOF	m	
G2C 442	0.57	heat shock cognate protein 80	PGSC0003DMT400057003	Environmental Information Processing	7500.35	4.76	80221	37	QTOF	m	

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G2C 457	0.57	transketolase 1	PGSC0003DMT400018062	Carbohydrate metabolism	728.48	6.22	80159	11	QTOF	m
G2C 465	0.57	tktc soltuame full chloroplastic short tk flags precursor	PGSC0003DMT400056799	Carbohydrate metabolism	113.86	5.91	79928	5	QTOF	s
G2C 468	0.61	tktc soltuame fullchloroplastic short tk flags precursor	PGSC0003DMT400056799	Carbohydrate metabolism	2358.69	5.91	79928	13	QTOF	s
G2C 508	0.61	heat shock protein sti like	PGSC0003DMT400012208	Environmental information processing ++	7259.62	5.82	65090	33	QTOF	m
G2C 455	0.65	transketolase 1	PGSC0003DMT400018062	Carbohydrate metabolism	2182.96	6.22	80159	15	QTOF	m
G2C 515	0.65	nadp dependent malic enzyme like	PGSC0003DMT400084201	Carbohydrate metabolism	231.54	5.62	64057	11	QTOF	m
G2C 675	0.65	v-type proton atpase subunit b2-like	PGSC0003DMT400008800	Energy metabolism	183	5.45	58410	6	MALDI	m
G2C 195	0.66	383130 ferredoxin-nadp reductase	PGSC0003DMT400009192	Energy metabolism	190	8.37	40432	6	MALDI	m
G2C 392	0.66	chloroplast rubisco activase	PGSC0003DMT400049256	Energy metabolism ++	334	8.10	48089	6	MALDI	m
G2C 668	0.67	chloroplast rubisco activase	PGSC0003DMT400049256	Energy metabolism ++	563	8.10	48089	6	MALDI	m
G2T 55	5.56	glyceraldehyde-3-phosphate dehydrogenase cytosolic	PGSC0003DMT400044944	Carbohydrate metabolism	57	6.34	36624	2	MALDI	m
G2T 149	3.39	tktc soltuame full chloroplastic short tk flags precursor	PGSC0003DMT400056799	Carbohydrate metabolism	1929.42	5.91	79928	3	QTOF	s
G2T 293	3.07	aspartic protease inhibitor 5-like	PGSC0003DMT400024601	Genetic information processing ++	105	8.04	23921	2	MALDI	m
G2T 298	2.14	glyceraldehyde 3 phosphate dehydrogenase chloroplastic like	PGSC0003DMT400030050	Energy metabolism	1833.09	8.38	42861	7	QTOF	s
G2T 324	2.89	annexin d1 like	PGSC0003DMT400045665	Environmental Information Processing ++	885.69	5.23	35823	11	QTOF	m
G2T 417	2.54	grip and coiled coil domain containing	PGSC0003DMT400046956	Uncharacterized ++	7953.63	4.08	85846	27	QTOF	1
G2T 202	2.46	aspartic protease inhibitor 5 like	PGSC0003DMT400024601	Genetic information processing ++	5425.74	7.77	23921	4	QTOF	m
G2T 81	2.42	700407 proteinase inhibitor ii precursor type a a	PGSC0003DMT400011562	Genetic information processing++	6643.65	6.79	16648	5	QTOF	m
G2T 213	2.40	ribulose bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	2783.69	730	40242	6	QTOF	s
G2T 550	2.40	gamma aminobutyrate transaminase 2 like	PGSC0003DMT400062385	Carbohydrate metabolism	5453.86	5.02	33499	11	QTOF	m
G2T 180	2.30	chloroplast stem loop binding protein of 41 kda chloroplastic like	PGSC0003DMT400069189	Genetic information processing++	1188.34	8.32	46007	6	QTOF	m

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G2T 387	2.12	2476393 glutamate 1 semialdehyde aminomutase	PGSC0003DMT400082447	Metabolism of cofactors and vitamins	4393.55	6.57	51401	7	QTOF	m	
G2T 387	2.12	saccharopine dehydrogenase isoform 2	PGSC0003DMT400088617	Environmental Information Processing ++	1054.27	6.55	46637	11	QTOF	m	
G2T 387	2.12	protein tic chloroplastic like	PGSC0003DMT400005492	Genetic information processing++	868.67	9.35	52530	8	QTOF	m	
G2T 626	1.92	ru large subunit binding protein subunit chloroplastic like	PGSC0003DMT400061700	Genetic Information Processing	3239.37	5.57	62995	14	QTOF	s	
G2T 208	1.87	700407 proteinase inhibitor ii precursor type a a	PGSC0003DMT400011562	Genetic information processing ++	9003.35	6.79	16648	6	QTOF	m	
G2T 208	1.87	peptidyl prolyl cis trans isomerase fkbp15 1 like	PGSC0003DMT400022512	Genetic information processing ++	11148.51	8.21	16019	5	QTOF	m	
G2T 208	1.87	aspartic protease inhibitor 5 like	PGSC0003DMT400024601	Genetic information processing ++	1953.03	7.77	23921	5	QTOF	m	
G2T 583	1.85	asr4 protein	PGSC0003DMT400017057	Environmental Information Processing ++	6697.71	4.74	33936	10	QTOF	m	
G2T 583	1.85	threonine dehydratase chloroplastic like	PGSC0003DMT400033801	Amino acid metabolism	2895.10	5.04	64723	13	QTOF	s	
G2T 583	1.85	heat shock cognate 70 kda protein 2 like	PGSC0003DMT400023039	Genetic Information Processing	2334.63	4.95	71193	16	QTOF	s	
G2T 583	1.85	heat shock cognate 70 kda	PGSC0003DMT400001180	Genetic Information Processing	776.72	4.95	71269	10	QTOF	s	
G2T 583	1.85	endoplasmin homolog	PGSC0003DMT400050556	Genetic Information Processing	493.08	4.70	90856	14	QTOF	s	
G2T 583	1.85	rhodanese like domain containing protein chloroplastic like	PGSC0003DMT400000605	No information	246.84	4.78	47067	7	QTOF	m	
G2T 328	1.83	peptidyl prolyl cis trans isomerase like	PGSC0003DMT400004125	Genetic information processing ++	2263.22	8.73	17913	3	QTOF	m	
G2T 652	1.80	cysteine protease inhibitor 8-like	PGSC0003DMT400026280	Genetic information processing++	79	5.52	24706	1	MALDI	m	
G2T 652	1.80	triosephosphate isomerase chloroplastic-like	PGSC0003DMT400004042	Carbohydrate metabolism	80	6.89	34647	2	MALDI	m	
G2T 669	1.74	protein disulfide isomerase like	PGSC0003DMT400010624	Genetic Information Processing	6943.30	4.62	54862	19	QTOF	m	
G2T 207	1.69	700407 proteinase inhibitor ii precursor type a a,	PGSC0003DMT400011562	Genetic information processing ++	8279.90	6.79	16648	6	QTOF	m	
G2T 58	1.64	leucine aminopeptidase	PGSC0003DMT400017373	Amino acid metabolism ++	58	8.52	19866	1	MALDI	m	
G2T 396	1.60	glyceraldehyde 3 phosphate dehydrogenase chloroplastic like	PGSC0003DMT400075608	Energy metabolism	2865.68	7.47	47896	11	QTOF	m	
G2T 42	1.59	hyoscyamine 6-dioxygenase-like	PGSC0003DMT400030676	Biosynthesis of other secondary metabolites	43	5.54	37857	2	MALDI	m	
G2T 650	1.52	triosephosphate isomerase chloroplastic-like	PGSC0003DMT400004042	Carbohydrate metabolism	80	6.89	34647	2	MALDI		

A = ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labelled with the same ID. G13 stands for the genotype 13 and C or T are standing for control or treatment with 0.2 M sorbitol, respectively.

B = Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (stress/ control).

C = Functional classification according to the KEGG Pathway Database (++ = if no classification was automatically annotated, the proteins were manually classified).

D = The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database, which was based upon the sequences from Solanum tuberosumgroup Phureja DM1-3, which was completely sequenced by the [12].

E = Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

F = Calculated MW obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

G = Number of peptides matched to the protein through the database search.

H = Method with which the protein was identified. MALDI = MALDI-TOF-MS/MS and QTOF = nLC-ESI-Q-TOF-MS/MS.

I = Molecular weight (MW) in gel as compared to the theoretically expected MW.

m: MW in gel corresponding to the theoretically expected MW ± 15 kDa

s: MW in gel lower than theoretically expected

l: MW in gel larger than theoretically expected

J = Mean relative spot volume obtained in the four gels of control or plants treated with 0.2 M sorbitol at day 11 illustrated by graphs. The first bar (purple) represents the mean normalized spot volume in the gel of the control plants of the tolerant genotype, the second bar (green) represents the mean normalized spot volume in the gel of the treated plants of the tolerant genotype, the third bar (light purple) stands for the mean normalized spot volume in the gels of the control plants of the sensitive genotypeand the fourth bar (light green) represents the mean normalized spot volume in the gel of the treated plants of the sensitive genotype.No graph is given if more than one protein per spot was identified.

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Table 4
Identification of proteins from spots with changed abundance in comparison between the treatment with 0.2 M sorbitol of the sensitive genotype and the treatment with 0.2 M of the tolerant genotype. A Student's t-test was performed (p-value ≤ 0.05) to determine significant changes in spot volume on the basis of normalised relative spot volume. Only alterations of at least 1.5-fold in spot volume were considered to represent true alterations in protein level.

ID[A]	Reg[B]	Protein name	PGSC numbers	KEGG[C]	Score[D]	PI Calc[E]	MW Calc[F]	Pep[G]	Method[H]	Size[I]	Normalized spot volume[J]
G2T 154	7.34	superoxide dismutase	PGSC0003DMT400070920	Environmental Information Processing	120	6.60	28305	2	MALDI	m	
G2T 207	6.97	700407 proteinase inhibitor ii precursor type a a	PGSC0003DMT400011562	Genetic information processing++	8279.90	6.79	16648	6	QTOF	m	
G2T 58	6.79	leucine aminopeptidase	PGSC0003DMT400017373	Metabolism of other amino acids	58	8.52	19866	1	MALDI	m	
G2T 293	3.25	aspartic protease inhibitor 5-like	PGSC0003DMT400024601	Genetic information processing++	105	8.04	23921	2	MALDI	m	
G2T 626	3.11	ru large subunit binding protein subunit chloroplastic like	PGSC0003DMT400061700	Genetic Information Processing	3239.37	5.57	62995	14	QTOF	s	
G2T 583	2.20	asr4 protein	PGSC0003DMT400017057	Environmental Information Processing ++	6697.71	4.74	33936	10	QTOF	m	
G2T 583	1.85	threonine dehydratase chloroplastic like	PGSC0003DMT400033801	Amino acid metabolism	2895.10	5.04	64723	13	QTOF	s	
G2T 583	1.85	heat shock cognate 70 kda protein 2 like	PGSC0003DMT400023039	Genetic Information Processing	2334.63	4.95	71193	16	QTOF	s	
G2T 583	1.85	heat shock cognate 70 kda	PGSC0003DMT400001180	Genetic Information Processing	776.72	4.95	71269	10	QTOF	s	
G2T 583	1.85	endoplasmin homolog	PGSC0003DMT400050556	Genetic Information Processing	493.08	4.70	90856	14	QTOF	s	
G2T 583	1.85	rhodanese like domain containing protein chloroplasticlike	PGSC0003DMT400000605	No information	246.84	4.78	47067	7	QTOF	m	
G2T 288	2.09	osmotin-like protein	PGSC0003DMT400001066	Environmental Information Processing ++	105	7.56	27158	9	MALDI	m	
G2T 274	1.87	chi2_soltu ame: full=endochitinase 2 flags: precursor	PGSC0003DMT400069033	Carbohydrate metabolism	286	6.18	34095	4	MALDI	m	
G2T 577	1.85	PREDICTED: uncharacterized protein At5g39570-like	PGSC0003DMT400003630	uncharacterised	410	4.67	40963	7	MALDI	m	
G2T 652	1.82	cysteine protease inhibitor 8-like	PGSC0003DMT400026280	Genetic information processing++	79	5.52	24706	1	MALDI	m	
G2T 396	1.81	glyceraldehyde 3 phosphate dehydrogenase chloroplastic like	PGSC0003DMT400075608	Carbohydrate metabolism	2865.68	7.47	47896	11	QTOF	m	
G2T 294	1.65	carbonic chloroplastic-like isoform x2	PGSC0003DMT400001297	Energy metabolism	406	6.40	34520	6	MALDI	m	
G2T 650	1.64	triosephosphate isomerase chloroplastic-like	PGSC0003DMT400004042	Carbohydrate metabolism	80	6.89	34647	2	MALDI	m	
G2T 81	1.61	700407 proteinase inhibitor ii precursor type a a	PGSC0003DMT400011562	Genetic information processing++	6643.65	6.79	16648	5	QTOF	m	
G2T 152	1.59	glycine-rich protein 2-like	PGSC0003DMT400004028	Genetic information processing++	188	4.82	13741	3	MALDI	m	
G2T 387	1.51	2476393 glutamate 1 semialdehyde aminomutase	PGSC0003DMT400082447	Metabolism of cofactors and vitamins	4393.55	6.57	51401	7	QTOF	m	
G2T 387	2.12	saccharopine dehydrogenase isoform 2	PGSC0003DMT400088617	Environmental Information Processing ++	1054.27	6.55	46637	11	QTOF	m	
G2T 387	2.12	protein tic chloroplastic like	PGSC0003DMT400005492	Genetic information processing++	868.67	9.35	52530	8	QTOF	m	

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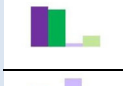
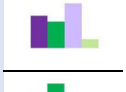
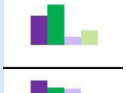
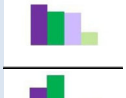
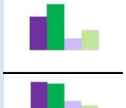
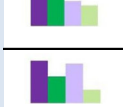
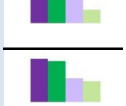
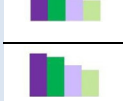
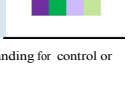
G2T 487	1.51	adp-glucose pyrophosphorylase small subunit	PGSC0003DMT400079823	Carbohydrate metabolism	235	6.73	57190	6	MALDI	m	
G13T 82	0.18	ribulose--bisphosphate carboxylase oxygenase small subunit	PGSC0003DMT400050381	Energy metabolism	184	7.56	20562	3	MALDI	m	
G13T 06	0.25	probable rhamnose biosynthetic enzyme 1-like	PGSC0003DMT400018192	Carbohydrate metabolism	282	6.75	75660	5	MALDI	m	
G13T 05	0.29	protein toc75-chloroplastic-like	PGSC0003DMT400078190	Environmental Information Processing ++	321	8.42	91403	7	MALDI	m	
G13T 557	0.33	utp:alpha-d-glucose-1-phosphate uridylyltransferase	PGSC0003DMT400034699	Carbohydrate metabolism	110	5.70	51774	2	MALDI	m	
G13T 478	0.35	threonine dehydratase chloroplastic-like	PGSC0003DMT400033801	Amino acid metabolism	189	5.22	64723	5	MALDI	m	
G13T 445	0.39	nadh dehydrogenase	PGSC0003DMT400034638	Energy metabolism	68	6.05	79799	2	MALDI	m	
G13T 07	0.43	probable rhamnose biosynthetic enzyme 1-like	PGSC0003DMT400054753	Carbohydrate metabolism	84	6.57	75521	2	MALDI	m	
G13T 235	0.48	glycolate oxidase	PGSC0003DMT400071115	Carbohydrate metabolism	108	9.26	40585	3	MALDI	m	
G13T380	0.49	ribulose--bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	176	7.23	40242	4	MALDI	m	
G13T 12	0.51	dynamin-related protein 1e-like	PGSC0003DMT400004319	Cellular process ++	138	7.62	68574	16	MALDI	m	
G13T 221	0.67	auxin-binding protein abp19a-like	PGSC0003DMT400036428	Cellular process ++	109	5.83	21506	1	MALDI	m	

A = ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labelled with the same ID. G13 stands for the genotype 13 and C or T are standing for control or treatment with 0.2 M sorbitol, respectively.

B = Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (stress/ control).

C = Functional classification according to the KEGG Pathway Database (++ = if no classification was automatically annotated, the proteins were manually classified).

D = The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database, which was based upon the sequences from Solanum tuberosumgroup Phureja DM1-3, which was completely sequenced by the [12].

E = Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

F = Calculated MW obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

G = Number of peptides matched to the protein through the database search.

H = Method with which the protein was identified. MALDI = MALDI-TOF-MS/MS and QTOF = nLC-ESI-Q-TOF-MS/MS.

I = Molecular weight (MW) in gel as compared to the theoretically expected MW.

m: MW in gel corresponding to the theoretically expected MW ± 15 kDa

s: MW in gel lower than theoretically expected

l: MW in gel larger than theoretically expected

J = Mean relative spot volume obtained in the four gels of control or plants treated with 0.2 M sorbitol at day 11 illustrated by graphs. The first bar (purple) represents the mean normalized spot volume in the gel of the control plants of the tolerant genotype, the second bar (green) represents the mean normalized spot volume in the gel of the treated plants of the tolerant genotype, the third bar (light purple) stands for the mean normalized spot volume in the gels of the control plants of the sensitive genotypeand the fourth bar (light green) represents the mean normalized spot volume in the gel of the treated plants of the sensitive genotype.No graph is given if more than one protein per spot was identified.

2.8. Mass spectrometry analysis of excised protein spots

Each protein spot that differed in abundance in one of the four comparisons was excised from the two gels, one from each experimental repetition. Excised protein spots were in-gel-digested with trypsin as described previously [23]. The acquisition of peptide mass fingerprinting and LIFT spectra was performed using a MALDI-TOF-MS/MS ultrafleXtreme™ (Bruker, Bremen, Germany). The calibration of spectra was performed using external calibration, with subsequent internal mass correction. The identification of proteins was performed using the MASCOT search algorithm (www.matrixscience.com, MASCOT version 2.5.1). When this procedure failed, spots were analyzed with a NanoAcquity LC coupled to a Q-TOF Premiere mass spectrometer (Waters Corporation, Eschborn, Germany). Peptides were separated using a 150 mm × 100 μm BEH (1.7 μm) C18 column with 3–35% acetonitrile over 30 min at a flow rate of 450 nl min^{-1}. The data acquisition was performed as described previously [24] with minor modifications. The identification of proteins was performed using ProteinLynx Global

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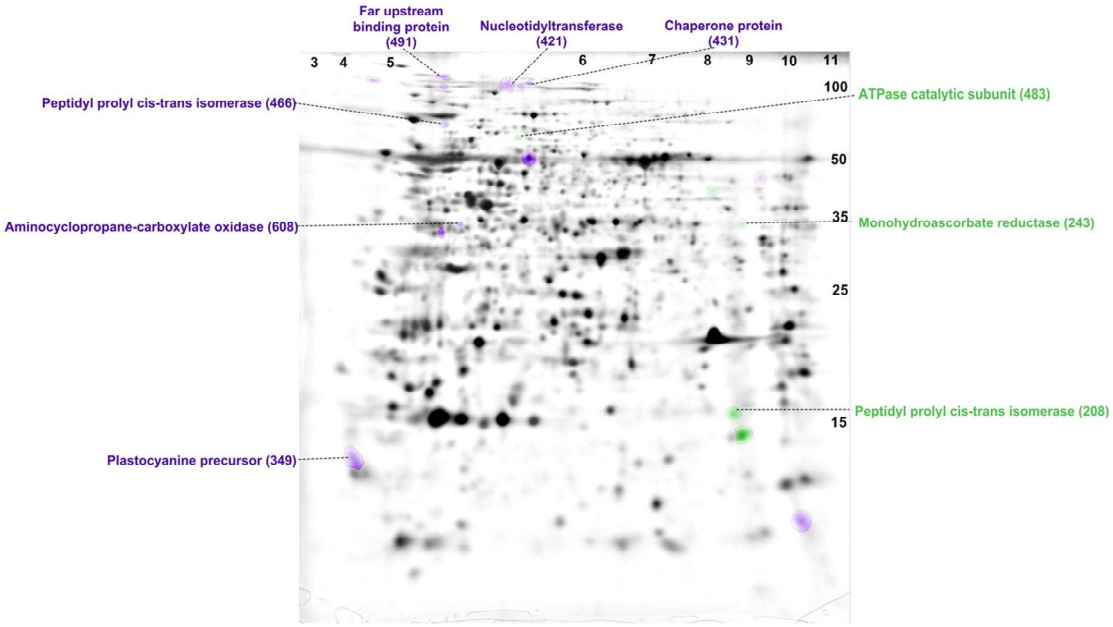

Fig. 3. Comparison 1 between treatment with 0.2 M sorbitol and the control within the tolerant genotype. Purple spots represent proteins higher abundant in gels from control plants, green spots represent proteins higher abundant in gels from plants treated with 0.2 M sorbitol. Numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis (Table 2). Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused inverted image over all gels. Numbers indicate the pI (horizontal) and MW (vertical), respectively.

Server 2.5.3 (Waters Corporation) and a potato protein database; the database contained sequences from S. tuberosum group Phureja DM1-3 (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml). The genome was completely sequenced by the Potato Genome Consortium in 2011 [12]. Identified proteins were functionally classified according to the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html). Identification was considered to be significant if the same result was obtained from two technical replicates. Spots

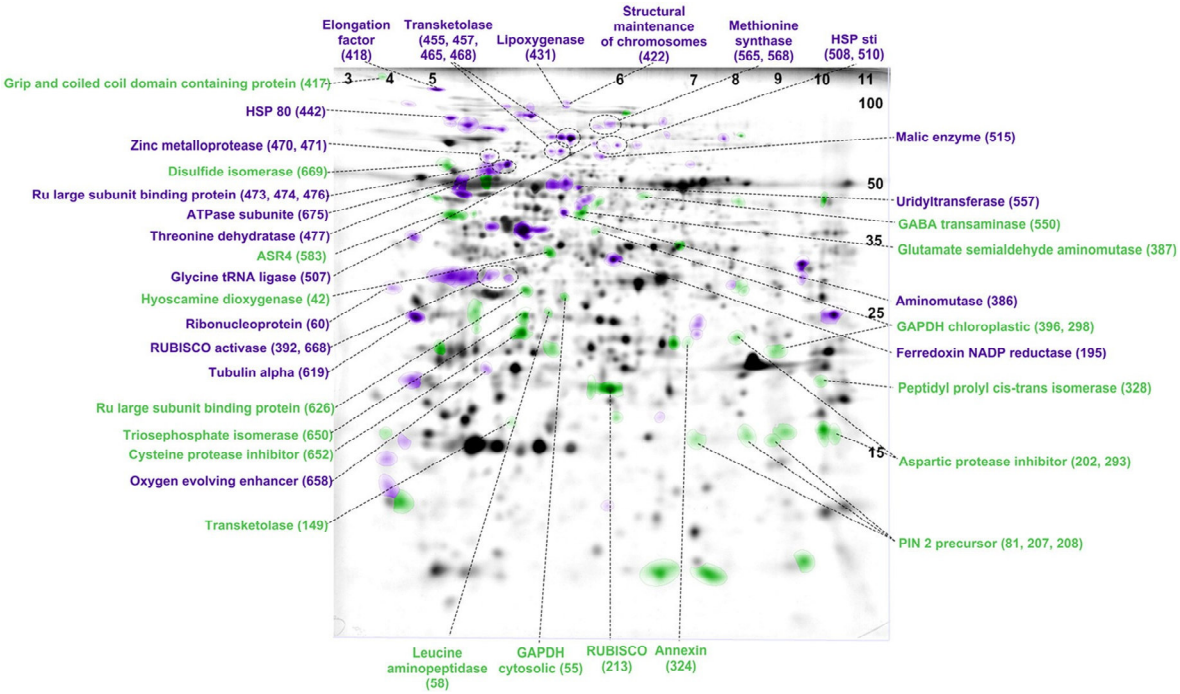

Fig. 4. Comparison 2 between treatment with 0.2 M sorbitol and the control within the sensitive genotype. Purple spots represent proteins higher abundant in gels from control plants, green spots represent proteins higher abundant in gels from plants treated with 0.2 M sorbitol. Numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis (Table 3). Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused inverted image over all gels. Numbers indicate the pI (horizontal) and MW (vertical), respectively.

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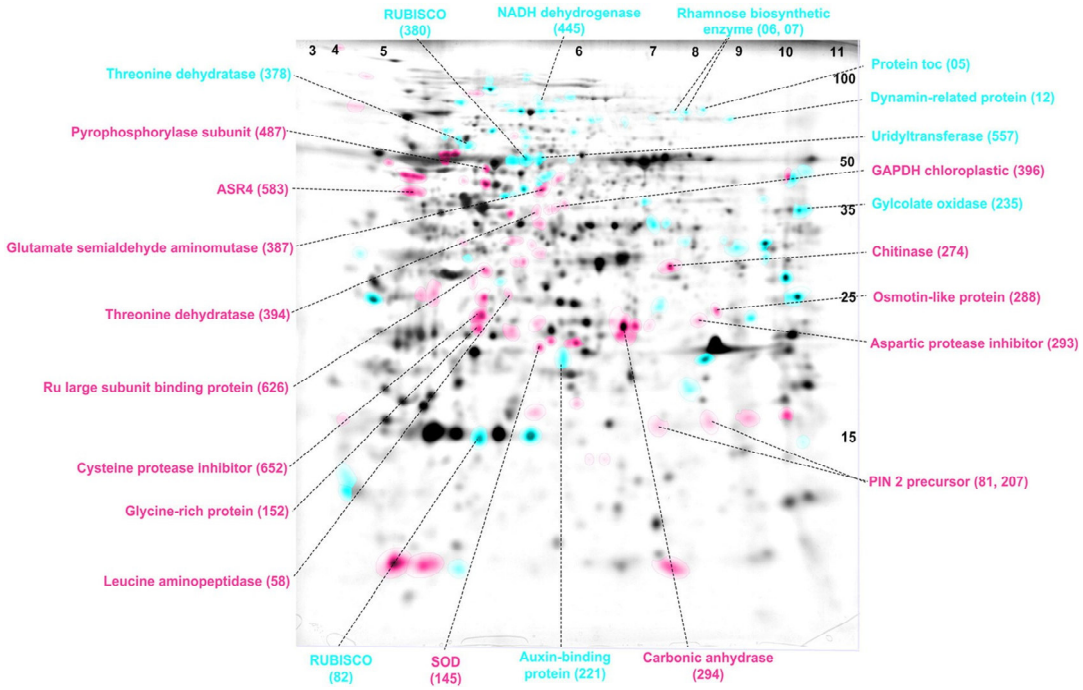

Fig. 5. Comparison 3 between treatment with 0.2 M sorbitol sensitive genotype and the treatment with 0.2 M sorbitol tolerant genotype. Pink spots represent proteins higher abundant in gels from plants treated with 0.2 M sorbitol of the sensitive genotype, blue spots represent proteins higher abundant in gels from plants treated with 0.2 M sorbitol of the tolerant genotype. Numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis (Table 4). Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused inverted image over all gels. Numbers indicate the pI (horizontal) and MW (vertical), respectively.

containing more than one identified protein were marked with an asterisk, e.g., spot 583*.

3. Results

An overview of the complete experiment as a workflow is given in Fig. 1. Samples used for protein extraction were taken after 11 days of culturing. At this time point, samples from both genotypes had formed roots also under osmotic stress conditions. After the extraction of soluble proteins, four high-resolution 2D IEF/SDS-PAGE gels were run for each genotype and treatment. Delta2D analysis revealed the separation of 679 spots on the gels. Spots which differed significantly in abundance were considered for digestion and mass spectrometry (Table 1).

Potato plants were grown for 21 days in vitro on control and osmotic medium before fresh mass (FM) and dry mass (DM) were analyzed (Fig. 2). A significant reduction in FM after the application of stress was observed in both genotypes at the end of the experiment. Conversely, DM was only reduced significantly in the sensitive genotype (Fig. 2). The genotypes therefore responded differently to osmotic stress in vitro, as shown previously in a larger test set (Bündig et al., in preparation), and were selected for proteomic comparison.

A total of the 351 spots were picked and analyzed from two of the four gels per genotype and treatment; a total of 100 proteins were identified and found in both gels. A number of spots were more abundant in two out of the four comparisons. For example, spots 207 and 293 in the sensitive genotype were identified when comparing treatment and control samples from the sensitive genotype and in comparisons between the sensitive genotype and the tolerant genotype. Thus, the total number of spots identified was 100; because some of the spots were differentially abundant in two comparisons, 122 spots are listed in Table 1.

In 13 spots, more than one protein was identified in both gel replicates (Tables 2–4, Supplementary Table 2). For these spots, abundances were not represented in the histograms in Tables 2–4. This is because abundance could only be assigned to a spot, not to one of the identified proteins.

Four comparisons between the genotypes and treatments were analyzed. Samples treated with 0.2 M sorbitol were compared to controls for the tolerant and sensitive genotypes to characterize the response of the two genotypes. It is noteworthy that 20 spots were differentially abundant in the tolerant genotype; in the sensitive genotype, 118 spots were differentially abundant (Table 1). Treatment and control samples from each genotype were compared to analyse genotypic differences, particularly under stress. The total number of differentially abundant spots between the treatment samples from the two genotypes was 134; 122 differentially abundant spots were identified in the comparison of control samples from the two genotypes (Table 1).

The relatively small number of differentially abundant proteins in comparison 1 was also evident in a PCA analysis of the gel patterns (Supplementary Fig. 2). Here, the control and treatment samples from the tolerant genotype overlapped; gel patterns of the control and treatment samples from the sensitive genotype clearly differed. Samples from the sensitive and tolerant genotypes could be differentiated by PCA analysis.

3.1. Comparison 1 - control vs stress treatment in the tolerant genotype

For the tolerant genotype, the proteome of plants treated with 0.2 M sorbitol was compared with the proteome of control plants. The identified proteins were matched to the corresponding spots in Fig. 3 and are shown in detail in Table 2. Of the 20 spots that differed in abundance, 9 were identified which were less abundant after stress (purple spots); these proteins, had a function in binding to RNA and single stranded DNA (spots 421 and 491) and in protein binding or processing (spots 466* and 431). Moreover, an enzyme involved in ethylene metabolism was identified (spot 608, aminocyclopropane carboxylate oxidase). In the proteome of treated plants, monohydroascorbate reductase was

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2.10-fold more abundant than in control plants (green spots in Fig. 3). Interestingly, spots 349, 466* and 491 (plastocyanin precursor, peptidyl prolyl cis trans isomerase fkpb62 like and far upstream binding protein 2 like, respectively) were also significantly more abundant in control plants of the tolerant genotype compared to control plants of the sensitive genotype (comparison 4, Supplementary Fig. 1 and Supplementary Table 1).

3.2. Comparison 2 - control vs stress treatment in the sensitive genotype

Comparison 2 was between the proteomes of the sensitive genotype; samples treated with 0.2 M sorbitol and control samples were compared, and a greater number of proteins (118) showed differential abundance than in the comparison 1 (Fig. 4 and Table 3). Of these 118 spots, 48 were identified. Nearly half of the identified proteins were localized to or associated with the chloroplast. Proteins that were less abundant after treatment with 0.2 M sorbitol (purple spots) were primarily of the KEGG classes 'energy metabolism', 'genetic information processing' and 'carbohydrate metabolism'. Proteins that increased in abundance under stress conditions were primarily of the KEGG class 'genetic information processing' (green spots). Most affected after treatment with 0.2 M sorbitol were proteins associated with photosystem 2 (PSII), including the ATP-dependant zinc metalloprotease ftsh (spots 470 and 471), oxygen evolving enhancer protein (spot 658) and the glutamate 1 semialdehyde aminomutase (spot 386); all of these proteins were significantly less abundant in treated samples. In addition, proteins regulating cell elongation, RNA stabilization and structural maintenance were less abundant after stress treatment; these included elongation factor ts (spot 418), structural maintenance of chromosomes protein 4 like protein (spot 422), glycine tRNA ligase mitochondrial like protein (spot 507) and 29 kDa ribonucleoprotein chloroplastic like protein (spot 60). An increase in spot volume was observed for a number of spots that were identified as protease inhibitors and protease inhibitor precursors. These include aspartic proteinase inhibitors (spot 202), cysteine protease inhibitor 8-like protein and precursors for proteinase inhibitor II type aa (PIN2) (spots 207, 208 and 81). An enzyme of the GABA (gamma-aminobutyric acid) shunt, gamma aminobutyrate transaminase 2 (spot 550), as well as annexin 1d (spot 324) and abscisic acid stress ripening protein 4 (ASR4) (spot 583*) were found to be more abundant. Of the identified proteins, 9 were also found in comparison 3 (treatment vs treatment): Spots 207, 293, 387*, 396, 58, 626, 650, 652* and 81 were all detected in higher levels in the sensitive genotype compared with the tolerant genotype under stress conditions.

3.3. Comparison 3 - proteomes under osmotic stress in the sensitive genotype vs. the tolerant genotype

Comparison 3 was between the proteomes of plants of the sensitive and tolerant genotypes treated with 0.2 M sorbitol; the results are shown in Fig. 5 and Table 4. Of 134 spots, 29 were identified. The proteins found to be more abundant in the sensitive genotype (pink spots) were primarily grouped into the following KEGG classes: ´genetic information processing´ and ´carbohydrate metabolism´. Spots that were more abundant in the tolerant genotype (blue spots) were primarily classified into the following KEGG classes: 'carbohydrate metabolism' and 'energy metabolism'. In the sensitive genotype, proteins involved in protein protection (protease inhibitors, HSPs and chaperons), a ROS (reactive oxygen species) detoxification enzyme (superoxide dismutase, spot 154) and proteins known to be stress induced (glycine-rich protein 2 like and osmotin-like protein; spots 152 and 288, respectively) were identified. In the tolerant genotype, proteins involved in growth, cell wall formation/stability and energy metabolism were identified (dynamin related protein, a probable rhamnose biosynthetic enzyme 1-like protein and NADH dehydrogenase; spots 12, 05/06 and 445, respectively). In the tolerant genotype, one detoxification enzyme was identified (glycolate oxidase, spot 235). Spots 154, 288, 577 and 583* (superoxide dismutase, osmotin-like protein, an uncharacterized protein and ASR4) were more highly abundant in the sensitive genotype. Surprisingly, these proteins were also found when the proteomes were compared under control conditions; in general, the proteins were less abundant in the treated plants (Supplementary Fig. 1 and Supplementary Table 1). Spots 12 and 82 (dynamin related protein and rubisco small subunit) were observed to be more abundant in the tolerant genotype when control sample proteomes were compared.

4. Discussion

In recent years, in vitro systems have been used to apply stress under controlled conditions. The use of these systems has been proposed for screening potatoes upon osmotic stress. In vitro systems can be used to preselect potato genotypes for drought tolerance [9]. The two genotypes used in this study were identified as tolerant (genotype 13) and sensitive (genotype 2) to drought stress in a rain-out shelter experiment (Bündig et al., manuscript in preparation).

The identification of proteins was relatively low (approximately 31%) in all comparisons. This result might be because only protein spots that were identified in two gels were considered to be true hits. When a spot was identified in one gel and the corresponding spot in the other gel was not identified, the results were not included. A number of protein spots did not generate any peptides in the MS analysis due to low abundance and subsequent low numbers of peptides, which were under the detection limit.

4.1. Small alterations in the tolerant proteome might indicate stress acclimatization

Only 5 protein spots were found to be more abundant after treatment with 0.2 M sorbitol in the tolerant genotype. Three of these proteins were identified as a monodehydroascorbate reductase (spot 243), a subunit of the v type proton ATPase (483*) and an immunophilin fkbp15–1 like protein (spot 208). The family of fkbp prolyl cis trans-isomerases act as chaperons and have been shown to operate in cellular responses to stress [25,26]. Monodehydroascorbate is involved in the glutathione-ascorbate cycle and helps in the detoxification of ROS by producing ascorbate, which is then used by ascorbate peroxidase to detoxify hydrogen peroxide [27].

In this comparison, 15 protein spots were found to be less abundant after the application of stress; of these, six were identified. A FKBP62 like protein was found to be less abundant after stress (spot 466*). This protein has been shown to bind to HSP90 in Arabidopsis thaliana, which then attaches to the heat shock transcription factor HsfA2. This complex is then transported to the nucleus under heat stress conditions and activates small HSPs [28]. This protein is also lower in abundance in the control proteome of the sensitive genotype compared to the control proteome of the tolerant genotype under comparison 4 (Supplementary Fig. 1). This indicates that the protein might not be a common component under control conditions in the sensitive genotype. The ethylene-forming enzyme ACC oxidase (1-aminocyclopropane-1-carboxylate oxidase; ACO) (spot 608) was also found to be less abundant after the application of stress in the tolerant genotype. This result was surprising, as both proteins are known to function in stress responses. These findings indicate that the control plants might already be confronted with mild stress during in vitro culture. These proteins are lower in abundance after the application of stress, indicating that they might not be of importance in the stress response at day 11.

4.2. Proteolysis and proteinase inhibitors as key elements of stress susceptibility in the sensitive genotype

Lower abundance was observed for proteins involved in tRNA elongation (elongation factor ts, spot 418) and the organization and

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dynamics of chromosomes (structural maintenance of chromosomes protein 4, spot 422). This reduction in protein abundance may reflect a loss in normal cell organization after stress treatment.

That a high number of identified proteins are associated with the chloroplast is not surprising, as chloroplasts are a major site of protein degradation under drought stress [29]. In the tolerant genotype, few chloroplast-associated proteins were affected by the stress treatment.

After stressing the sensitive genotype with 0.2 M sorbitol in vitro, many proteinase inhibitors were more abundant compared to the proteome under control conditions. Two spots were identified as aspartic proteinase inhibitor proteins (spots 202 and 293), three spots were PIN2 precursors (spots 207, 208*, and 81) and one spot was identified as a cysteine proteinase inhibitor. PIN2 was found to be present in potato foliage after mechanical damage, and expression of the pin2 gene was shown to be induced by ABA [30]. It was demonstrated through an ABA-deficient mutant that an increase in ABA concentration is necessary for the up regulation of PIN2 in potato and tomato leaves. Aspartic and cysteine proteinase inhibitors act upon the proteolytic activity of endopeptidases. The presence of these inhibitors indicates that the plant is actively regulating proteolysis. The proteinase inhibitors were only more abundant in the stressed proteome of the sensitive genotype. Environmental stressors such as drought, salt and high or low temperatures affect various classes of proteases. Proteolytic enzymes were shown to be involved in the response to drought in an expression analysis of genes coding for putative proteases [31,32]. Protein breakdown has been recognized as essential for the adaptation of plants to environmental conditions [1]; proteolysis must be regulated to counteract unfavourable protein degradation, such as the degradation of photosystem proteins. That rubisco activase (spots 392 and 668) is less abundant after stress treatment contributes to the total picture of severe proteolysis in the sensitive genotype. It was shown that rubisco activase and rubisco are commonly degraded by cysteine proteinases in the leaf [33]. After stress treatment, a metalloprotease ftsh protein was found to be less abundant and a leucine aminopeptidase (also in the metalloprotease class) was more abundant. These findings demonstrate that some members of the Solanaceae family contain additional leucine aminopeptidases in addition to the common leucine aminopeptidase, which is constitutively expressed in all plant species [34,35]. These enzymes are present in the reproductive organs and are up-regulated under severe stressors such as osmotic stress, wounding and pathogen attack [35,36,37,38,39]. Aminopeptidases are involved in protein turnover and are thought to mobilize C and N resources from cells subjected to senescence [40]. Leaf senescence is a common response upon drought stress in the field, as a reduction in canopy size reduces water loss [41]. A reduction in mass was also observed under in vitro conditions, suggesting that C and N are also mobilized in this scenario.

Increased abundance under stress was found for annexin 1 (spot 324), which was shown to be part of the ROS signalling network in A. thaliana; this protein may act at the crossroads of Ca^{2+}, ABA and ROS signalling [42]. On an mRNA level, STANN1 (S. tuberosum annexin 1) significantly increased in the leaves of potato after drought stress. STANN1 was the only annexin that responded to drought stress of the nine annexin proteins in potato [43]. Annexins in A. thaliana are thought to mediate osmotic stress [42] responses, and annexin 1 was shown to have a peroxidase activity [44]. Whether this is also the case for the identified annexin in potato remains to be further investigated.

After stress treatment, one enzyme of the GABA shunt was more abundant in stress treated plants of the sensitive genotype. Gamma aminobutyrate transaminase 2 (spot 550) uses GABA to produce either glutamate or alanine depending on the amino acid receptor. It is well known that the GABA concentration increases in plants under various stress conditions and that GABA can be used as a storage and transport compound for nitrogen [45]. A reduction in GABA via this enzyme might reflect an increased demand for nitrogen, which is highly unlikely due to large nitrogen supply in the medium. A reduction might therefore reflect a reduced need for GABA and an increased demand for downstream products.

Two proteins were identified as less abundant after osmotic stress; both of these proteins are associated with photosystem 2 (PSII). The glutamate 1 semialdehyde aminomutase protein (spot 386) was found to be less abundant; this protein takes part in the biosynthesis of chlorophyll. However, this protein was also found to be more abundant in another spot (spot 387*) under stress conditions. Oxygen evolving enhancer protein 1 (OEE1) (spot 658) was also found to be less abundant after osmotic stress. This protein is thought to stabilize the manganese cluster in the PSII, which is the primary site of water splitting [46]. A lack of this protein results in a complete loss of the ability to evolve oxygen at PSII; transgenic bacterial cell cultures lacking this protein were unable to grow photosynthetically [47]. In seaweed (Capsosiphon fulvescens), a purified protein identified as OOE1 was shown to act as an antioxidant against oxygen radicals [48]. A reduction in the abundance of these proteins might therefore indicate damage to PSII and reflects a general down-regulation of photosynthetic activity under osmotic stress conditions [49].

Lower abundance under stress conditions was also observed for heat shock protein cognate 80 (spot 442), HSP sti like protein (spots 508 and 510) and the rubisco large subunit binding protein (spots 473, 474 and 478). These proteins are thought to be molecular chaperons that assist in protein folding and refolding. Chaperones are essential for the proper functioning of proteins and are distributed ubiquitously in all living organisms [50]. An increase in abundance of a chaperone in the control proteome was only visible for the rubisco large subunit binding protein (spot 626). Following a BLAST search (BLASTKOALA from the KEGG database) of the sequence, it was found that this protein belongs to the GroEL chaperon family. This protein family is involved in protein folding [51]. Nevertheless, an overall lower abundance of chaperons after stress treatment might be a sign of severe damage to proteins.

The identification of proteins either less abundant or more abundant after stress suggests that growth and PSII inhibition and the degradation of numerous proteins occurs (reflected as a decrease in the abundance of chaperons) in the sensitive genotype; protein protection (protease inhibitors), drought-mediated transcriptional regulation and an increase in plant antioxidant enzymes, which are at least partially ABA responsive, are of higher priority. It is therefore tempting to speculate that the sensitive genotype suffers from severe protein degradation and subsequently invests most of its energy in counter measurements. That many of the identified proteins are located in or associated with the chloroplast might suggest that stress-senescence occurs in response to osmotic stress. This is surprising because photosynthesis rates are low in in vitro cultured plants; energy is supplied by the sucrose in the medium.

4.3. Comparison of genotypes after stress treatment suggests differential management of protection mechanisms

The comparison between the proteomes of stressed plants of both genotypes could point to possible tolerance mechanisms. A number of protein spots from the sensitive genotype were higher in abundance in this comparison and more abundant in the treated plants compared to control samples of the sensitive genotype (comparison 2). These included rubisco large subunit binding protein (spot 626), aspartic protease inhibitor (spot 293), PIN2 precursor (spots 207 and 81), cysteine proteinase inhibitor (spot 652*), glutamate 1 semialdehyde aminomutase (spot 386), leucine aminopeptidase (spot 58) and ASR4 (spot 583*). In addition, an osmotin-like protein (spot 288) was more abundant. Osmotin and osmotin-like proteins have been shown to be induced by different stressors, including ABA and wounding, through a transgenic approach in potato [52]. SOD (spot 154) was found to be more abundant in the proteome of the sensitive genotype after stress treatment compared to the tolerant genotype after stress treatment. In the tolerant genotype, glycolate oxidase (spot 235) was more abundant.

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Both proteins produce hydrogen peroxide (H_2O_2). Glycolate oxidase (or (S)-2-hydroxy-acid oxidase) functions in photorespiration where O_2 is used [53], and SOD produces O_2. Photorespiration leads to the formation of glycine, which is required for glutathione production [24] and is important for ammonia metabolism [54]. Other proteins that were more abundant in the tolerant genotype after stress treatment were TOC75 (a subunit of the apparatus for protein import into the chloroplast, spot 05) [55], a dynamin-related protein (a GTPase that functions in membrane trafficking and is involved in organelle division, spot 12) [56] and a probable rhamnose biosynthetic enzyme (produces a precursor of the primary cell wall in A. thaliana, spots 06 and 07) [57]. Because the dynamin-related protein was also found after comparing the genotypes under control conditions, this might be a protein that displays genotypic differences.

The proteins identified in this comparison suggest that 11 days after plants were transferred to stress medium containing 0.2 M sorbitol, the sensitive genotype displayed a severe stress reaction; at this time point, the tolerant genotype seemed to have resumed to normal cell growth and might have already overcome the stress. The most divergent responses are comprised of differences in the abundance of HSPs, proteinase inhibitors, different ROS scavenging enzymes and a more abundant set of proteins promoting cell growth in the tolerant genotype.

4.4. Concluding remarks

This study revealed the proteomic responses to stress in starch potato genotypes sensitive or tolerant to osmotic stress in vitro for the first time. The fact that only a small set of proteins was identified in each comparison might distort the overall picture. After a review of the literature, common stress responses were observed through the identified proteins. Our data suggests that the differences between the sensitive and the tolerant genotype on day 11 after stress treatment might be due to differences in the time needed for stress acclimatization. The two genotypes addressed protein degradation differently; the sensitive genotype responded strongly to proteolysis by increasing the abundance of proteinase inhibitors and altering the abundance of ROS scavenging and producing enzymes. It is tempting to speculate that while the sensitive genotype responded strongly to the applied stress, the tolerant genotype might have already overcome the stress and was returning to normal growth. The identified proteinase inhibitors and their precursors (spot volumes based on control/treatment samples of the sensitive genotype; spot 81, 0.070/0.169; spot 202, 0.113/0.278; spot 207, 0.176/0.282; spot 208*; spot 293, 0.053/0.162; spot 652*), the ASR4 protein (spot 583*) and annexin d1 (spot 324, 0.025/0.072) in the sensitive genotype should be studied further. For the tolerant genotype (spot volumes based on treatment samples of the tolerant/sensitive genotypes), the probable rhamnose biosynthetic enzyme (spot 12, 0.038/0.019), the glycolate oxidase (spot 235, 0.37/0.186) and the dynamin-related protein (spot 06, 0.063/0.016; spot 07, 0.028/0.012) are interesting biomarker candidates. To further elucidate the stress responses of starch potatoes, a time series over the first days of osmotic stress should be performed, and the specific proteins involved in growth regulation and the response to osmotic stress and drought stress should be closely observed under rainout-shelter conditions. Further insights can be expected from analyses involving LC-MS-based proteomic approaches. These types of assays should allow to overcome some of the limitations of gel-based approaches, such as relatively low sensitivity, the low solubility of membrane proteins, and the restriction to hydrophilic proteins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.04.048.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

The study was conducted with funding provided by the Federal Ministry of Food and Agriculture (BMEL) through the Agency of Renewable Resources (FNR), funding nos. 22023511 (LUH) and 22023411 (IPK). The authors would like to thank Dagmar Lewejohann, Dr. Christin Lorenz and Annegret Wolf for their support and Prof. Dr. Hans-Peter Braun for partial use of his proteomic laboratory.

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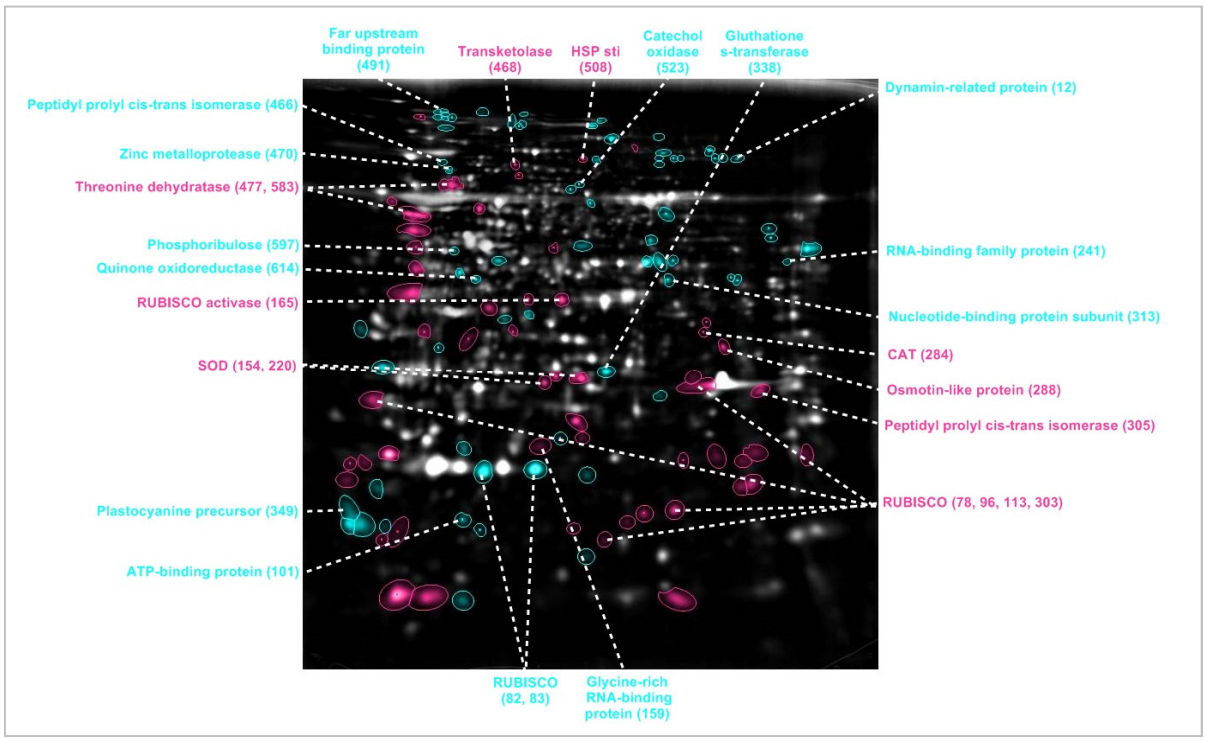
Supplementary data



Supplementary Figure 1: Comparison of control sensitive genotype versus the control of the tolerant genotype. Pink spots represent proteins higher abundant in gels from control plants of the sensitive genotype, blue spots represent proteins higher abundant in gels from control plants of the tolerant genotype. Numbers behind the protein names give the spot ID of the corresponding spots after Delta2D analysis. Only the first hit, which matches the identified protein, is shown here. Spots are marked on a fused inverted image over all gels.

Supplementary Table 1: Identification of proteins from spots with changed abundance in comparison between the control of the sensitive genotype and the control of the tolerant genotype. A Student´s t-test was performed (p-value ≤0.05) to determine significant changes in spot volume on the basis of normalized relative spot volume. Only alterations of at least 1.5-fold in spot volume were considered to represent true alterations in protein level.

ID[A]	Reg[B]	Protein name	PGSC numbers	KEGG[C]	Score[D]	PICalc[E]	MWCalc[F]	Pep[G]	Method[H]	Size[I]	Normalized spot volume[J]
G2C 154	6.30	superoxide dismutase	PGSC0003DMT400070920	Signal transduction	121	6.60	28305	2	MALDI	m	
G2C 284	4.78	catalase isozyme 1-like protein	PGSC0003DMT400075611	Carbohydrate metabolism	66	6.56	56417	7	MALDI	s	
G2C 96	3.64	ribulose- -bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	123	7.23	40242	3	MALDI	s	
G2C 113	2.61	ribulose bisphosphate carboxylase small chain chloroplastic-like	PGSC0003DMT400032975	Energy metabolism	101	7.60	20306	2	MALDI	m	
G2C 468	2.61	tktc soltuame full chloroplastic short tk flags precursor	PGSC0003DMT400056799	Carbohydrate metabolism	2358.69	5.91	79928	13	QTOF	m	
G2C78	2.43	ribulose- -bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	46	7.23	40242	2	MALDI	m	
G2C 508	2.37	heat shock protein sti like	PGSC0003DMT400012208	Environmental information processing ++	7259.62	5.82	65090	33	QTOF	m	
G2C 583	2.27	threonine dehydratase chloroplastic-like	PGSC0003DMT400033801	Amino acid metabolism	50	5.22	64723	2	MALDI	s	

G2C159	2.25	glycine-rich rna-binding protein grp1a-like	PGSC0003DMT400084266	Genetic information processing++	185	7.79	15550	3	MALDI	m
G2C303	2.11	ribulose- -bisphosphate carboxylase oxygenase large subunit	PGSC0003DMT400083063	Energy metabolism	71	7.23	40242	1	MALDI	s
G2C 577	2.05	PREDICTED: uncharacterized protein At5g39570-like	PGSC0003DMT400003630	uncharacterized	141	4.67	40963	7	MALDI	m
G2C 477	1.80	threonine dehydratase chloroplastic like	PGSC0003DMT400033801	Amino acid metabolism	1265.12	5.04	64723	12	QTOF	m
G2C 165	1.71	chloroplast rubisco activase	PGSC0003DMT400049256	Energy metabolism ++	206	8.10	48089	4	MALDI	s
G2C 288	1.67	osmotin-like protein	PGSC0003DMT400001066	Environmental information processing ++	83	7.56	27158	7	MALDI	m
G2C 305	1.60	peptidyl-prolyl cis-trans isomerase cyp20- chloroplastic-like	PGSC0003DMT400013412	Genetic information processing++	179	9.16	26510	3	MALDI	m
G2C 220	1.58	superoxide dismutase	PGSC0003DMT400042937	Signal transduction	76	7.14	25316	1	MALDI	m
G13C 83	0.08	ribulose bisphosphate carboxylase small chain chloroplastic-like	PGSC0003DMT400032975	Energy metabolism	333	7.60	20306	4	MALDI	m
G13C 523	0.09	catechol oxidase chloroplastic	PGSC0003DMT400076054	Amino acid metabolism	52	6.63	66404	2	MALDI	m

G13C 82	0.14	ribulose- -bisphosphate carboxylase oxygenase small subunit	PGSC0003DMT400050381	Energy metabolism	284	7.56	20562	4	MALDI	m
G13C 466	0.35	peptidyl prolyl cis trans isomerase fkbp62 like	PGSC0003DMT400051809	Genetic information processing++	2086.31	5.05	63456	16	QTOF	m
G13C 491	0.45	far upstream element binding protein 2 like	PGSC0003DMT400031096	Genetic information processing++	1569.80	4.72	82586	9	QTOF	l
G13C 597	0.49	145429 phosphoribulose	PGSC0003DMT400024090	Carbohydrate metabolism	250	5.82	44838	6	MALDI	m
G13C 470	0.53	atp-dependent zinc metalloprotease ftsh chloroplastic-like	PGSC0003DMT400044601	Environmental information processing	220	6.14	74370	22	MALDI	m
G13C 12	0.55	dynamin-related protein 1e-like	PGSC0003DMT400004319	Cellular process ++	157	7.62	68574	16	MALDI	m
G13C 313	0.57	guanine nucleotide-binding protein subunit beta-like	PGSC0003DMT400074094	Genetic information processing++	46	6.71	35953	2	MALDI	m
G13C 101	0.58	20 kdachloroplastic-like	PGSC0003DMT400004324	Environmental Information Processing	57	8.58	14891	1	MALDI	m
G13C 241	0.63	2631191 rna-binding family protein isoform 1	PGSC0003DMT400044866	Genetic Information Processing	199	4.76	53563	3	MALDI	s
G13C 338	0.64	glutathione s-transferase-like	PGSC0003DMT400037918	Metabolism of other amino acids	57	5.98	23817	2	MALDI	m

G13C 349	0.66	plastocyanin precursor	PGSC0003DMT400092049	Energy metabolism	366	5.04	16970	3	MALDI	m	
G13C 375	0.66	atp synthase subunit beta	PGSC0003DMT400002642	Energy metabolism	126	4.78	44598	2	MALDI	s	
G13C 614	0.66	quinone oxidoreductase-like protein chloroplastic-like	PGSC0003DMT400079606	Carbohydrate metabolism ++	370	6.48	41008	6	MALDI	m	

A = ID represents the number of a protein spot in the 2D PAGE gels. Corresponding spots of all gels are labelled with the same ID. G2 stands for the genotype 2, G13 for the genotype 13 and C is standing for control.

B = Regulation of a spot according the comparison between groups. Regulation is given as the ratio between spot abundance (control sensitive genotype / control tolerant genotype).

C = Functional classification according to the KEGG Pathway Database (++ = if no classification was automatically annotated, the proteins were manually classified).

D= The protein score obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database, which was based upon the sequences from *Solanum tuberosum* group Phureja DM1-3, which was completely sequenced by the [9].

E= Calculated PI obtained either via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

F = Calculated MW obtained via the MASCOT search algorithm (www.matrixscience.com) or through the ProteinLynx Global Server 2.5.3 (Water Corporation) against a potato protein database.

G = Number of peptides matched to the protein through the database search.

H = Method with which the protein was identified. MALDI = MALDI-TOF-MS/MS and QTOF = nLC-ESI-Q-TOF-MS/MS.

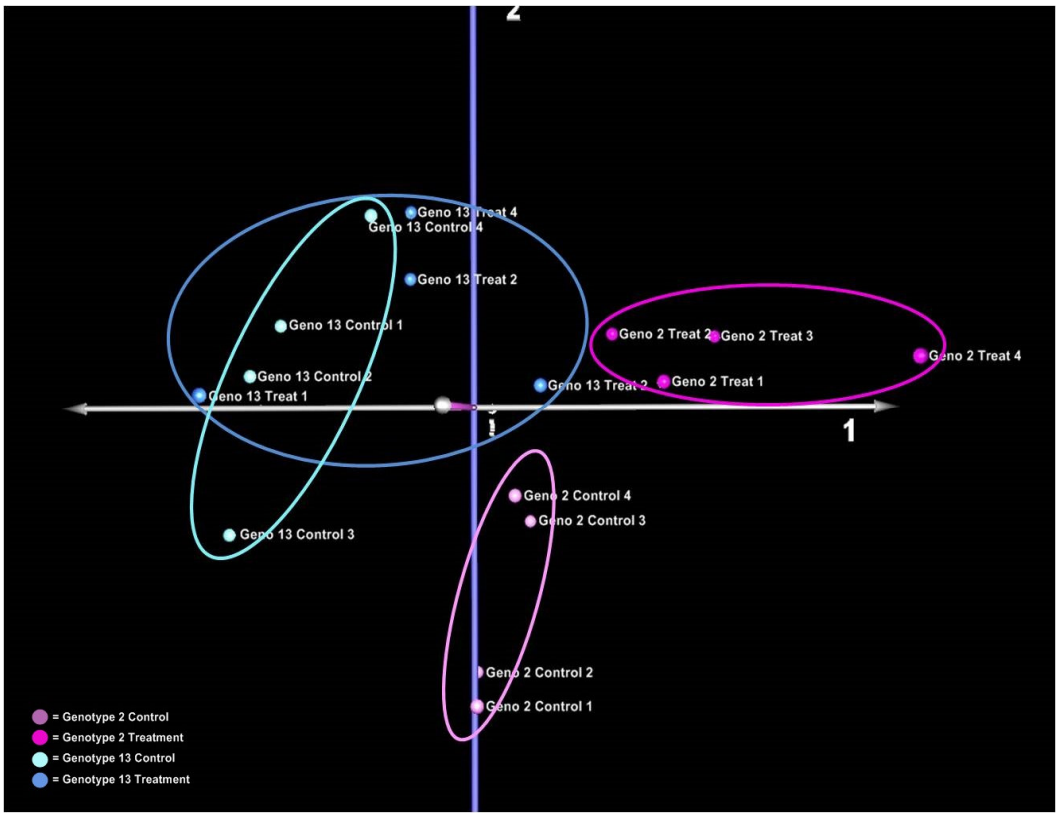
I= Molecular weight (MW) in gel as compared to the theoretically expected MW.

m: MW in gel corresponding to the theoretically expected MW ± 15 kDa

s: MW in gel lower than theoretically expected

l: MW in gel larger than theoretically expected

J= Mean relative spot volume obtained in the four gels of control or plants treated with 0.2 M sorbitol at day 11 illustrated by graphs. The first bar (purple) represents the mean normalized spot volume in the gel of the control plants of the tolerant genotype, the second bar (green) represents the mean normalized spot volume in the gel of the treated plants of the tolerant genotype, the third bar (light purple) stands for the mean normalized spot volume in the gels of the control plants of the sensitive genotype and the fourth bar (light green) represents the mean normalized spot volume in the gel of the treated plants of the sensitive genotype



Supplementary Figure 2: Global sample analysis by PCA. Based on 2D IEF/SDS PAGE gels for the sensitive genotype 2 and gels for the tolerant genotype 13 have been analyzed by PCA using Delta2D software 4.4 (Decodon, Greifswald, Germany). The 3D plot of the samples demonstrates that sample replicates were grouped together, whereas the different genotypes were separated. Larger differences in gel patterns were observed in the sensitive genotype 2. Genotype samples are indicated as follows: rose = Genotype 2 Control; pink = Genotype 2 Treatment; aqua = Genotype 13 Control; blue = Genotype 13 Treatment.

For Supplementary table 2 and 3 see disc in the back of the thesis.

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2.3 Changing compositions of metabolites in *Solanum tuberosum* L. subjected to osmotic stress *in vitro*. Is sorbitol taken up?

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Type of article:	Research article
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Status	Accepted; Plant Cell, Tissue and Organ Culture

Changed composition of metabolites in *Solanum tuberosum* subjected to osmotic stress *in vitro*. Is sorbitol taken up?

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Acknowledgements: The study was conducted with funding provided by the (BMEL) through the Agency of Renewable Resources (FNR e.V.), funding no Federal Ministry of Food and Agriculture.: 22023511. The authors would like to thank Birgit Lippmann for GC/MS support and Dr A. Schum for *in vitro* cultures of the studied potato genotypes.

Key message

The experiments display that various metabolites are regulated differently in an osmotic stress tolerant and a sensitive genotype, respectively. The regularly used osmoticum sorbitol is taken up from the medium.

Keywords

Abiotic stress; in vitro; proline; *Solanum tuberosum*; sorbitol; starch potato, water deficiency

Abstract

Climate change and the increasing world population will lead to an increased water shortage. This gives rise to the need for plant cultivars which are drought tolerant. *Solanum tuberosum* L. is important not only as a nutritive rich food, but starch potatoes are of great value for the industry. Two starch genotypes of *S. tuberosum* L. divergently responding to osmotic stress were subjected to medium containing 0.2 M sorbitol *in vitro*. A targeted metabolomics approach was performed in which 42 metabolites were analysed 11 days after the transfer of the plants to the experimental medium. The sensitive genotype displayed stress responses comprising higher abundant metabolites such as phenylalanine, proline and sucrose and a decrease e.g. in GABA and fumaric acid. These can be used for protein build up, nitrogen storage and the protection through osmotic active compounds. In contrast, the tolerant genotype showed a higher abundance in compounds used as osmolytes (citric acid and proline), which might give rise to acclimatisation to the stress. Interestingly, in chromatograms of both genotypes a high sorbitol peak was detected, whereas control plants or plants treated with 4.8% PEG 8000 did not accumulate this substance. Conclusively, sorbitol is taken up during *in vitro* growth, which raises the question for the fate and effect of the incorporated sorbitol.

Introduction

Economic importance of potato (*S. tuberosum* L.) has led to an increased interest in its response upon biotic and abiotic stress. Especially the starch from starch potatoes is of economic interest as it is used for the production of biopolymers, ethanol, adhesives and as a gelling agent in the pharmaceutical industry. Interest is given to a sustainable production without high inputs of water. Potato plants are known to be drought sensitive due to their shallow root system (Iwama and Yamaguchi 2006). As a consequence, its response under salt and drought stress gathered interest. In this context, i*n vitro* systems have been proposed to be a valuable technique to distinguish the most tolerant and the most sensitive potato genotypes for their performance upon osmotic stress (Gopal and Iwama 2007), because field and pot trials are labour and cost intense and other stresses such as heat might mask drought specific responses.

Osmotic agents such as PEG (polyethylene glycol) and sorbitol have been applied in *in vitro* systems in several studies to simulate water deficiency (Wang et al. 1999; Veselov et al. 2002; Gopal and Iwama 2007; Karimi et al. 2013). These osmotic agents are usually chosen because they reduce the osmotic

potential without bearing toxicity for the plants. Because of the viscosity of medium supplemented with different levels of PEG, 0.2 M sorbitol was found to be more suitable to use for potato screenings under osmotic stress (Gopal and Iwama 2007). Typical stress responses occurring under drought conditions in field grown plants are growth reduction or even arrest and an increase in osmolytes. These responses have also been reported for plants subjected to osmotic stress *in vitro* (Hsiao and Acevedo 1974; Schafleitner et al. 2007; Skirycz and Inzé 2010).

Abiotic stress affects plant metabolism because of an inhibition of specific enzymes, shortage of substrates, higher demands for defined metabolites and a number of other reasons (Obata and Fernie 2012). Metabolite research in potato has mainly focused on the tubers grown in soil (Roessner et al. 2000), how they respond to major pests (Price et al. 1976) and abiotic stress (Geigenberger et al. 1997). Metabolite analysis of the canopy has recently come into focus as stress signals such as the hormone ABA have been shown to be transported across the plant as a root-to-shoot signal transduction pathway (for reviews see Davies and Zhang 1991; Sauter et al. 2001). Thus, a closer look into shoot metabolite composition of *in vitro* grown starch potato plants might lead to a better understanding of osmotic stress tolerance.

This work is part of a bigger study comprising an osmotic stress experiment with 18 *S. tuberosum* genotypes and two wild type species (Bündig et al, under revision) and a proteomic comparison of the two most divergently responding genotypes (Bündig et al, 2016). The present study (i) focuses on comparing major metabolites in a targeted metabolite profiling approach of two divergently responding starch potato genotypes in response to osmotic stress applied by 0.2 M sorbitol *in vitro* and (ii) addresses the question, if sorbitol is taken up by the plant during stress application.

Material and methods

Plant material

Two genotypes of *S. tuberosum* L. divergently responding to osmotic stress, Eurobravo (EUROPLANT Pflanzenzucht GmbH, sensitive to osmotic stress *in vitro*) and Maxi (Bayerische Pflanzenzuchtgesellschaft e.G.& Co KG, tolerant to osmotic stress *in vitro*), were used in this study. *In vitro* plant material of the two genotypes was kindly provided by Dr. A Schum, Julius-Kühn Institute, Groß Lüsewitz. The plant material was grown *in vitro* on solid MS-medium (3% sucrose; 7.5 g/ l plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands) pH = 5.8) (Murashige and Skoog 1962) at constant 18°C in a 16 h light/ 8 h dark cycle with a photosynthetically active photon flux density of approx. 35 µmol m^{-2} s^{-1}. 500 ml plastic vessels were used (material PP, used in the food industry) with ca. 80 ml of solid medium per vessel. Nodal cuttings were used for propagation.

In vitro stress treatment

Plants were grown on MS-medium for 2 weeks before they were transferred to the experimental media. Only shoot tips of about 1 cm length were transferred to solid MS-medium with and without 0.2 M sorbitol as treatment and control, respectively. After 11 days of culture, eight vessels per genotype and treatment with five shoots each were analysed for each repetition of the experiment. 11 days represent the time point when nearly all plants were rooted. Data was collected from two independent experiments. Around 50 mg of plant material (shoot tips) was harvested after around 8 h of light and immediately frozen in liquid nitrogen. The material was then ground with metal beads (stainless steel, 7 mm diameter) to a fine powder for subsequent preparation of polar phase metabolites. Fresh and dry mass was determined after 11 days. To analyse the dry mass, plant material from five additional vessels per repetition was separated into roots and shoots after 11 days, weighed (fresh mass, FM) and dried in an oven at constantly 70°C for 48 hours (dry mass, DM). Genotypes were chosen due to their performance after 21 days of culture under the above mentioned conditions in a larger test set (data not shown). For the 21 day culture, plants were grown as described above for the 11 day culture, but harvested after 21 days .

Metabolite analysis

The metabolite extraction was performed according to Niessen et al. (2012). 1 ml of pre-chilled (-20 °C) extraction mix (1:2.5:1 H_2O:methanol:$CHCl_3$) with an additional 5 µl ^{12}C ribitol was added to the ground powder. The samples were vortexed for 10 sec and shaken for 5 min at 4 °C with a subsequent centrifugation step of 15.7 xg for 2 min at 4 °C. Afterwards 500 µl were transferred into a new reaction tube and 250 µl of ultra pure H_2O was added. The sample was vortexed and centrifuged at 15.7 xg for 2 min at 4 °C. The top layer was collected and 250 µl, 25 µl or 80-fold diluted supernatants were transferred into a glass vial and dried down in a speed vac concentrator until dry. Most of the substances were analysed with the 25 µl samples. After the samples were dry they were transferred to a glass vial and closed with a magnetic lid including a septum (8 mm, thickness 1.3 mm, hardiness 45 °). Derivatisation, addition of standards and sample injection were performed as described by Lisec et al. 2006. The derivatization was performed by the MultiPurposeSampler (Gerstel, Mühlheim an der Ruhr, Germany), which is operated by the Maestro-Software. 30 µl methoxyamin in pyridine was added to the samples and shaken for 90 min at 40 °C. Afterwards the samples were per-sylilated with 50 µl of N-methyl-N-tri-methylsilyltrifluoracetamid + fatty acid methylesters (MSTFA (Sigma-Aldrich, Munich, Germany) + FAMEs) mixture (1.5 ml MSTFA and 37 µl FAMES (0.4 mg/ ml in chloroform)) and shaken for 45 min at 40 °C. Of each sample, one µl was injected in a split less mode. The carrier gas was helium at a constant flow rate of 2 ml/ min. An Agilent 7890 A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) was used with a 30 m VF-5ms capillary column with an integrated pre-column of 10 m and a diameter of 0.25 mm (Agilent VF-5ms 30 x 0.25 (0.25)+10 m EZ-Guard). The admittance temperature was 200 °C and the inlet was a KAS 4 Liner with glass wool. The oven temperature was set to 85 °C for 2 min and heated up stepwise 15 °C/ min until the final temperature of

330 °C was reached. This temperature was held for 6 min. The total runtime was set to 25 min with a subsequent cooling period of 10 min to 85 °C.

The subsequent MS was a Pegasus TOF-MS (Leco, St. Joseph, MI, USA) and the transfer temperature was set to 250 °C. The ion source was also heated to 250 °C. The masses from 70 m/z to 600 m/z were detected in a scan rate of 20 scans/ s. The detector voltage varies with the age of the detector between 1500 and 1800 V. The tuning was performed with Perfluorotributylamine (PFTBA) (Leco, St. Joseph, MI, USA). Chromatografic runs and mass spectra were processed and analysed with the LECO® ChromaTOF® software optimized for Pegasus HT (Leco, St. Joseph, MI, USA) and the TAGFINDER software (Luedemann et al. 2008). Metabolites were only considered when a correlation between two specific masses of at least 0.900 ($R^2 \geq 0.900$) and a number (n) above six (out of eight) was detected for each repetition. The RTI (retention time index) was calculated based on the retention time of each substance. As reference, fatty acid methylesters (FAMEs) of different length were used (FAMEs = C8:0, C9:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0 methylesters; all from Sigma-Aldrich, Munich, Germany; except C:24 methylester VWR, Hanover, Germany). Condensate water and shoot tips from plants treated with 4.8% PEG were processed as described for shoot material from plants treated with 0.2 M sorbitol. Relative and absolute quantification of molecules is based on the peak height of one mass specific for each metabolite on FM (Supplementary Table 2 and Supplementary Figure 1). The absolute values were calculated based on a standard curve of the pure substance on the same column. Afterwards, the concentration of the metabolites was recalculated on DM basis by multiplying each value with a genotype and treatment specific empiric factor obtained from FM und DM measurements after 11 d.

Results

The potato genotypes were chosen due to their differential response to osmotic stress after 21 days *in vitro* and to drought under field conditions as was shown within a larger test set before (Bündig et al. in revision). In this experiment, the plants were grown on MS-medium and MS-medium supplemented with 0.2 M sorbitol for 11 days *in vitro* (Fig1). At this time point, the genotypes showed a significant reduction of 54% and 55% in fresh mass, and a significant reduction of 14% and 15% in dry mass due to the stress treatment (Fig2).

Metabolic profiles resulting from the polar phase extracts of the two genotypes displayed very similar patterns (Fig3). Different concentrations of the polar phase metabolites had to be measured for recognition of low abundant molecules like isoleucine and proper quantification of high abundant molecules like sucrose, respectively. For each dilution, all eight replicates were measured. In a targeted approach, 26 of 42 metabolites could be identified. The identification was based on two specific masses for each metabolite and the retention time index. Of the 26 metabolites, 23 could be quantified by aligning one specific mass each to standard curves (Supplementary Table1). Three compounds could not be properly quantified due to an overlay with another molecule or due to the absence in some

chromatograms (low n number ≤ 6). The overlay of other molecules was identified by a low correlation (R^2 <0.900 for two putatively specific masses). One peak at RTI 670, which represents sorbitol according to the described specifications, showed up only in the treated samples and was marked with a question mark in Fig3. This peak was not observed under control conditions.

When comparing the absolute levels of metabolites in control plants of the sensitive genotype to those of the tolerant genotype fumaric acid, glucose, trehalose and glycine were detected in higher concentration in the tolerant genotype (Table1). In treated plants of the tolerant genotype it was noted that significantly more citric acid, fumaric acid, GABA, oxo-proline and malic acid were detected, whereas more isoleucine, phenylalanine, serine and urea were detected in the sensitive genotype. The metabolites arginine, isocitric acid, lysine, ornithine, spermine, tyrosine and raffinose of the targeted approach were not detectable within the samples.

A ratio between metabolites from treated plants and control plants displayed changes in some of these compounds (Table1, Fig4). A good reproducibility between the repetitions was noticed (Supplementary Fig2). After calculation of the data on the basis of the DM the ratio between stress/ control changed in comparison to the data calculated on FM. The DM was calculated based on an empirical factor. Changes occurred predominantly in the ratio of α-ketogluterate and ascorbate, which were not significantly altered or higher abundant on FM basis, respectively. A higher abundance was also found for glycine, phenylalanine and sucrose in the tolerant and isoleucine and myo-inositol for the sensitive genotype on basis of the FM (Supplementary Table2).

Changes in metabolite content are displayed as the ratio between treatment and control in Fig4. Most metabolite concentrations decreased. The major exception is proline, which increased in both genotypes after osmotic stress application. However, the sensitive genotype also had a higher content of glycine (ratio 3.75), phenylalanine (ratio 1.38), proline (ratio 7.24) and sucrose (1.25) (all significant), whereas the tolerant genotype only displayed an significant increase in proline (11.39) (Fig4, Table1). All other metabolites were lower abundant in both genotypes with a similar decrease being found in α-ketoglutarate (sensitive genotype ratio 0.43, tolerant genotype ratio 0.41), ascorbate (sensitive genotype ratio 0.78, tolerant genotype ratio 0.74), aspartate (sensitive genotype ratio 0.30, tolerant genotype ratio 0.30) and succinate (sensitive genotype ratio 0.40, tolerant genotype ratio 0.38) for both genotypes. For the metabolites spermidine*, putrescine* and phosphoric acid* no satisfactory correlation between two specific masses was found ($R^2 \leq 0.900$) or a low identification number of the substance in the chromatograms ($n \leq 6$) occurred and therefore, these metabolites will further be marked with an asterisk (see Supplementary Table 3 for R^2 values and n numbers). In the sensitive genotype, fumaric acid had the most pronounced decrease of all metabolites (ratio 0.10). In the tolerant genotype this was found for glucose (ratio 0.23) (Table1).

Both genotypes in the stress treatment applied by sorbitol displayed an unknown peak at around RTI 670 (Fig3). This peak was identified as sorbitol. To analyse if sorbitol was taken up by the plant or would only be in the surrounding condensed water touching the medium and the plants, condensed water that covered the medium and the walls of the vessels was compared to the control and treated plant extracts (Fig5) and plants treated with PEG. The peak was not visible in plants treated with PEG (Supplementary Fig3). The overall chromatogram only changed slightly when comparing the 80-fold diluted samples from plants treated with PEG or sorbitol (Supplementary Fig4). In the condensed water from vessels with and without 0.2 M sorbitol the peak only appeared in the condensed water from vessels containing medium with 0.2 M sorbitol (Fig5a), whereas no peak at this position was observed for condensed water from vessels with MS-medium. The peak appears at the specific position of sorbitol and specific masses match the sorbitol standard.

Unfortunately, the putative sorbitol peak is overlapping with several other peaks. Due to its size, it bears a broad spectrum of masses at a great intensity and by this, it overpowers the height of the other peaks (see control samples Fig3). Therefore, a correlation of two specific masses was not satisfactory (R^2 value below 0.500).

Discussion

Response upon osmotic stress

The environmental shift will give rise to more rainfall during winter time and less during summer in temperate regions (Haverkort and Verhagen 2008). This and an increasing population will cause water to become even scarcer in the near future (Chaves et al. 2003). This has led to an increased demand for starch and table potato cultivars with enhanced drought stress tolerance. *In vitro* systems have been shown to be a powerful tool to screen potato cultivars for extreme tolerance or susceptibility (Gopal and Iwama 2007).

After 11 days of culture, a reduction in growth was observed in osmotically stressed plants (Fig1), but regarding the DM no difference between the genotypes was visible (Fig2). The reduction at day 11 of 54% and 55% in FM and the DM reduction of 14% and 15% indicate that most of the lost weight in the FM was due to water loss. However, after 21 days the tolerant genotype displayed nearly the same DM as the control plants (-7%), while the sensitive genotype nearly arrested growth and had a growth reduction of 53%. Conclusively, at some stage between 11 d to 21 d adaptation takes place and plants of the tolerant genotype continue growth (Table1, 21 d data taken from Bündig et al. in revision). The sampling time point at 11 d after transferring the plants to the experimental medium was chosen because at this time point plants were rooted and early stress responses could be analysed.

Differences in metabolites between genotypes after stress application

The highest increase in concentration was observed for proline in both genotypes (Table1, Fig4). Proline is a major compatible solute and is up-regulated under various stress conditions (Delauney and Verma 2008). Moreover, it plays a role in the stabilisation of membranes and prevents the degradation of proteins and enzymes (Farooq et al. 2009). Recently, it was proposed to act as an antioxidant (Das and Roychoudhury 2014). Therefore, an increase in proline reflects the osmotic stress applied. The sensitive genotype accumulated more sucrose, which is readily available *in vitro* through uptake from the medium. Sucrose works as an osmolyte and helps to prevent protein denaturation (Kim et al. 2003). It is the major transport sugar and was shown to accumulate under various stress conditions (Rolland et al. 2006). Moreover, sucrose is the source of raffinose, a sugar with a protective role in osmotically stressed *Arabidopsis* plants (Taji et al. 2002). However, raffinose was not found in any genotype, which displays that under this stress condition it might not be relevant for the plant. Nevertheless, in line with the data from Kim et al. (2003) and Rolland et al. (2006), the accumulation of sucrose in the sensitive potato cultivar is a clear stress avoidance mechanism. However, from this it cannot be concluded that the tolerant genotype does not have avoidance mechanisms as these might comprise other factors.

The increase of phenylalanine in the sensitive genotype might be due to its function as a precursor of phenylpropanoid derivatives, including lignin and flavonoids. Flavonoids, best known as the red and purple anthocyanins are known to rise after stress application (Winkel-Shirley 2002). In *Arabidopsis thaliana* they were shown to accumulate in a stress dependent manner (Sperdouli and Moustakas 2012). Anthocyanins can either simply screen visible radiation and therefore help to reduce excessive excitation energy or can quench ROS as they represent a powerful antioxidant (Rice-Evans et al. 1995; Smillie and Hetherington 1999). In the first reaction step of the phenylpropanoid pathway, phenylalanine is converted by the phenylalanine ammonia-lyase (PAL), which has been reported to be up-regulated after drought in young maize seedlings (Bardzik et al. 1971). This reaction has been described as the rate-limiting step in the synthesis of phenylpropanoid derivatives (Zhang et al. 2013). However, if an increase in phenylalanine in the sensitive genotype might reflect a subsequent increase in downstream products of the phenylpropanoid pathway, should be further be tested.

Glycine was also more abundant in the sensitive genotype after stress. GDC (Glycine decarboxylase complex) is the enzyme complex which catalyses the reaction from glycine to serine during photorespiration in mitochondria. It was shown that high levels of glycine accumulated in drought stressed leaves of barley GDC mutants. This was proposed to be the result of higher photorespiration combined with a decrease of photosynthesis under drought conditions (Wingler et al. 2002). An increase of glycine is therefore probably due to an increased photorespiration in the sensitive genotype. Glycine can also be used as a component to produce glutathione for ROS scavenging, therefore contributing to detoxification responses upon stress (Noctor and Foyer 1998) or to produce glycine-rich proteins, which have been shown to be more abundant after stress application (Mousavi and Hotta 2005). A glycine-rich protein 2-like (PGSC0003DMT400004028) was also found to be more abundant in the sensitive

genotype in the comparison between proteomes of the two genotypes after treatment with 0.2 M sorbitol (Bündig et al. 2016). The ratio between glycine (gly) and serine (ser) was found to be an indicator for higher photorespiration activity (Novitskaya et al. 2002) and is increased in both genotypes. Unfortunately, any further evaluation of the gly/ser ratio is hampered by a huge variation between the replicates. For the tolerant genotype it was shown that in a proteomic comparison a glycolate oxidase was also more abundant after stress application (Bündig et al. 2016), leading to the conclusion that while the calvin cycle might also be affected, an increase in photorespiration clearly takes place. Thus, both genotypes display a probable increase in photorespiration.

Startling decrease of classic stress metabolites after stress application

Surprisingly, the lesser abundant metabolites identified in this study, such as ascorbate, GABA and trehalose are also known to be active components of stress response upon drought stress in many plants. For instance, ascorbate is used in the glutathione-ascorbate cycle, which helps to detoxify hydrogenperoxide (H_2O_2) (for review see Noctor and Foyer 1998). A decrease in ascorbate might show a previously increased detoxification rate. Unfortunately, dehydroascorbate, the product of ascorbate peroxidase, could not be clearly distinguished from other peaks. This should further be tested as ascorbate is a major part of the ROS detoxification network.

A decrease in GABA through an increase in the GABA shunt leading to alanine or glutamate might contribute to an N remobilisation as GABA was proposed to be a transient nitrogen (N) storage compound (Shelp et al. 1999). Even though inorganic N supply is provided by the medium, a remobilisation of organic N might take place. A reduction might also reflect a reduced need for GABA and an increased demand for downstream products even beyond alanine or glutamate. A higher abundance in gamma aminobutyrate transaminase (PGSC0003DMT400062385), the enzyme leading to GABA depletion as part of the GABA-shunt, was found to be more abundant in the sensitive genotype in a comparative proteomic view of the two genotypes (Bündig et al. 2016). The enzymes of the GABA shunt were shown to be important during stress responses, such as the enzyme producing succinate. *Arabidopsis thaliana* ssadh-1 mutants (succinic-semialdehyde dehydrogenase) were shown to accumulate higher levels of H_2O_2 than wild type plants (Bouché et al. 2003). An increase of the GABA shunt activity could therefore contribute to higher detoxification rates as NAD^+ is used to produce NADH.

Trehalose was also decreasing after stress application. Thus, trehalose itself obviously does not fulfil any role in osmotic stress protection in potato and its decrease seems to have no significant impact on the response of the tolerant phenotype. Its protective function has largely been replaced by sucrose in most plants (El-Bashiti et al. 2005). Many plants only accumulate very small amounts of trehalose and it was proposed that rather trehalose-6-phosphate is the responsive element to drought as it is a main signalling molecule in plants upon stress (Smeekens et al. 2010). Therefore, a reduction of trehalose in

potato plants after osmotic stress might reflect an increased need for either trehalose-6-phosphate as a precursor or other downstream products.

Fumaric acid showed the highest decrease in the sensitive genotype of all analysed metabolites under osmotic stress conditions. Fumaric acid is proposed to be a carbon storage compound, which can like other compounds of the TCA-cycle be used as a carbon backbone for amino acids (Chia et al. 2000). Because α-ketoglutarate is converted to fumarate in the TCA cycle, a reduction in α-ketoglutarate might lead to a depletion of other compounds in the TCA cycle e.g. fumarate. This leads to a picture where α-ketoglutarate is drawn from the TCA cycle and subsequently glutamate is produced. Glutamate, which was also found to be lower abundant, is the primary compound in proline synthesis, which is shown to be strongly enhanced. However, glutamate can also be used as a substrate for the GABA shunt and its decrease might support an increased flux through the GABA shunt.

The strongest reduction in the tolerant genotype was found for glucose. Sugars not only provide important energy and act as a carbon source, but were also shown to be important signalling molecules (Rolland et al. 2006). The ratio between glucose/ sucrose decreased from 0.34 to 0.13 in the sensitive and from 0.53 to 0.17 in the tolerant genotype. The ratio between glucose (gluc) to sucrose was shown to decrease after salt stress application in tomato leaves of a sensitive cultivar, indicating a blockage of the utilisation of sucrose by the plant (Khelil et al. 2007). If a blockage in the exploitation of sucrose over time is also present in both genotypes should be tested further.

Comparing the treated plant extracts reveals different stress responses

Comparison of the extracts from plants treated with 0.2 M sorbitol between the genotypes displayed a higher amount of citric acid, oxo-proline and a lesser down-regulation of GABA in the tolerant genotype. Oxo-proline was proposed to act as an osmoprotectant. It is an intermediate of glutathione breakdown and is a precursor of glutamate (Ohkama-Ohtsu et al. 2008). A higher amount under stress conditions in the sensitive genotype compared to tolerant genotype was found for the amino acids isoleucine and phenylalanine. As was mentioned before, a higher abundance of phenylalanine might be due to its importance as a precursor for the phenylpropanoid pathway. However, a higher abundance in both amino acids was shown to be present during energy depletion when dark phases were prolonged. This was probably due to protein breakdown (Gibon et al. 2006). As was proposed after proteomic analysis of these two potato genotypes, proteolysis takes place in the sensitive genotype (Bündig et al. 2016) and an increase in these two amino acids therefore contributes to the overall picture of protein breakdown.

Because an accumulation of a certain metabolite can always be the cause of an up-regulation of upstream reactions or a down-regulation of downstream reactions, combining these data with transcriptomic and proteomic data (first data available as indicated above) will help to distinguish specific pathways in potato shoots affected by osmotic stress *in vitro*.

Sorbitol is taken up by potato plants *in vitro*

A very high peak was observed at about RTI 670 (Fig3). The peak appears at the specific position of sorbitol and specific masses match the sorbitol standard. To determine if sorbitol is derived from externally attached condensed water or if it was taken up, plant samples were compared with the condensed water from the wall of the vessels, which was in contact with the medium with and without 0.2 M sorbitol. The peak also showed up in condensed water touching the medium from vessels containing 0.2 M sorbitol in the medium (Fig5a), but neither in the control extracts from plants nor in the condensed water touching the medium from control vessels. The peak was also absent in plants treated with PEG (Supplementary Fig3). The peak height from the condensed water and the plant extract from the vessels with MS-medium containing 0.2 M sorbitol displayed nearly the same height (Fig5b). Because the plant sample was diluted 80-fold and the condensed water 10-fold, it can be assumed that the sorbitol found in the treated plant extract was not only a result of external sorbitol, but sorbitol must have been taken up by the plants.

An ideal osmotic agent would be non-toxic, non-penetrating yet reducing the osmotic potential of the medium. Therefore, it was proposed that mannitol as an osmoticum would not be a good choice, as it was shown to be taken up by potatoes (Lipavska and Vreugdenhil 1996). However, sorbitol and PEG of high molecular weight (>6000) have been thought to be good candidates to use for reducing the osmotic potential of the medium for potato selection, where sorbitol was later favoured (Gopal and Iwama 2007). PEG was identified by Gopal and Iwama (2007) as being problematic in *in vitro* cultures as a high viscosity of the medium supplemented with PEG might limit O_2 movement resulting in an O_2 deficiency in the roots. It was proposed by Criel et al. (2006) that sorbitol was taken up by potatoes *in vitro*, but no clear evidence had been documented. The presented data clearly demonstrates that sorbitol is taken up. This leads to the question if sorbitol is a suitable choice for experiments similar to the one conducted.

However, it should further be analysed if sorbitol can also be taken up by unwounded plants *in vitro* as in this experimental set up sorbitol was taken up by potato shoot tips. As these were cut and immediately transferred to medium containing 0.2 M sorbitol, this might lead to an accumulation of sorbitol before rooting. Shoot tips should therefore first be rooted and then subjected to osmotic stress in further studies.

These results demonstrate that the two starch potato genotypes, divergently reacting to 0.2 M sorbitol *in vitro* deal differently with the applied stress. While the sensitive genotype displays a picture where amino acids from protein degradation, an increased demand for energy and an externally supplied osmolyte through the higher accumulation of sucrose is most important, the tolerant genotype only shows an increase in a major osmoprotecting compound. This appears to give rise to the suggestion that the tolerant genotype might have adapted to the applied stress *in vitro* after 11 days, whereas the sensitive genotype still suffers from osmotic stress and takes countermeasures in building up new metabolites and proteins for protection. In a further step it should be analysed if the current findings hold true for other

genotypes with known tolerance. However, the main result of the conducted experiments was that both genotypes displayed an uptake of sorbitol. If this is also metabolised or only stored in potatoes should be tested further.

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Figures and Tables

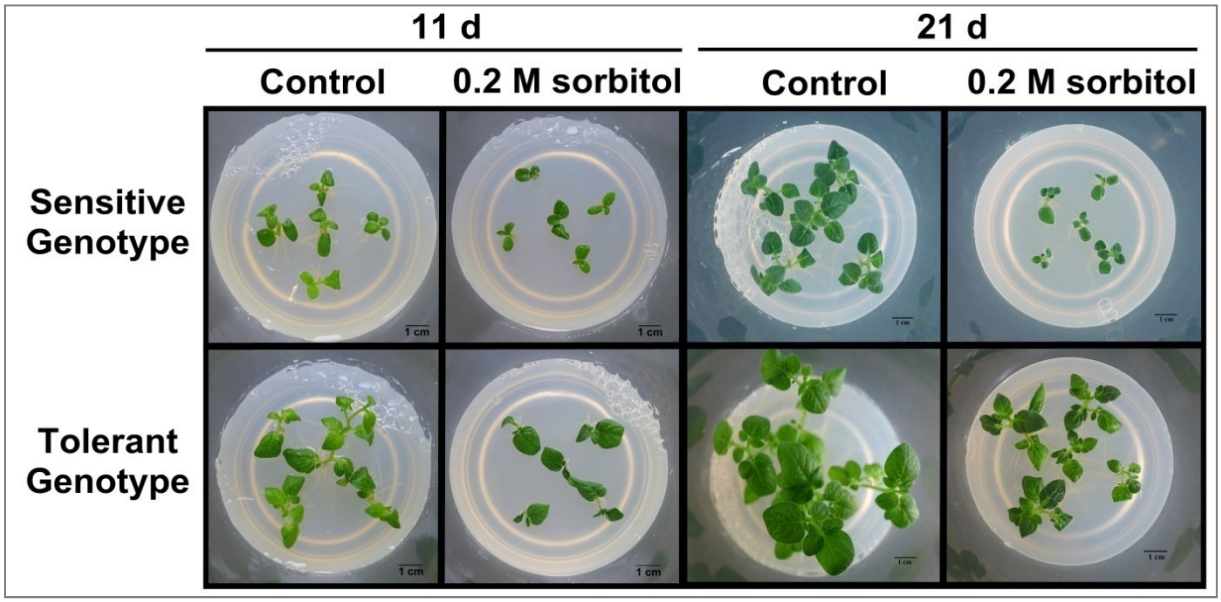


Figure 1: Plants of the two contrasting genotypes after 11 or 21 days of culture on control medium or medium supplemented with 0.2 M sorbitol. Bars indicate 1 cm. Sensitive genotype = Eurobravo, tolerant genotype = Maxi.

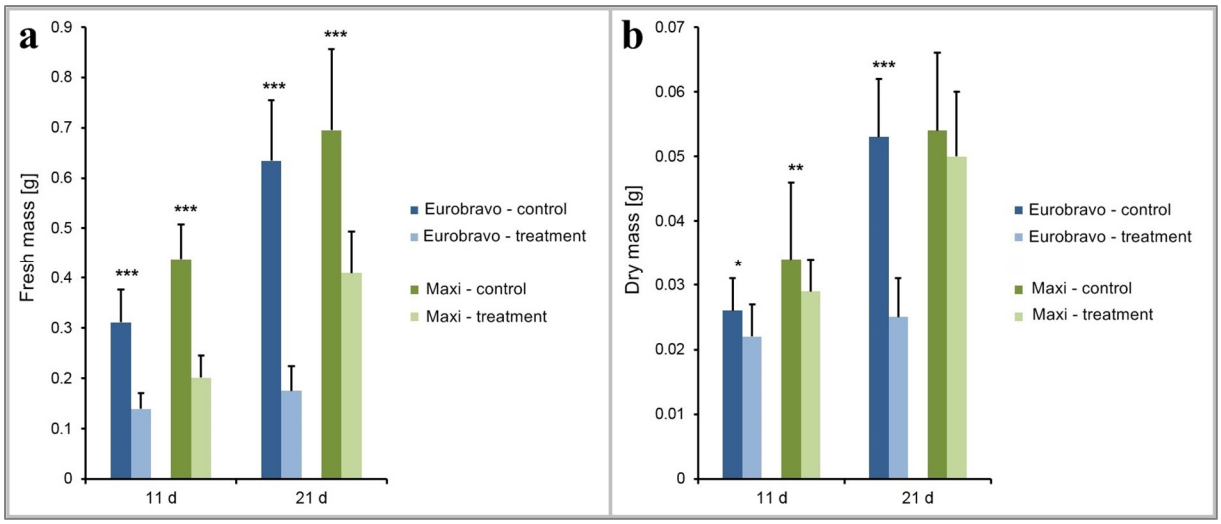


Figure 2: Measured fresh mass and dry mass ± SD after 11 and 21 days after transfer of the plants to medium with and without 0.2 M sorbitol. **a** = fresh mass; **b** = dry mass. 21 day data taken from the in vitro screening in a larger test set (Bündig et al. under revision). Asterisks behind the 0.2 M sorbitol values display significant difference according to student´s t-test between control and treatment on day 11 and 21, respectively (*** ≤ 0.001, ** ≤0.01, *≤ 0.05). 11d n = 10; Maxi control n = 9; 21 days n = 20; Eurobravo control n =19.

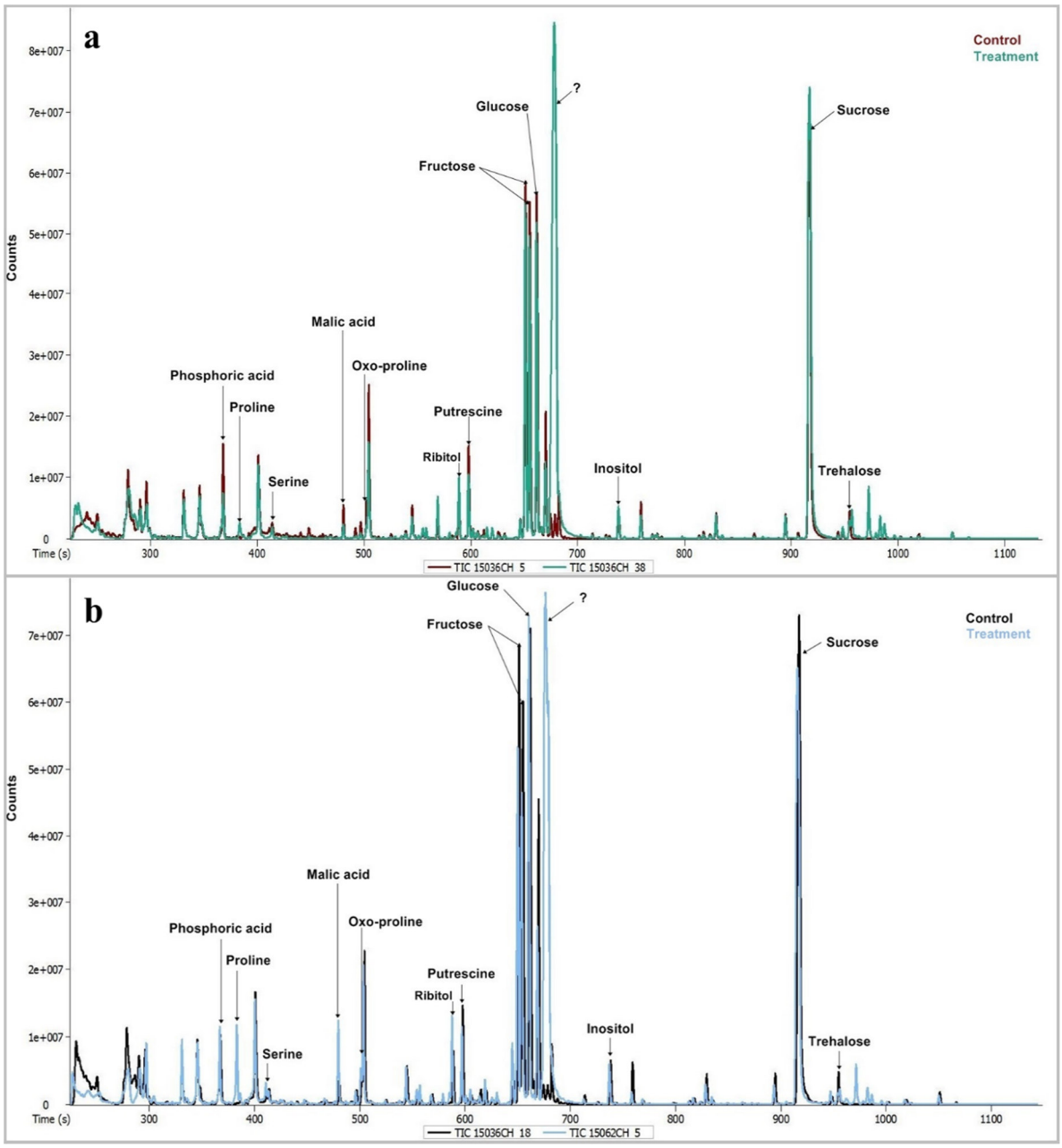


Figure 3: Exemplary chromatograms in the TIC (total ion count) view of samples for both genotypes with indication of some of the 28 compounds detected. **a** = sensitive genotype Eurobravo (red = control, green = treatment with 0.2 M sorbitol), **b** = tolerant genotype Maxi (black = control, blue = treatment with 0.2 M sorbitol. 25 µl of sample volume was analysed.

Table 1: Mean values and standard deviation of detected metabolites in µmol/ g DM in shoot samples after 11 days of culture on medium with and without 0.2 M sorbitol *in vitro*. The ratio between the 0.2 M sorbitol treatment and the control is given. Asterisks behind the ratio display significant difference between control and treatment (*** ≤ 0.001, ** ≤0.01, *≤ 0.05). For putrescine, spermidine and phosphoric acid the peak height over all samples is given. Green = more abundant after stress; orange = less abundant after stress, yellow = significant differences between genotypes. n = see Supplementary Table 3 for individual n numbers. Sensitive genotype = Eurobravo, tolerant genotype = Maxi.

	Sensitive genotype			Tolerant genotype		
	Control [A] Mean ± SD	0.2 M sorbitol [B] Mean ± SD	Ratio Stress:control	Control Mean ± SD	0.2 M sorbitol Mean ± SD	Ratio Stress:control
Alpha-ketoglutarate (198)	16.60 ± 6.92	7.13 ± 1.61	0.43***	12.94 ± 4.93	5.28 ± 1.77	0.41***
Ascorbate (173)	4.63 ± 1.12	3.61 ± 0.90	0.78**	5.89 ± 1.18	4.36 ± 0.98	0.74***
Asparagine(188)	63.46 ± 33.41***	135.71 ± 52.38***	2.14	326.11 ± 338.02	11.83 ± 12.06	0.04***
Aspartate (232)	6.40 ± 2.20	1.93 ± 0.34	0.30***	5.75 ± 2.21	1.70 ± 0.43	0.30***
Citric acid (183)	1.99 ± 0.94	0.91 ± 0.24**	0.46***	1.25 ± 0.52	1.61 ± 0.51	1.29
Fructose (217)	152.36 ± 38.78	78.62 ± 26.41*	0.52***	253.77 ± 49.95	94.49 ± 20.01	0.37***
Fumaric acid (245)	3.51 ± 1.76**	0.34 ± 0.10***	0.10***	6.35 ± 1.99	3.35 ± 1.23	0.53***
GABA (174)	2.44 ± 1.14	0.61 ± 0.09***	0.25***	3.40 ± 1.25	1.20 ± 0.34	0.35***
Glucose (319)	114.69 ± 35.93***	53.22 ± 20.07	0.46***	251.88 ± 57.02	58.41 ± 49.59	0.23***
Glutamate (246)	25.60 ± 10.29	13.71 2.12±	0.54***	35.70 ± 12.35	12.26 ± 2.32	0.34***
Glycine (174)	1.20± 0.49***	4.49± 1.71	3.75***	4.82 ± 3.87	3.53 ± 1.51	0.73
Isoleucine (158)	0.67 ± 0.21	0.59 ± 0.14***	0.88	0.65 ± 0.28	0.32 ± 0.09	0.50***
Malic acid (233)	11.10 ± 4.97	3.91 ± 0.80***	0.35***	12.28 ± 4.47	8.56 ± 3.19	0.70**
myo-Inositol (305)	7.58 ± 2.09	7.00 ± 0.74	0.92	10.13 ± 3.64	7.99 ± 1.74	0.79*
oxo-proline (258)	39.56 ± 18.09	14.22 ± 4.78*	0.36***	42.41 ± 17.96	22.16 ± 9.19	0.52***
Phenylalanine (218)	0.87 ± 0.20	1.20 ± 0.17***	1.38***	0.69 ± 0.29	0.56 ± 0.15	0.81
Proline (142)	1.22 ± 0.63	8.85 ± 3.40	7.24***	0.87 ± 0.35	9.87 ± 5.75	11.39***
Serine (204)	3.93 ± 1.12	2.49 ± 0.57***	0.63***	4.18 ± 1.80	1.30 ± 0.38	0.31***
Succinate (247)	1.43 ± 0.91	0.58 ± 0.28	0.40***	1.37 ± 0.78	0.52 ± 0.23	0.38***
Sucrose (437)	334.10 ± 109.49	416.73 ± 108.58	1.25*	471.62 ± 96.10	337.68 ± 29.51	0.72**
Threonine (219)	1.79 ± 0.60	0.74 ± 0.18	0.41***	1.40 ± 0.59	0.52 ± 0.15	0.37***
Trehalose (191)	2.18± 0.71**	1.69 ± 0.96	0.78	4.46 ± 2.67	2.17 ± 0.90	0.49**
Urea (171)	1.60 ± 0.91	0.75 ± 0.43***	0.47***	1.12 ± 0.73	0.30 ± 0.17	0.26***
Putrescine	2257*10^6 ± 1021*10^6	1226*10^6 ± 446*10^6	0.54**	2187*10^6± 1473*10^6	924*10^6± 313*10^6	0.42***
Spermidine	4.7*10^6± 5.5*10^6	5.1*10^6 ± 4.8*10^6	1.08	14*10^6± 15*10^6	6.8*10^6± 7.6*10^6	0.49
Phosphoric acid	24445*10^6± 8726*10^6	9730*10^6 ± 5247*10^6	0.40***	23008*10^6 ± 11674*10^6	10250*10^6± 3751*10^6	0.45**

A = Asterisks display significant difference between the control of the sensitive and the control of the tolerant genotype (*** ≤ 0.001, ** ≤0.01, *≤ 0.05) For putrescine, spermidine and phosphoric acid the difference was calculated based on the peak height over all samples.

B = Asterisks display significant difference between the treated plants of the sensitive and the plants treated with 0.2 M sorbitol of the tolerant genotype (*** ≤ 0.001, ** ≤0.01, *≤ 0.05) For putrescine, spermidine and phosphoric acid the difference was calculated based on the peak height over all samples.

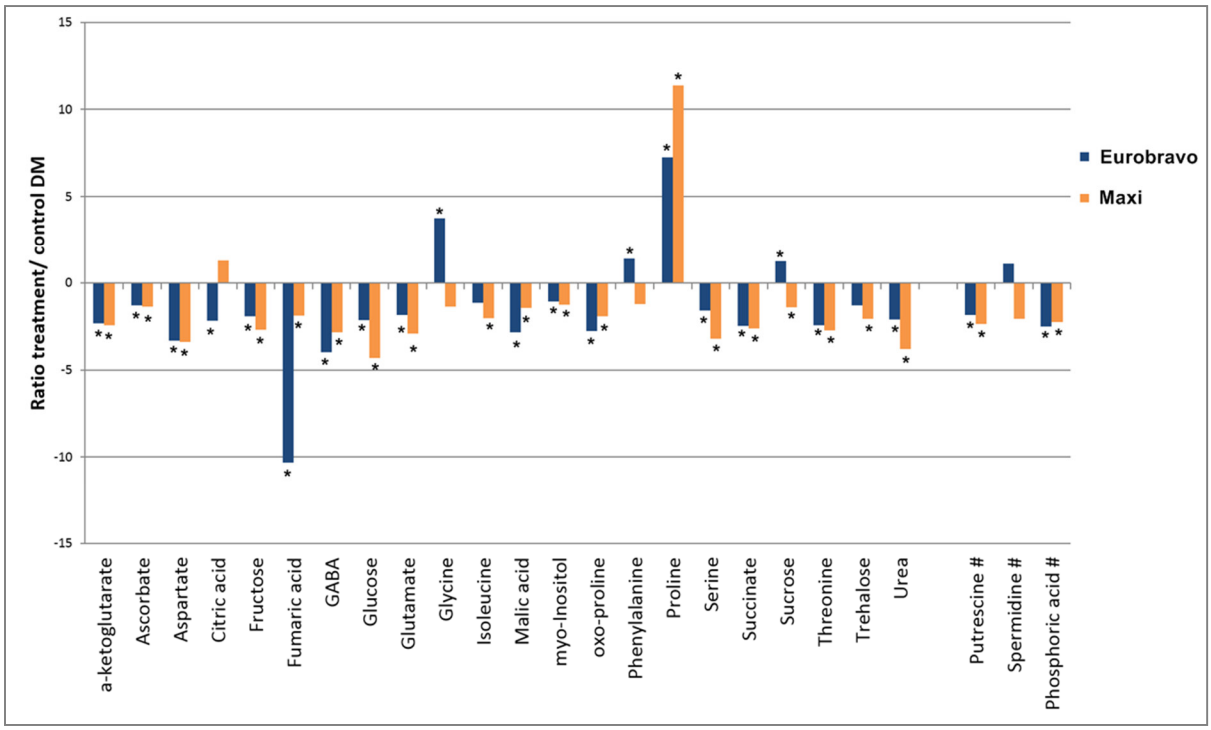


Figure 4: Changes in metabolite content after treatment with 0.2 M sorbitol compared to control samples. Ratios were based on values in µmol/ g DM. Ratios below 1 were inversed to display decrease. Blue = sensitive genotype Eurobravo, Orange = tolerant genotype Maxi. # = ratio based on peak height. * = indicates significant differences between control and treatment.

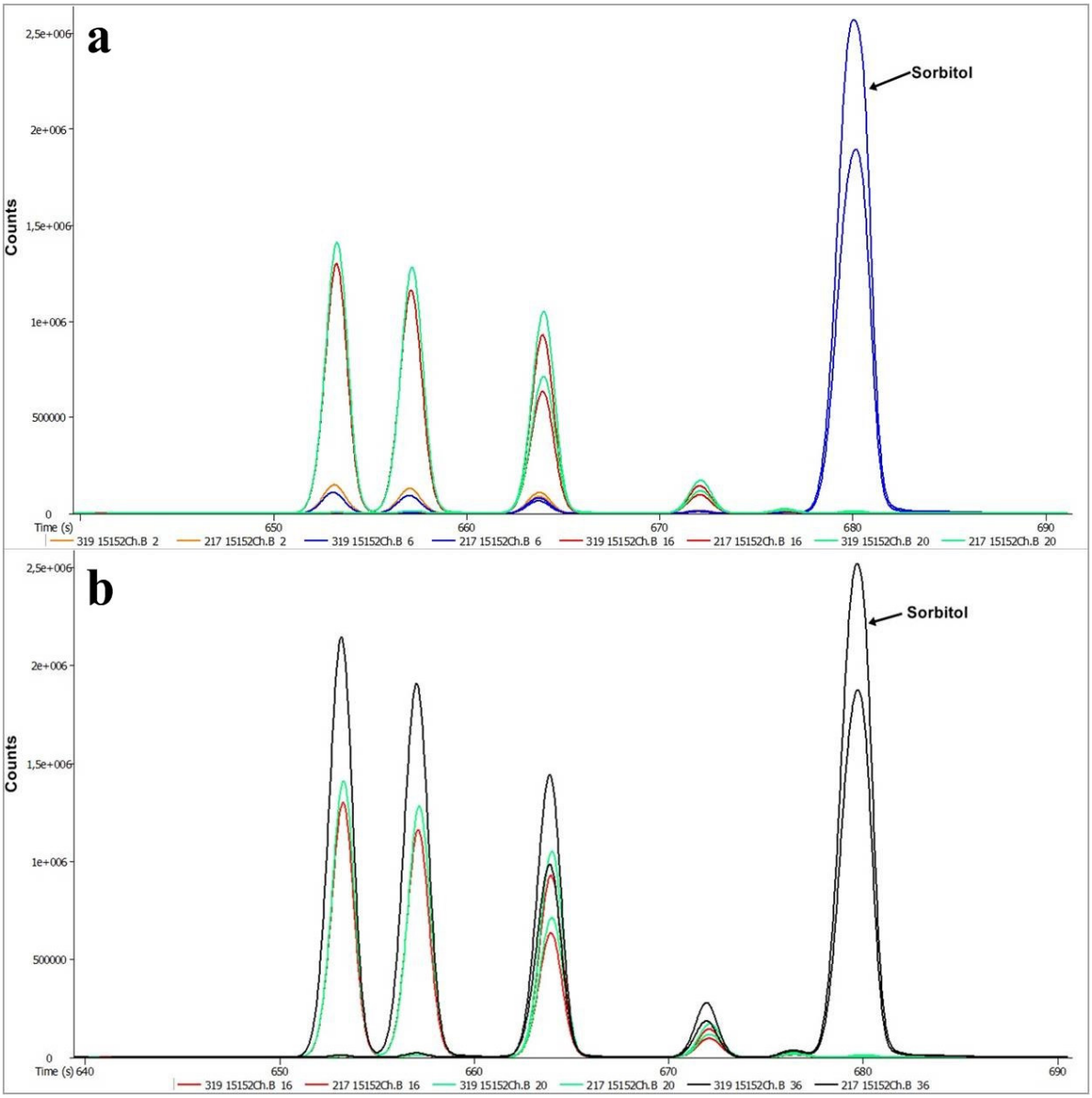


Figure 5: Chromatograms of condensed water from vessels with medium containing 0.2 M sorbitol and the 80-fold diluted extracts from shoots treated with 0.2 M sorbitol. **a** = condensed water from vessels with control medium (2 samples, orange lines) and condensed water from vessels containing medium with 0.2 M sorbitol (2 samples, blue lines); **b** Condensed water sample from vessels with control medium (orange lines), plant samples grown on control medium (pink lines), condensed water samples from vessels containing medium with 0.2 M sorbitol (blue lines) and mixed extracts from plants grown on control medium plus 80 fold-diluted plant samples treated with 0.2 M sorbitol (green lines). Each time 25 µl were analysed.

Supplementary data

Supplementary Table 1: List of metabolites considered in the targeted metabolite approach. For the first 36 metabolites a standard curve was drawn from pure substances. For substances which could be detected in the chromatograms (marked with an X) a concentration was calculated.

Metabolite	Detected [A]	Mean RTI [B]	Dilution factor [C]
1. Alanine (190)	No clear peak	-	
2. β-alanine (248)	No clear peak	-	
3. Arginine (142)	Not detected	-	
4. Ascorbate (173)	X	632345	80-fold
5. Asparagine (188)	X	543530	10-fold
6. Aspartic acid (232)	X	477898	10-fold
7. Citric acid (183)	X	617211	10-fold
8. Citrulline (157)	Not calculable	-	
9. Cysteine (220)	Not calculable	-	
10. Dehydroascorbate (303)	No clear peak	-	
11. Fructose (217)	X	639234	80-fold
12. Fumaric acid (245)	X	389288	10-fold
13. GABA (174)	X	484339	10-fold
14. Glucose (319)	X	649283	80-fold
15. Glutamate (246)	X	527613	10-fold
16. Glycine (174)	X	-	10-fold
17. Isocitric acid (245)	Not detected	-	
18. Isoleucine (158)	X	355821	10-fold
19. a-ketoglutarate (198)	X	507304	10-fold
20. Leucine (158)	No clear peak	-	
21. Lysine (174)	Not detected	-	
22. Malic acid (233)	X	-	10-fold
23. Methionine (176)	No clear peak	-	
24. Myo-Inositol (305)	X	727800	10-fold
25. Ornithine (142)	Not detected	-	
26. oxo-Proline (258)	X	483172	10-fold
27. Phenylalanine (218)	X	534187	10-fold
28. Proline (142)	X	360629	10-fold
29. Serine (204)	X	392012	10-fold
30. Shikimic acid (204)	Not calculable	-	
31. Succinate (247)	X	369163	10-fold
32. Sucrose (437)	X	910814	80-fold
33. Threonine (218)	X	405870	10-fold
34. Trehalose (191)	X	945781	250 µl
35. Urea (171)	X	328515	10-fold
36. Valine (144)	No clear peak	-	10-fold
37. Putrescine (174)	X (relative value)•	583255	10-fold
38. Spermidine (144)	X (relative value)	790581	250 µl
39. Spermine (144)	Not detected	-	
40. Phosphoric acid (299)	X (relative value)	332260	10-fold
41. Tyrosine (280)	Not detected	-	
42. Raffinose (437)	Not detected	-	

A= Overview of detected samples from the test set.

X: Detected and concentration calculated

No clear peak: No clear peak could be matched to the substance; calculation was not possible

Not detected: Substance was not detectable in the sample.

Not calculable: The R^2 for two masses of these substances were below 0.900. Therefore, these substances were not considered as a pure peak (see Supplementary table 4 for R^2 values).

X (relative value): No standard curve was available for this substance; therefore, values are given as peak height.

•: The R^2 value for the metabolite was lower than 0.900 in one of the comparisons; calculations are made based on all measurements, but should be handled with care.

B = Mean retention time index (RTI) over all chromatograms for the given substance.

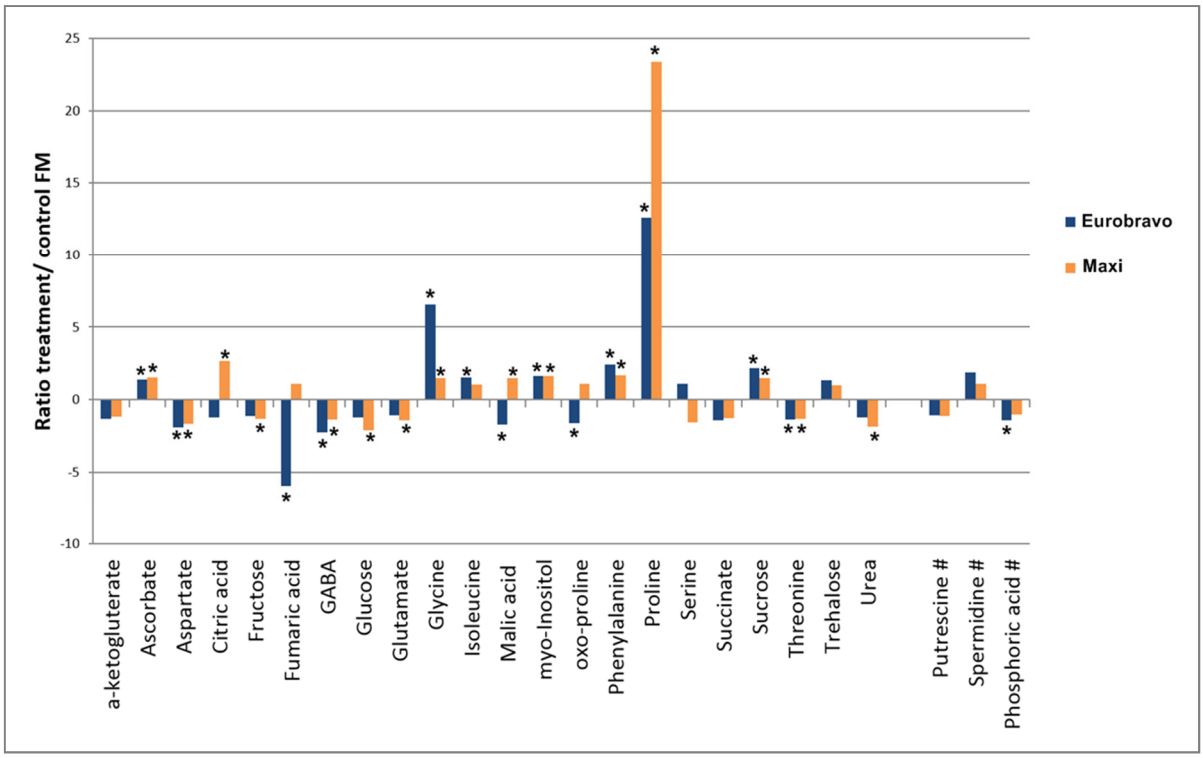
C= Volume analysed for the compound. 250 µl = original volume. 10-fold = 10-fold dilution of the 250 µl and 80-fold = 80-fold dilution of the 250 µl

Supplementary Table 2: Mean values and standard deviation of detected substances in µmol/ g F M in shoot samples after 11 days of culture on medium with and without 0.2 M sorbitol *in vitro*
The ratio between the 0.2 M sorbitol treatment and the control is given. Asterisks behind the ratio displ ay significant difference between control and treatment (*** ≤ 0.001, ** ≤0.01, *≤ 0.05). For putrescin e, spermidine and phosphoric acid the peak height over all samples is given. Green = more abundant af ter stress; red = less abundant after stress, grey = equal after stress. Green = more abundant after stress; orange = less abundant after stress, yellow = significant differences between genotypes. n = see Supple mentary Table 3 for individual n numbers. Sensitive genotype = Eurobravo, tolerant genotype = Maxi.

	Sensitive genotype			Tolerant genotype		
	Control [A] Mean ± SD	0.2 M sorbitol [B] Mean ± SD	Ratio Stress:control	Control Mean ± SD	0.2 M sorbitol Mean ± SD	Ratio Stress:control
alpha-ketoglutarate (198)	1.53± 0.64	1.14± 0.26	0.75	1.01± 0.38	0.85± 0.28	0.84
Ascorbate (173)	0.43± 0.10	0.58± 0.14	1.36***	0.46± 0.09	0.70± 0.16	1.52***
Asparagine(188)	5.84± 3.07**	21.71± 8.38***	3.72***	25.44± 26.37	1.89±1.93	0.07***
Aspartate (232)	0.59± 0.20	0.31± 0.05	0.52***	0.45± 0.17	0.27± 0.07	0.61***
Citric acid (183)	0.18± 0.09**	0.15 ± 0.04**	0.80	0.10± 0.04	0.26± 0.08	2.65***
Fructose (217)	14.02± 3.57	12.58± 4.23	0.90	19.79± 3.90	15.12± 3.20	0.76**
Fumaric acid (245)	0.32± 0.16	0.05± 0.02***	0.17***	0.50± 0.16	0.54± 0.20	1.08
GABA (174)	0.22± 0.10	0.10± 0.01	0.44***	0.27± 0.10	0.19± 0.05	0.72*
Glucose (319)	10.55± 3.31***	8.52± 3.21	0.81	19.65± 4.45	9.35± 7.93	0.48***
Glutamate (246)	2.35± 0.95	2.19 ± 0.34	0.93	2.78± 0.96	1.96± 0.37	0.70**
Glycine (174)	0.11± 0.05***	0.72± 0.27	6.53***	0.38±0.29	0.56± 0.24	1.50**
Isoleucine (158)	0.06± 0.02	0.09 ± 0.02***	1.53***	0.05± 0.02	0.05± 0.01	1.02
Malic acid (233)	1.02 ± 0.46	0.59 ± 0.20***	0.57**	0.93± 0.35	1.37± 0.51	1.47**
myo-Inositol (305)	0.70± 0.19	1.12± 0.12	1.61***	0.79± 0.28	1.28± 0.28	1.62***
oxo-proline (258)	3.64± 1.66	2.27± 0.76*	0.63**	3.31± 1.40	3.55± 1.47	1.07
Phenylalanine (218)	0.08± 0.02	0.19± 0.03***	2.41***	0.05± 0.02	0.09± 0.02	1.67***
Proline (142)	0.11± 0.06*	1.42± 0.54	12.60***	0.07± 0.03	1.58± 0.92	23.36***
Serine (204)	0.36± 0.10	0.40± 0.09	1.10	0.33± 0.14	0.21± 0.06	0.64
Succinate (247)	0.13± 0.08	0.09± 0.04	0.70	0.11± 0.06	0.08± 0.04	0.79
Sucrose (437)	30.74± 10.07	66.68± 17.37	2.17***	36.79± 7.50	54.03± 4.72	1.47***
Threonine (219)	0.16± 0.06	0.12± 0.03	0.72*	0.11± 0.05	0.08± 0.02	0.75*
Trehalose (191)	0.20± 0.07	0.27± 0.15	1.35	0.35± 0.20	0.35± 0.14	1.00
Urea (171)	0.15 ± 0.08*	0.12± 0.07***	0.82	0.09± 0.06	0.05± 0.03	0.54**
Putrescine (174)	$208*10^6$± $94*10^6$	$196*10^6$±$71*10^6$	0.94	$165*10^6$± $114*10^6$	$148*10^6$± $50*10^6$	0.90
Spermidine (144)	$0.4*10^6$± $0.5*10^6$	$0.8*10^6$± $0.7*10^6$	1.88	$1.0*10^6$± $1.2*10^6$	$1.1*10^6$± $1.2*10^6$	1.06
Phosphoric acid (299)	$2249*10^6$± $803*10^6$	$1557*10^6$± $840*10^6$	0.69*	$1718*10^6$± $921*10^6$	$1640*10^6$± $600*10^6$	0.95

A = Asterisks display significant difference between the control of the sensitive and the control of the tolerant genotype (*** ≤ 0.001, ** ≤0.01, *≤ 0.05) For putrescine, spermidine and phosphoric acid the difference was calculated based on the peak height over all samples.

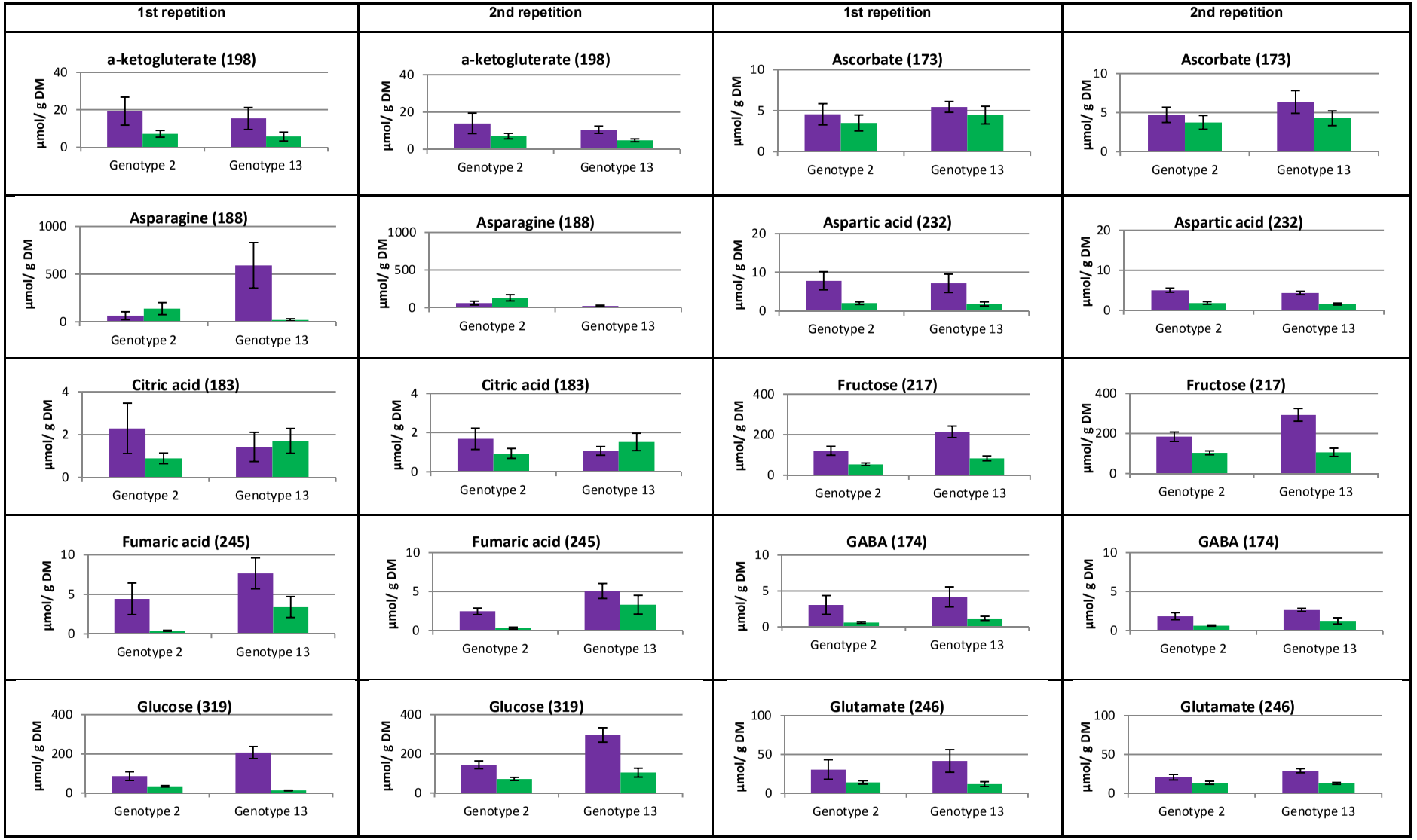
B = Asterisks display significant difference between the treated plants of the sensitive and the plants treated with 0.2 M sorbitol of the tolerant genotype (*** ≤ 0.001, ** ≤0.01, *≤ 0.05). For putrescine, spermidine and phosphoric acid the difference was calculated based on the peak height over all samples.

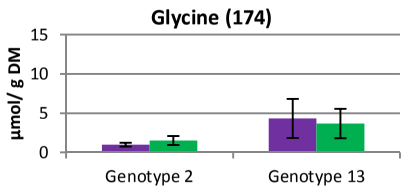
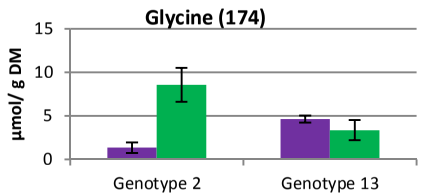
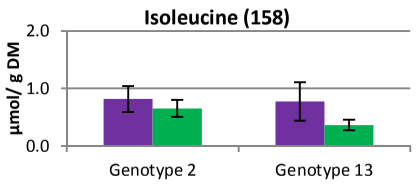
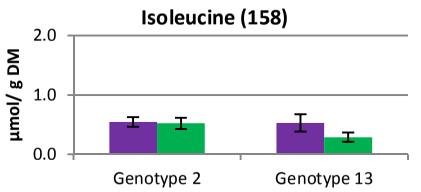
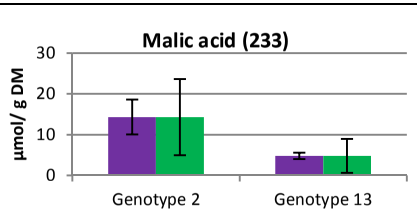
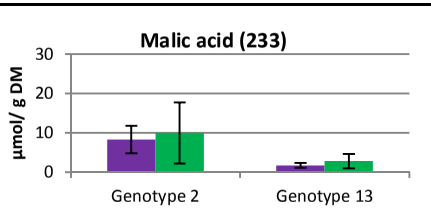
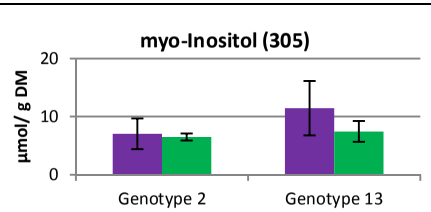
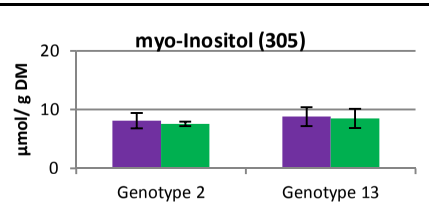
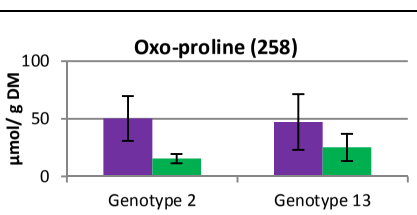
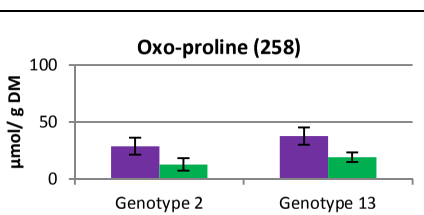
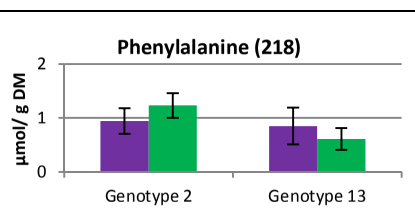
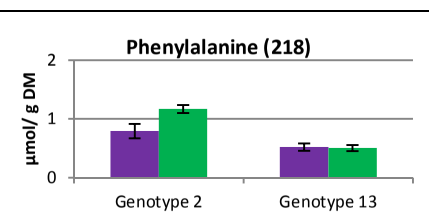
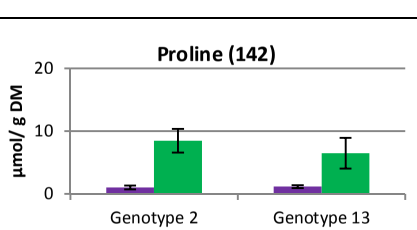
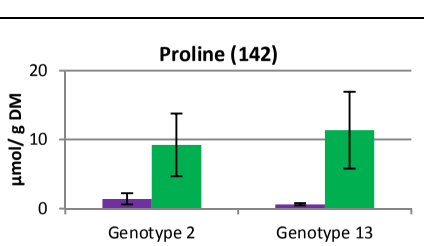
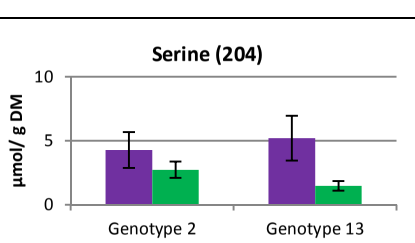
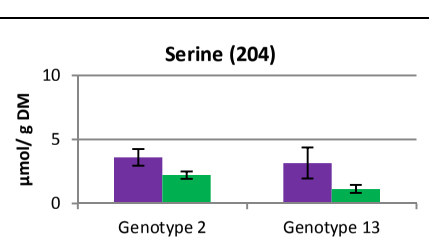
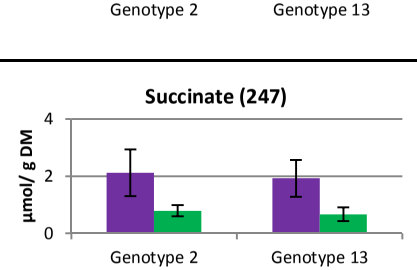
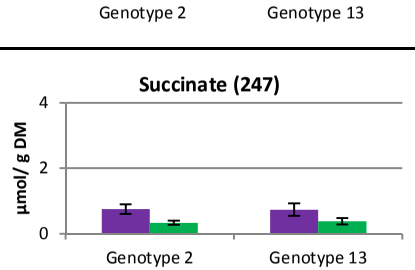
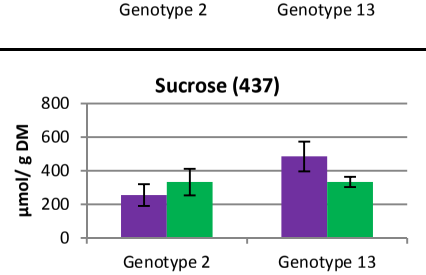
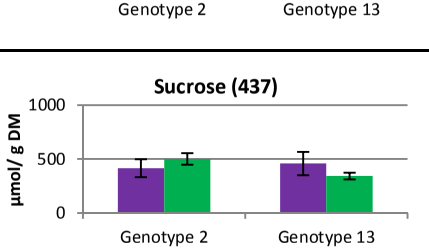


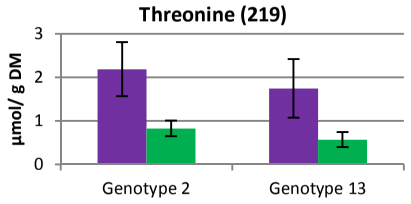
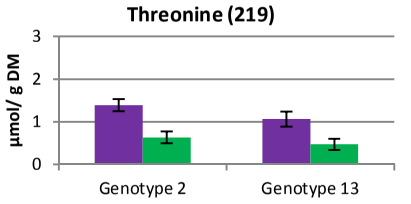
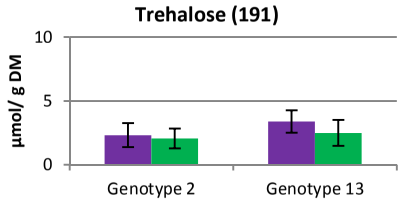
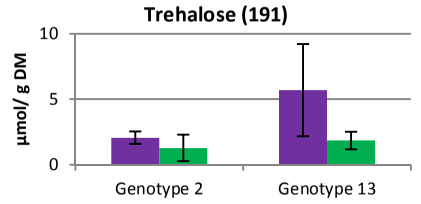
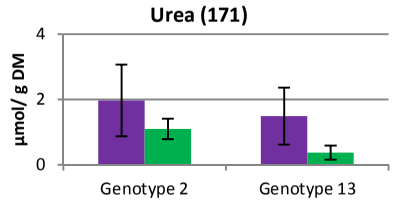
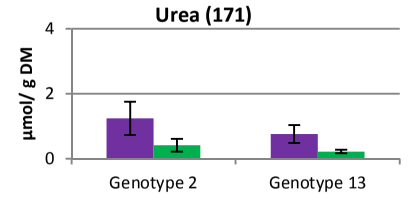
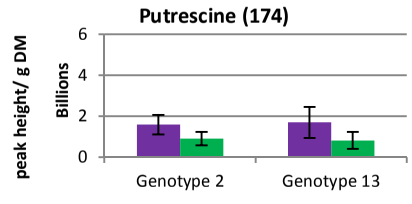
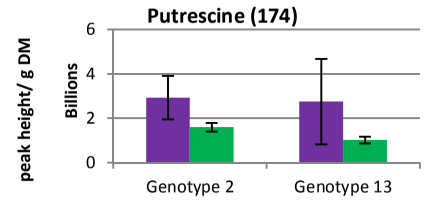
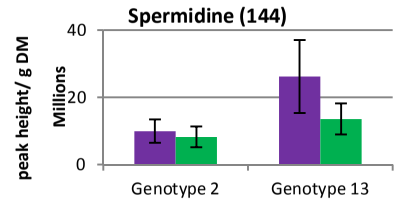
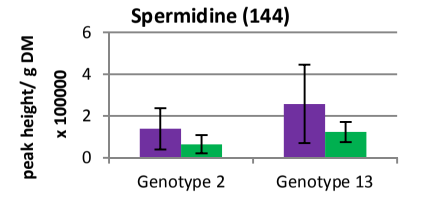
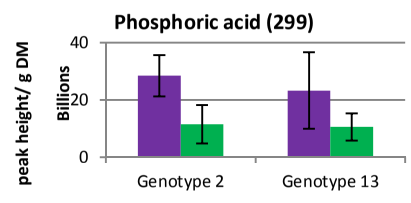
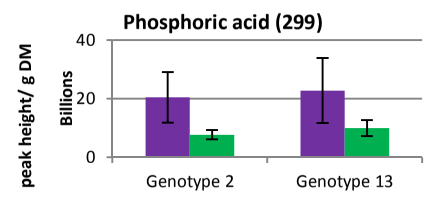
Supplementary Figure 1: Changes in metabolite content after treatment with 0.2 M sorbitol compared to control samples

Ratios were based on values in µmol/ g FM. Ratios below 1 were inversed to display decrease. Blue = Sensitive genotype Eurobravo, Orange = Tolerant genotype Maxi. # = ratio based on peak height. * = indicates significant differences between control and treatment.

Supplementary Figure 2: Metabolite concentration or peak height in the first and second repetition of the experiment. For n number see Supplementary Table 3. Purple bars represent control samples and green bars represent samples treated with 0.2 M sorbitol. Number after the respective metabolite represents the specific mass on which the calculation was based. Genotype 2 = Eurobravo; Genotype 13 = Maxi.

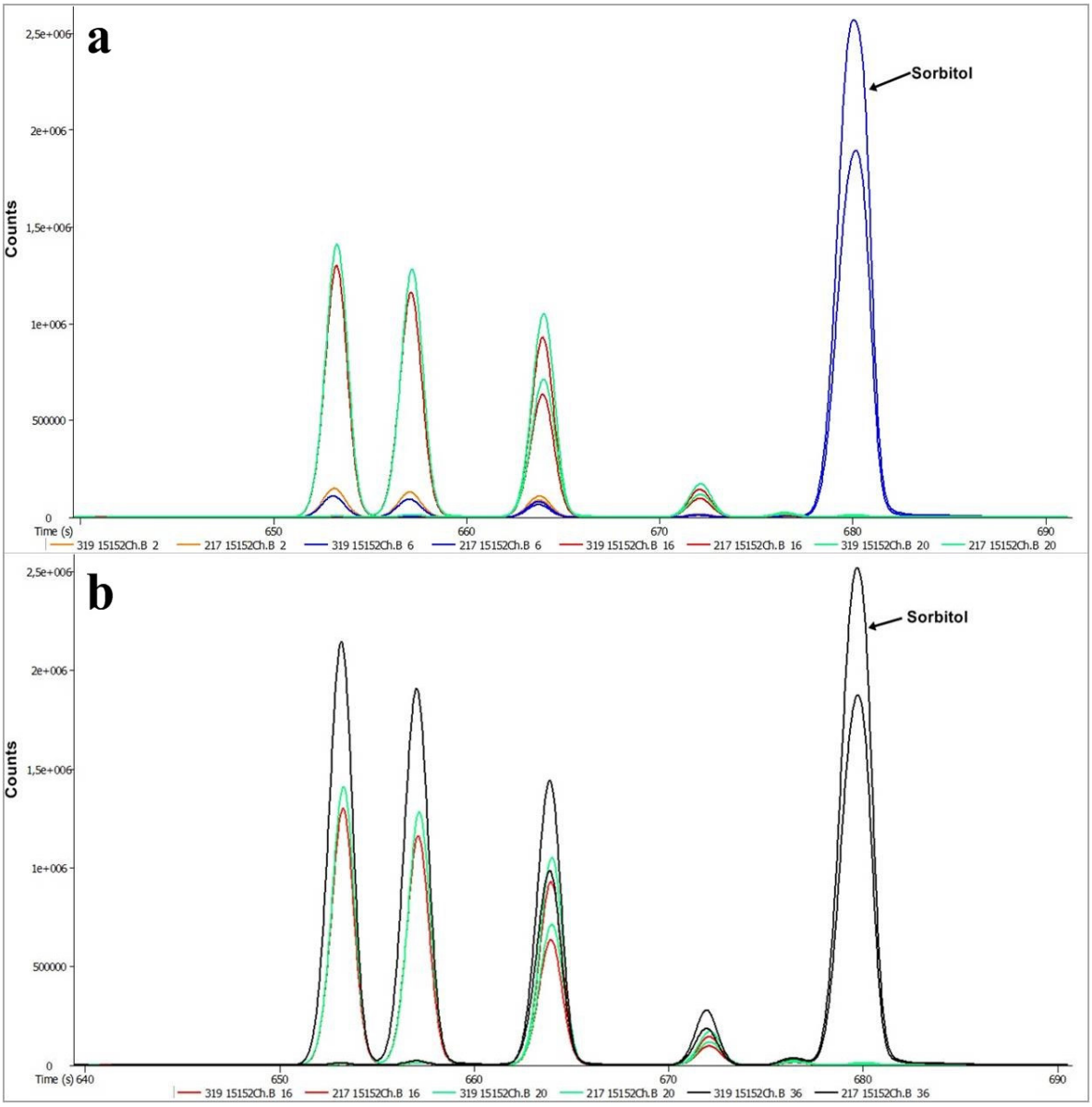


1st repetition	2nd repetition	1st repetition	2nd repetition
			
			
			
			
			

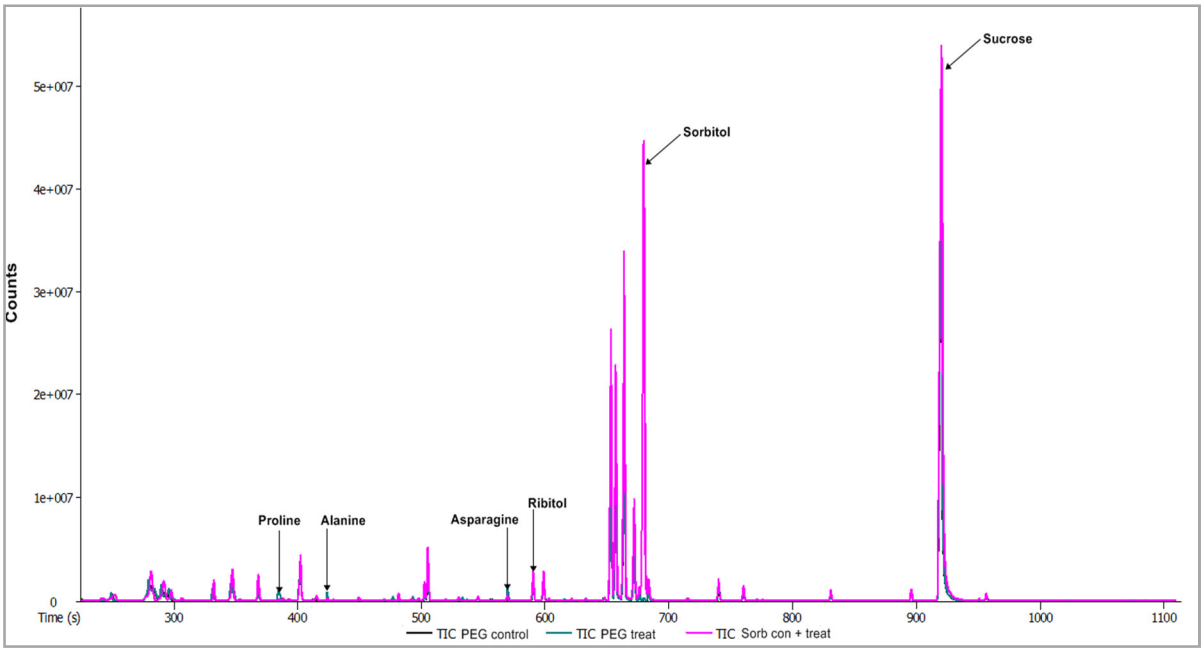
1st repetition	2nd repetition	1st repetition	2nd repetition
			
			
			
			

Supplementary Table 3: R^2 values for all identified metabolites for the first and the second repetition of the experiment. R^2 was based on the specific masses stated behind the respective metabolite. Underlined numbers are those on which further calculation was based. Red numbers indicate low R^2 values. n = 8 unless otherwise stated

Metabolite	Eurobravo				Maxi			
	1st repetition		2nd repetition		1st repetition		2nd repetition	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
α-ketoglutarate (198/288)	0.993	0.995	0.990	0.911 (n = 7)	0.951	0.960	0.923	0.960
Ascorbate (173/157)	0.984	0.998	0.995	0.994	0.994	0.996	0.994	0.979
Asparagine (188/216)	0.998	0.998 (n = 7)	0.999	0.999	0.968 (n = 7)	0.998	0.934	0.997
Aspartate (232/218)	0.999	0.999	0.999	0.993 (n = 7)	0.998	0.998	0.991	0.999
Citric acid (183/273)	0.997	0.955	0.985	0.992 (n = 7)	0.980	0.980	0.963	0.997
Fructose (217/307)	0.998	0.999	0.998	0.999	0.999	0.999	0.998	0.999
Fumaric acid (245/143)	0.977	0.904 (n = 7)	0.975 (n = 7)	0.987 (n = 7)	0.912	0.986	0.980	0.984
GABA (174/304)	0.989	0.925	0.957	0.982	0.932	0.975 (n=7)	0.970	0.997 (n = 7)
Glucose (319/160)	0.997	0.995	0.998	0.999	0.999	0.997	0.997	0.999
Glutamate (246/363)	0.998	0.995	0.996	0.995 (n = 7)	0.948	0.976	0.996 (n = 7)	0.991
Glycine(174/248)	0.909 (n = 7)	0.935 (n = 7)	0.991	0.983 (n = 7)	0.953	0.919 (n = 7)	0.995	0.997
Isoleucine (158/232)	0.989 (n = 7)	0.936	0.943	0.977 (n = 7)	0.971	0.929 (n=7)	0.967	0.968
Malic acid (233/245)	0.999	0.999	0.999	0.998	0.999	0.999	0.998	0.999
Myo-Inositol (305/265)	0.999	0.998	0.999	0.998 (n =7)	0.996	0.993	0.984	0.999
Oxo-proline (258/156)	0.993	0.971	0.927	0.982	0.964	0.951	0.948	0.995
Phenylalanien (218/192)	0.999	0.999	0.992	0.999 (n = 7)	0.994	0.995	0.987	0.997
Proline (142/216)	0.998 (n=7)	0.908	0.990	0.941	0.968 (n = 7)	0.993	0.994	0.996
Serine (204/218)	0.999	0.997	0.999	0.998 (n = 7)	0.993	0.997	0.986	0.999
Succinate (247/172)	0.995	0.958	0.900	0.928 (n = 7)	0.912	0.982	0.992 (n = 7)	0.969
Sucrose (437/361)	0.999	0.993	0.996	0.993	0.998	0.992	0.997	0.988
Threonine (219/291)	0.997	0.989	0.986	0.980 (n = 7)	0.973	0.978 (n = 7)	0.980 (n = 7)	0.986
Trehalose (191/361)	0.978	0.994	0.978 (n = 7)	0.992	0.990	0.987	0.990	0.989
Urea (171/189)	0.983	0.959 (n = 7)	0.997	0.976 (n = 7)	0.994	0.986	0.955 (n = 7)	0.972 (n = 7)
No quantification (low R^2 values; low n numbers)								
Putrescine (174/361)	0.970	0.898	0.900	0.943 (n = 7)	0.909 (n = 7)	0.968	0.855	0.941
Spermidine (144/174)	0.951 (n = 7)	0.997	0.979	0.998 (n = 5)	0.968	0.998	0.962	0.995
Phosphoric acid (299/211)	0.985	0.991	0.988	0.977 (n = 7)	0.973 (n = 6)	0.929	0.927	0.935



Supplementary Figure 3: Comparison of chromatograms from plants treated with 4.8% PEG or 0.2 M sorbitol and condensed water from vessels with medium with 0.2 M sorbitol. a = condensed water from vessels with control medium (orange lines), condensed water from vessels containing medium with 0.2 M sorbitol (blue lines), plant extracts from plants grown on control medium (red lines) and plants grown on 4.8% PEG 8000 (80-fold dilution, green lines); **b** 80-fold diluted plant extracts from plants grown on control medium (red lines) and plants grown on 4.8% PEG 8000 (green lines) and extracts from 80 fold-diluted plant samples treated with 0.2 M sorbitol (black lines).



Supplementary Figure 4: TIC overview of chromatograms from 80-fold diluted plant extracts treated either with 4.8 % PEG or 0.2 M sorbitol. 80-fold diluted plant extracts from plants grown on control medium (black lines) and plants grown on 4.8 % PEG 8000 (blue lines) and 80 fold-diluted plant samples treated with 0.2 M sorbitol (pink lines).

3 Chapter 3 - Conclusion and outlook

To investigate the effects of water deficiency on starch potato cultivars, *in vitro* trials with sorbitol as an osmotic agent, were performed. Three main chapters were processed with different research foci (Chapter 2, three manuscripts). The main results are discussed directly in the manuscripts. This chapter focuses on the main conclusions drawn over all chapters and on an additional outlook for further studies based on the obtained results.

3.1 Morphological responses of *S. tuberosum* genotypes to osmotic stress *in vitro*

Plants have successfully been stressed *in vitro* through the addition of 0.2 M sorbitol to solid MS medium. When stressing the plants, a reduction in FM and DM was observed. This decrease was more pronounced after 21 days of stress than after 11 days of stress for the sensitive cv. Eurobravo compared to the tolerant cv. Maxi, where it was not detectable (Chapter 2.1). When facing abiotic stresses, plants respond in growth by modulating both cell division and cell expansion. Growth reduction upon stress rapidly occurs, but growth recovers and adapts once stress responses become stable (Skrycz and Inze 2010). It was therefore concluded that the tolerant genotype started growth again after acclimatization to the stress whereas the sensitive genotype responded more strongly in terms of long-term growth reduction. Calculation of the SSI of the total DM together with the root-to-shoot ratio were shown to be good parameters to distinguish the more tolerant genotypes from the more sensitive genotypes. However, in total the analysed test set was to homogenous upon their response and more extreme responding genotypes would have been desirable.

The osmotic potential of shoot tips of stressed plants compared to control plants displayed an overall stable increase between middle-range and sensitive genotypes, but a higher increase was found for the tolerant cv. Maxi. If this holds true for other more tolerant genotypes has to be analysed. It was proposed by Wingler et al. 1999 that leaf water potential of barley plants under climate chamber conditions would be between -1.0 MPa and -2.0 MPa under moderate drought stress conditions. The leaf water potential of around -1.0 MPa to -1.5 MPa might therefore be a sign for moderate drought stress (Chapter 2.1). If this holds true, the responses identified would comprise those to moderate drought stress and would be different to those which might be observed under mild drought stress conditions. However, the systems are not identical and the ranges might be altered *in vitro*.

3.2 Metabolites and adjustment

A combined approach of *in vitro* culture, proteomics and metabolomics techniques was chosen for this study, as this can contribute to identify novel metabolites and proteins involved in the stress response. This might help to select genotypes with desired traits at an early stage of the breeding cycle.

The metabolite proline was found to be increased after stress application in both genotypes detected by GC/MS or photometrically (Chapter 2.1 and Chapter 2.2). A higher increase was identified in the

tolerant cv. Maxi on day 11 compared to the sensitive cv. Eurobravo. However, a higher concentration was found in the identification with the GC/MS technique (stress/ control ratio: Photometer cv. Eurobravo 6.9 and cv. Maxi 8.0; GC/MS cv. Eurobravo 7.2 and cv. Maxi 11.4). Nevertheless, the overall tendency that the tolerant genotype had a slightly higher increase was found with both techniques. No significant differences in their proline concentration were identified when the control or the treated plants between the genotypes were compared. The measurement with the GC/MS is however thought to be more sensitive. Nevertheless, the absolute values of the control and treated samples were similar between the two techniques due to high standard deviations in the GC/MS measurements (absolute values control photometer cv. Maxi 1.5 ± 0.7 µmol/ g DM and cv. Eurobravo 1.7 ± 0.6 µmol/ g DM; GC/MS cv. Maxi 0.9 ± 0.4 and cv. Eurobravo 1.2 ± 0.6 µmol/ g DM; and for the treated samples photometer cv. Maxi 5.2 ± 0.9 µmol/ g DM and cv. Eurobravo 4.5 ± 0.6 µmol/ g DM; GC/MS cv. Maxi 9.9 ± 5.8 µmol/ g DM and cv. Eurobravo 8.9 ± 3.4 µmol/ g DM). A correlation of tolerance to proline concentration was however not observed (Chapter 2.1).

In the metabolite analysis higher concentration in specific amino acids presumably from protein degradation, a higher demand for energy and an externally supplied osmolyte (sucrose) were identified as factors contributing to the overall picture of a severe stress response of the sensitive genotype. The tolerant genotype displayed a significant increase only in proline and a slight increase in citric acid (not significant). These two metabolites may function as osmolytes. As a decrease in all other metabolites was identified, it was concluded that both genotypes suffer from stress, but to a different extend (Chapter 2.3).

3.3 Comparison between proteomic and metabolite profiling data sets

A general tendency towards lower alterations in spot abundance and metabolite regulation was observed in the tolerant genotype during the analysis of the proteomic and metabolite data (Chapter 2.2 and 2.3). This might be a sign for adjustment. If this holds true for other potato cultivars with known stress performance, should be tested. However, in winter wheat cultivars differing in their drought stress tolerance, which were analysed for their reversible changes in leaf pigment and protein content, it was shown that these changes were least expressed in the tolerant cultivar (Simova-Stoilova et al. 2010).

Through the identification of proteinase inhibitors and higher concentrations of amino acids in the sensitive genotype on day 11, it was proposed that an increased protein breakdown takes place. The effect of drought stress on proteolytic activities in *Phaseolus* and *Vigna* leaves from sensitive and resistant plants was shown to correlate with the stress tolerance of the plants (Roy-Macauley et al. 1992). Relative small changes of the protease activity were identified in a drought-resistant winter wheat cultivar, whereas high values were detected in a cold-resistant but drought sensitive cultivar (Simova-Stoilova et al. 2010). It was concluded that, under water deficit during the early developmental stage of winter wheat, a lower proteolytic activity and a decreased expression of specific cysteine protease genes (through identification with gene expression analysis) could be used as an identification marker for

drought resistance of winter wheat cultivars (Simova-Stoilova et al. 2010). As higher abundance of protease inhibitors and precursors of such were found in potato shoot tips of the sensitive cultivar, this leads to an overall picture, where proteolysis in the sensitive genotype is present against which countermeasures have to be taken. As a higher proteolysis activity was found in other sensitive plants, this might be a good indicator of stress susceptibility for potato plants. If this hypothesis holds true has to be tested further.

From the obtained data (Chapter 2.2 and 2.3) it was proposed that a higher photorespiration after osmotic stress application was present in both genotypes. This is a valuable sign for moderate stress, as it was shown that barley plants under moderate drought stress increased the flux through the photorespiration pathway (Wingler et al. 1999). Glyoxylate is an intermediate of photorespiration. It was shown that a higher content of glyoxylate negatively correlated with the Rubisco activation state (Häusler et al. 1996). As a higher abundance in glycolate oxidase was found in the tolerant genotype it is tempting to speculate that this genotype might have a higher glyoxylate content leading to a reduction in Rubisco activity. As Rubisco activase (PGSC0003DMT400049256) was identified to be less abundant in the sensitive genotype, a reduction in Rubisco activity in the sensitive genotype might have also taken place (Chapter 2.2). However, the glyoxylate content was not analysed, as this compound leaves the column very early and is therefore masked by the noise signal at the beginning of the chromatographic run.

As drought, salinity, extreme temperature and oxidative stress often arise at the same time, they often also induce similar damage. The interference of ion homeostasis and ion distribution in cells is often a result of drought or salinity, as they are both comprising osmotic stress (Zhu 2001). As these environmental stresses can inflict the same damage on plant tissue, they often activate similar cell signalling pathways (Shinozaki and Yamaguchi-Shinozaki 2000; Knight and Knight 2001). Oxidative stress resulting from an increased production of ROS (mainly hydrogen peroxide and singlet oxygen) was shown to accompany stress responses. As high levels of ROS are toxic for the plant an active detoxification machinery helps to reduce damaging effects. It was shown that the tolerant genotype Maxi and the sensitive genotype Eurobravo deal differently with oxidative stress. While SOD which was higher abundant in the sensitive genotype is one of the main enzymes for detoxification of ROS, glycolate oxidase, which was found to be higher abundant in the tolerant genotype, is part of the photorespiration and produces H_2O_2. On the other hand, a monodehydroascorbate oxidase was identified when comparing the control and the stressed proteome of the tolerant genotype. Monodehydroascorbate reductase is an enzyme within the detoxification machinery helping in the regeneration of ascorbate (Groß et al. 2013). On FM basis ascorbate was slightly induced in both genotypes and slightly reduced on DM basis, but to an equal extent in both genotypes.

The identification of the protein glycolate oxidase involved in ROS production together with the probable rhamnose biosynthetic enzyme in the tolerant genotype suggests a reorganisation of the cell wall. It was implicated that a cell wall remodelling takes place under abiotic stress. At the onset of

abiotic stress, a hardening of the cell wall takes place through a crosslinking between phenolic compounds and glycoproteins through ROS and peroxidases. However, as stress persists the functions of ROS changes. As ROS levels stay high, hydroxyl radicals are produced which act in polymer cleavage. Together with expansins and other modifying enzymes this results in cell wall loosening and will allow further growth under stress conditions (for review see Tenhaken 2015 and references therein).

As most of the proteins identified in the sensitive genotype were associated with or located at the chloroplast, it can be assumed that a major site of reorganisation within the cell is located here. This was however rather surprising. Photosynthesis in plants cultured *in vitro* is thought to be neglectable, as it is downregulated because of the presence of sucrose in the medium and an insufficient CO_2 supply (Kozai 1991). It was also shown that chloroplast ultrastructure varied *in vitro* from that of *in vivo* plants. In *Dianthus* it was observed that a poor thylakoid stacking and lower chloroplast number was present in *in vitro* grown plants compared to those observed after acclimatisation or forced ventilation of the vessels (Majada et al. 2002). Nevertheless, as most of the proteins identified in the sensitive genotype were associated with or located at the chloroplast, it can be assumed that a major site of reorganisation within the cell is located here. Damage of the PS2 might have also occurred in the sensitive genotype as a decrease in abundance was identified for an oxygen enhancer protein after stress application. A damage of PS2 through drought stress in *Prunella vulgaris* was described before as it is the site where photodamage occurs (Chen et al. 2016). This medicinal plant was described as drought sensitive. As potato plants are also thought to be relative drought sensitive, this might underline the hypothesis that the two genotypes divergently respond to osmotic stress where the sensitive cultivar is more affected. However, together with an increase in photorespiration, which consumes energy in form of ATP and reducing equivalents, produces CO_2 and NH_3 and therefore consumes a vast amount of excess excitation energy of photosynthesis, further damage of the photosystem might be reduced (Lovelock and Winter 1995, Wingler et al. 2000). This might be especially beneficial for the sensitive genotype as the chloroplast protein abundance is highly affected by osmotic stress *in vitro*. The chloroplast might therefore comprise an interesting site of action for further studies.

Nonetheless, because not all protein spots could be assigned to an identified protein, other responses might also be present. To specifically determine the proteins not identified before, further measurements would be needed, as a number of proteins was only identified in a single repetition and was therefore neglected.

3.4 Sorbitol uptake

Sorbitol as the osmoticum of choice was found to be taken up in this experimental set up. As it is unclear if the osmoticum was taken up only at the beginning of the experiment when shoot tips were wounded or if an uptake was also present after the formation of roots, plants which have previously been rooted should be stressed and analysed. However, because sorbitol was identified in the *in vitro* shoots a

response upon the internal sorbitol might also have taken place. If sorbitol can also be metabolised by potato should be further analysed.

In woody Rosaceae the major photosynthetic product and translocatable carbohydrate is sorbitol (Wang et al. 2013). This can be converted to fructose by sorbitol dehydrogenase. Molecular evidence has been reported for a sorbitol dehydrogenase in tomato (Ohta et al. 2005). For potato only predictions based on sequence similarities are available (sorbitol or alcohol dehydrogenase, LOC102595131 form NCBI database; PGSC0003DMT400081907 from PGSC database). Nevertheless, as fructose concentration was reduced after stress application, even though sorbitol was shown to be taken up by the shoot tips, no conclusion about an activity of a sorbitol dehydrogenase in potato can be drawn.

3.5 Further perspectives

Drought in field gradually increases over time. The uppermost soil layers dry out first exposing the plant to a steadily increasing water shortage. In our test system we stressed the shoot tips by subjection to an osmotic shock resulting in a severe stress at the start of the experiment. As the plant had to root, acclimatise to the new medium and additionally respond to the applied stress consisting of osmotic stress and the injury by cutting the shoot tip, multiple stress might have taken place at the beginning of the experiment. On day 11 after the start of the experiments when the proteomic analyses and metabolite profiling took place due to reasons of comparability to other PROKAR experiments, the plants still suffered from stress, but probably only from the osmoticum applied. The stress resulting from fresh medium, the injury through cutting and also rooting should have been neglectable at this time point. Because these stress factors cannot be excluded and an osmotic shock does not simulate the natural occurring drought it was suggested that an alternative system for further analysis might be used.

The proposed adjustment for the stress system would comprise three changes to better simulate naturally occurring drought. First solid medium should be changed to liquid medium. This would have two advantages. The plants could be rooted before facing osmotic stress and omitting the agar would also reduce the osmotic potential to a less negative value in the controls. It was shown that agar is lowering the osmotic potential of the medium without an osmoticum being present (Gopal et al. 2008). The second change of the system would comprise, that after rooting of the plants the stress could be applied in a gradient, adding sterile sorbitol solution or PEG to the medium over time. This would allow simulating a partial increase of water shortage over time as can be seen under field conditions. As it was shown that sorbitol in our test system was taken up by the potato plants, rooting the plants and subsequently checking for sorbitol uptake would allow a conclusion if sorbitol can be taken up by intact roots or is limited to the uptake by wounded plants. This could either be analysed through a sorbitol assay or by analysis by HPLC (high performance liquid chromatography). Nonetheless, because sorbitol is taken up in this experimental set up an alternative osmoticum could also be analysed. As PEG is commonly used, this osmoticum could be an alternative choice to reduce the osmotic potential in the medium. However, as PEG of high molecular weight is usually a mixture of different MW e.g. PEG 8000 (MW range 7000-

9000) low MW PEG in the mixture might also be taken up. If this is the case for potato plants has to be tested further.

After rooting the plants stress application could be monitored through a time series through proteomic and metabolite analysis. This would allow a better identification of earlier responses during the progression of water shortage. Because 11 days of stress application seems to be too long to identify specific stress response of the more tolerant genotype, earlier time points should be checked after stress application in liquid medium. Together these changes would lead to a clearer test system.

It was not possible to correlate *in vivo* and *in vitro* responses on morphological level with just one parameter (calculated SSI *in vitro* DM and SSI *in vivo* yield). As plants in pot trials were allowed to naturally ripen no DM or FM was available at the end of the pot experiment in Groß Lüsewitz. Further tests in a green house could give similar stress responses after 11 days of drought and to have a look if the relative reduction in mass would be similar.

Further analysis to better characterise stress responses would include the analysis of the internal phytohormones especially ABA and the antagonistic cytokinins. This would contribute to the overview of internal stress responses, as ABA is known to inhibit growth (Farooq et al. 2009) and mediate major stress responses.

As the responses were only analysed on proteom and metabolite level, further identification of stress responses in starch potatoes upon osmotic stress *in vitro* could be obtained by analysing specific genes of interest (GOI) with gene expression analysis (realtime-PCR/ qPCR). These GOI could be deduced from the proteins identified through proteom analysis. However, it was stated that the abundance of transcript and protein might not correlate, as post-transcriptional, translational and protein degradation might alter the abundance (Vogel and Marcotte 2013).

Further steps could also include that candidate proteins could be mutated leading to knock-down or knock-out lines through RNAi and CRISPR/Cas techniques. These mutant lines could then be monitored for their morphological, proteomic and metabolomic response upon osmotic stress *in vitro* and drought stress in the greenhouse or under a rain out shelter. These mutants would be important for functional analysis of the GOIs deduced from the interesting proteins.

In conclusion the obtained results build a fundament for deeper analyses of specific osmotic stress responses of potato *in vitro*.

3.6 Conformance of thesis objectives

The three main objectives of this thesis were described in Chapter 1.13. A conformation to which extent the thesis objectives were achieved is given below.

1. Analysing if screening a set of potato genotypes *in vitro* would allow a classification of the variance in tolerance to osmotic stress between the genotypes.

   Osmotic stress was successfully applied to potato plants *in vitro*. Growth reduction was documented and compared between the genotypes. After 21 days on medium contacting 0.2 M sorbitol a classification into tolerant and sensitive genotypes was achieved. The root:shoot ratio and the SSI of the total DM were identified as suitable parameters to distinguish more tolerant and more sensitive genotypes. However, a correlation of the SSI for *in vitro* DM to SSI of tuber yield in pot trials was not detected. Adjustments of the test system seem necessary.

2. Identifying differentially abundant proteins in genotypes previously classified as tolerant or sensitive and screening these for probable biomarkers which confer stress tolerance

   Separation of the proteome of divergently responding genotypes was successfully achieved. Differentially abundant spots were picked and 100 proteins were identified. With the identified proteins it was concluded that the genotypes deal differently with protein degradation, where the sensitive genotype had a higher abundance in protease inhibitors, but also in terms of ROS scavenging and producing enzymes. Together with the fact that more protein spots displayed altered abundance after stress application in the sensitive genotype these findings gave rise to the speculation that the sensitive genotype still suffers from stress, whereas the tolerant genotypes already was in a stage of acclimatisation. The identified proteinase inhibitors and precursors of such (PGSC0003DMT400024601, PGSC0003DMT400026280 and PGSC0003DMT400011562), the ASR4 protein (PGSC0003DMT400017057) and annexin d1 (PGSC0003DMT400045665) in the sensitive genotype, but also the probable rhamnose biosynthetic enzyme (PGSC0003DMT400018192), the gylcolate oxidase (PGSC0003DMT400071115) and the dynamin-related protein (PGSC0003DMT400004319) in the tolerant genotype were proposed as interesting candidates for further detailed analyses.

3. Performing a metabolite profiling approach on targeted metabolites to gain insight into early stress responses of the divergently responding genotypes

   With the analysis of specific metabolites by GC/MS, an identification of specific responses was achieved. It was stated that both genotypes displayed a higher photorespiration as the sensitive genotype, accumulates more glycine and the tolerant genotype showed a higher abundance in glycolate oxidase in the proteomic analysis. However, it was also determined that the sensitive genotype displayed more severe stress responses and that compared to the tolerant genotype it suffered from protein breakdown. It was shown that sorbitol was taken up by both genotypes tested in this experimental setup.

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„Antworten von verschiedenen Stärkekartoffelgenotypen auf osmotischen Stress *in vitro* durch Sorbitol“

19 th triennial conference EAPR, 06.-11.07.2014, Brussels, Belgium

“Response of different potato genotypes to *in vitro* osmotic stress and first proteomic comparisons”

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Oral presentation

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Danksagung

Ich möchte an dieser Stelle allen danken die mich auf meinem Weg die letzten Jahre begleitet und unterstützt haben:

Ich möchte mich als erstes ganz besonders bei Prof. Dr. Traud Winkelmann für die Überlassung des Themas, die exzellente Betreuung, sowie fachliche und moralische Unterstützung während meiner Doktorandenzeit bedanken.

Vielen Dank an Prof. Dr. Hans-Peter Braun für die Bereitschaft als Korreferent zur Verfügung zu stehen. Weiterhin möchte ich mich dafür bedanken das ich in Ihrer Gruppe die Ansätze und Methoden der Proteomik erlernen und benutzten durfte.

Prof. Dr. Thomas Debener möchte ich für seine Bereitschaft den Prüfungsvorsitz zu übernehmen danken.

Ich möchte mich ebenfalls bei meinen Projektpartnern für die gut organisierte und erfolgreiche Zusammenarbeit bedanken. PD Dr. Hans-Peter Mock möchte ich dafür danken, dass ich in seinem Labor arbeiten durfte, bei Anna Maria Jozefowicz für gute Diskussionen und Hilfestellung bei der MS und Annegret Wolf für ihre Unterstützung in Gatersleben. Ich möchte mich auch bei Frau Dr. Annegret Schum, Prof. Dr. Sylvia Seddig und Herrn Philipp Meise für das *in vitro* Pflanzenmaterial bedanken, welches eine Versuchsdurchführung zu Beginn erheblich beschleunigt hat.

Ein herzliches Dankeschön geht ebenfalls an Prof. Dr. Christoph Peterhänsel und Dr. Christian Blume für ihre Unterstützung beim experimentellen Design der Metaboliten Analyse.

Der gesamten AG Winkelmann möchte ich ganz herzlich für die schöne Zeit danken. Ihr habt den Laboralltag sehr bereichert. Ein spezieller Dank geht dabei an meinen „Co-Doktoranden/innen“ Svenja Ratjens, Philipp Braun, Bunlong Yim, Mona Quambusch, Stefan Weiß, Emmanuel Omondi, James Wanjiku, Felix Mahnkopp und Malte Selig. Prof. Dr. Heike Bohne würde ich gerne speziell für die Diskussionen über Trockenstressantworten danken.

Vielen herzlichen Dank für die Unterstützung, die fachlichen Diskussionen und die Einführung in Kaffeegenüsse Brasiliens an Dr. Clarissa Caprestano.

Ein besonderer Dank gilt Dagmar Lehwejohann und Birgit Lippmann, welche mir die notwendigen Methoden für die Proteomik und die Metabolit Analysen beigebracht haben.

Dr. Christin Lorenz möchte ich für ihre Unterstützung und hilfreiche Diskussionen bezüglich der Proteomik danken.

Sabine Reinecke möchte ich für ihre Unterstützung im Organisatorischen danken.

Ich möchte meiner Familie für ihre Unterstützung danken. Ich bin froh, dass es euch gibt und dass ihr auch in stressigen Zeiten immer ein offenes Ohr hattet. Im speziellen würde ich gerne Inge Hadden und Gary Hadden danken. Danke Gregor Bündig!