Improvement of osmotic and salt tolerance in potato (*Solanum tuberosum* L.) by homologous protein overexpression

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Korreferent: PD. Dr. Bernhard Huchzermeyer

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Dedicated to my beloved parents, my wife,

my children, Gehad and Ahmad

SUMMARY

Osmotic and/or salt stress tolerance is of pivotal interest for crop improvement through conventional breeding as well as through genetic engineering. Especially for improved osmotic/salt stress tolerance in economically relevant crop plants genetic engineering could be a useful tool because it may allow the overexpression of genes which are in nature expressed on demand. In agricultural systems some environmental factors are predictable e.g. the increase of soil salinity and drought. Therefore a pre-adaptation of plants through constitutive gene expression of certain proteins in transgenic plants may help to stabilize the yield.

In this work a strategy should be developed to improve the salt and/or osmotic tolerance of potato cell cultures and plants by homologous overexpression of a specific protein. Output of the work should be a deeper insight into cellular mechanisms of osmotic stress tolerance which may help to get a better understanding of osmotic stress tolerance in entire plants.

The cellular mechanisms of osmotic/salt stress tolerance are the basis of a coordinated stress response of differentiated cells in entire plants. Based on previous literature, after initial studies with *in vitro* plants and already known differences in cryopreservation experiments, undifferentiated cell cultures of the cultivars (Desiree, Unicopa, and Ijsselster) were selected as a model system for further comparison of osmotic and salt tolerance. Osmotic and salt tolerance of these cell cultures was characterized by growth tests and the study of biochemical key reactions against osmotic and salt stress including proline, free sugar and amino acid accumulation as well as biochemical protection against oxidative stress. It could be shown that differences concerning these aspects exist between the different cell cultures.

By comparison of the proteome pattern of the cell culture of cultivar ´Desiree´ under normal and osmotic/salt stress conditions several de novo induced proteins could be detected and identified by mass spectrometry. One of the proteins PR10a (STH2) was selected for overexpression. On the basis of sequence information obtained from data bases the corresponding gene (gDNA as well as cDNA) was amplified from potato, inserted into specific dicistronic transformation vectors and overexpressed in tobacco as a model system and finally in potato cell cultures and plants.

A characterization of osmotic and salt tolerance of the transgenic plants and cell cultures based on growth and biochemical tests, confirmed that the homologous overexpression of the *pr10a* (*sth2*) gene leads to improved salt and osmotic tolerance. It further demonstrated that constitutive PR10a overexpression influences other stress reactions and the proteome pattern of cell cultures grown under normal non-stress conditions.

Finally it was demonstrated that increased osmotic tolerance conferred by PR10a overexpression also improves cryotolerance under standard freezing conditions.

Keywords: potato, Agrobacterium, PR10a, cell suspension, osmotolerance, salt tolerance

Kurzfassung

Die Salz- und die Trockentoleranz von Pflanzen sind von steigender ökonomischer Bedeutung. Ihre Verbesserung kann sowohl durch Züchtung als auch mit Hilfe molekularbiologischer Methoden erreicht werden. Eine mögliche Strategie zur Verminderung von Ernteverlusten bei vorübergehender Trockenheit kann die Präadaptation von Pflanzen durch konstitutive Überexpression von stressinduzierten Proteinen sein.

In dieser Arbeit soll versucht werden, durch die homologe Überexpression eines stressinduzierten Proteins eine Erhöhung der Trocken- beziehungsweise der Salztoleranz bei Kartoffelpflanzen und Kartoffelzellkulturen zu erreichen. Ziel der Arbeit ist es dabei auch ein tieferes Verständnis der biochemischen Mechanismen zu erreichen, die bei Kartoffelpflanzen und Zellkulturen zu einer erhöhten Salz- bzw. Trockentoleranz führen.

Als Modelsysteme für die Arbeit wurden Zellkulturen der Kartoffelsorten ´Unicopa´, ´Desiree´ und ´Ijsselster´ verwendet. Die Auswahl der Sorten beruhte auf Literaturdaten und bekannten Resultaten von Kryokonservierungsexperimenten. Da die Kryotoleranz zumeist auch eine Funktion der Osmotoleranz ist, waren unterschiedliche Grade der Osmotoleranz bei den verwendeten Kartoffelsorten wahrscheinlich. Zunächst wurde die Osmo und Salztoleranz der verschiedenen Zellkulturen auf der Basis von Wuchsstudien und biochemischen Tests charakterisiert. Als biochemische Parameter wurden die Bildung von Prolin, löslichen Zuckern und freien Aminosäuren sowie die Reaktion gegen oxidativen Stress untersucht. Unter geeigneten Testbedingungen wurden Proteomunterschiede zwischen Zellkulturen der Sorte ´Desiree´ unter Einfluss von osmotischem und Salzstress untersucht. Unter Stressbedingungen induzierte Proteine wurden mit Hilfe von Massenspektrometrie und Datenbankvergleichen identifiziert. Für eine homologe Überexpression wurde das PR10a (STH2) Protein ausgewählt.

Die aus Datenbanken ermittelte Sequenzinformation wurde zur Amplifikation des Gens (gDNA und cDNA) aus Kartoffeln genutzt und die erhaltenen Sequenzen in besondere dicistrinische Transformationsvektoren eingebaut. Durch Transformation mit diesen Vektoren wurden transgene Tabakpflanzen, Kartoffelzellkulturen und Kartoffelpflanzen

erhalten. Die osmotische und Salztoleranz der erhaltenen Zellkulturen und Pflanzen wurde wieder durch Wuchs- und biochemische Test untersucht.

Es konnte gezeigt werden, dass die konstitutive Überexpression des PR10a (STH2) Proteins in allen Fällen zu einer Steigerung der Salz- bzw. osmotischen Toleranz der Kulturen und Pflanzen führt. Darüberhinaus wurden wichtige Indizien dafür gefunden, dass die Überexpression des PR10a Proteins andere Stoffwechselwege beeinflusst und das Protein daher vermutlich regulatorische Funktion hat. Schließlich konnte gezeigt werden, daß die durch PR10a Überexpression erhöhte Salz- und osmotische Toleranz auch zu einer Erhöhung der Kryotoleranz unter Standardbedingungen führt.

Keywords: Kartoffel, Agrobakterium, Suspensionszellen, Osmotoleranz, Salztoleranz

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Abbreviations

APS	Ammonium persulfate
A. thaliana	*Arabidopsis thaliana*
A.tumefaciens	*Agrobacterium tumefaciens*
AtNHX1	*Arabidopsis thaliana* Na^+/H^+ exchanger
CCLR	Cell Culture Lysis Reagent
B5	B5 basal medium
BAP	6-Benzylaminopurine
bar	BASTA® (bialaphos) resistance gene
bp	Base pair
BSA	Bovine Serum Albumin
°C	Degree Celsius
CaMV	Cauliflower mosaic virus
cDNA	Complementary cDNA
cm	Centimeter
CTAB	Cetyl Tri-methyl ammonium bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo nucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
E. coli	*Echerichia coli*
EDTA	Ethylene diamine tetra acetate
FW	Fresh weight
g/l	Gram per litre
GFP	Green fluorescence protein
h	Hour
HPLC	High performance liquid chromatography
IPTG	Isopropyl-1-thio- β-*D*-galactoside
kb	Kilo base pair
kDa	Kilo Dalton
l	Liter
LAR	Luciferase Assay Reagent
LB	Left border
LB medium	Luria Bertani
M	Molar
mA	Milliamber
MAS	Manopine Synthase promoter
mg	Milligram
mg/l	Milligram per litre
ml	Milliliter
mM	Milli mole
mRNA	Messenger RNA
MS	Murashige and Skoog medium
NAA	Naphthylacetic acid
Na+/H+	Sodium/proton

ng	Nanogram
N.benthamiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
nm	Nano meter
nptII	Neomycin-phospho-transferase (gene)
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pfu-Polymerase	*Pyrococcus furiosus* polymerase
PPT	Phosphinothricin
PR-proteins	Pathogenesis-related proteins
RB	Right border
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
sec	Seconds
Taq	*Thermus aquaticus*
T-DNA	Transferred DNA
TEMED	*N,N,N',N',*-Tetramethylethylenediamine
Ti-Plasmid	Tumour-inducing plasmid
Tris	Tris-(hydroxymethyl)-Aminomethan
u	Unit enzyme
µ1	Micro liter
µM	Micro mole
UV	Ultra violet
V	Volt
(v/v)	Volume per Volume
vir	Virulence
Vol.	Volume
(w/v)	Weight per Volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-*D*-galactopyranosid
YEB	Yeast extract broth
2,4-D	2,4-dichlorophenoxyacetic acid

1. INTRODUCTION

1.1. Overview

Potato (S*olanum tuberosum L*.) is the most important non-cereal food crop of the world. It ranks 4th in the world after wheat, rice and maize (FAOSTAT, FAO, Rome, 2006). Drought and salt stress are serious problems for agricultural productivity especially in the arid zones. Probably global warming may further increase the problem.

Abiotic stress is counteracted in plants by a series of morphological, physiological, biochemical and molecular responses to restore plant cellular homoeostasis and growth. Apart from signal perception and transduction these include production of osmotic substances, water and ion movements, a variety of functional and structural stress-induced proteins, free radical scavenging and many others (Wang *et al.,* 2001).

Increased drought or salt tolerance of plants can be achieved either by plant breeding or also by genetic engineering. Prerequisite for both approaches is the deeper understanding of the network of mechanisms leading to improved salt and/or drought tolerance.

One possible way to investigate this network is the overexpression of genes involved in stress tolerance in a suseptable plant and the analysis of the physiological consequences. It has been shown that the overexpression of a single mono-functional protein, the membrane bound Na^+/H^+ ion pump At-NHX1 (Blumwald and Poole 1985, Z. Ali 2007) already leads to improved salt tolerance. Even better targets should be those proteins exerting key regulatory functions influencing various protective mechanisms (Oh S.J. *et al.,* 2007).

Biochemical investigations are often more easily performed using plant cell cultures (Singh *et al.,* 1985; Murota *et al.,* 1994). This is also true for proteome studies as in plant cell cultures often photosynthetic proteins are missing which complicate the proteomic analysis of leaf material (Jain *et al.,* 2001).

The intention of this study was to investigate osmotic and /or salt tolerance mechanisms realized in potato using cell cultures as model systems. Furthermore, the study aimed to identify key proteins induced by osmotic and salt stress and to overexpress these proteins in plant cell cultures in order to modify salt and/or osmotic tolerance. Plant cell cultures and plants overexpressing the target protein were used as model systems for future studies on osmotic and salt tolerance

Objectives:

Objectives of this study are:

* The investigation of cellular salt and osmotic tolerance strategies of S*olanum tuberosum* using cell cultures as model systems and further analysis of physiological and biochemical responses of potato cell cultures to salt and osmotic stress.

    The investigated responses include biomass accumulation, cell viability, proline accumulation, analysis of oxidative stress reaction by determination of glutathion pools, analysis of the pools of the soluble sugars glucose, fructose and sucrose and free amino acids.

* The identifcation of key proteins induced by osmotic and salt stress by proteome analysis to identify possible target proteins for gene overexpression.

    Transformation should be carried out using dicistronic transformation vectors allowing the coexpression of a physically independent reporter gene together with a target gene for easy expression monitoring

* Characterization of consequences of gene overexpression on osmotic and salt tolerance of the transformed cell cultures.

* Preliminary studies on the influence of the overexpressed gene on cryotolerance.

1.2. Effects of abiotic stress in plants

Abiotic stress leads to a series of damages that adversely affect plant growth and productivity (Wang *et al.,* 2001a). Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage: for instance, drought and salinity induce disruption of homeostasis and ion distribution in the cell (Serrano *et al.,* 1999; Zhu 2001a), damage to plants include membrane disorganization, increase in levels of toxic metabolites, inhibited nutrient uptake and photosynthesis, generation of reactive oxygen species (ROS) and ultimately cell and plant death (Hasegawa 2000).

Differentiated plants are classified as glycophytes and halophytes according to their response to salinity. While halophytes can accumulate high levels of salt in their tissues, a high NaCl concentration in tissues is toxic for the growth of glycophytes. In most glycophytes, the increment of the external NaCl concentration increases the amount of Na^+ and Cl^- in both shoot and roots, whereas K^+ and Ca^{2+} decrease with the progressive rise in salinity (Ashraf and Orooj, 2006). An increase of Na^+ uptake leads to a decrease of K^+ content in the cytoplasm which subsequently causes physiological disorders as several enzymes need K^+ for their activity. Oversupply of Na^+ displaces K^+, thereby inactivating these enzymes (Bhandal and Malik 1988; Haro *et al.,* 1993).

It is well documented that high salinity leads to osmotic stress, oxidative stress and changes in ion homeostasis (Zhu, 2001). Oxidative stress, which frequently accompanies salinity or drought stress may cause denaturation of functional and structural proteins (Smirnoff 1998).

Another effect of salt stress is the reduction of photosynthesis (Sudhir and Murthy, 2004). The chlorophyll content decreased in salt susceptible plants such as tomato, potato and pea, whereas chlorophyll content increased in salt tolerant plants such as mustard and wheat (Sudhir and Murthy 2004; Venkatesan *et al.,* 2005). Moreover, the efficiency of photosystem II does not decrease in salt resistant plants and is reduced in salt sensitive species (Wang *et al.,* 2007).

Increase of solutes as a result of salt stress decreases the water potential in the rhizosphere, and subsequently decreases or even disrupts water uptake of intact plants followed by water deficit in the plant. Generally differentiated plants respond to increased salinity in the soil by stomatal closure that is induced by abscisic acid (ABA) signaling. This is followed by a decrease in evaporation to prevent loss of turgor and recovery of osmotic homeostasis with

the accumulation of inorganic ions, amino acids, sugars and other metabolites (Ashraf and Orooj 2006; Katerji *et al.*, 2000).

1.3. Salt tolerance mechanisms

As a consequence, the diverse environmental stresses often activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000; Knight and Knight 2001; Zhu 2001b, 2002) and cellular responses, such as the production of stress proteins, up-regulation of antioxidant formation and accumulation of compatible solutes (Vierling and Kimpel 1992; Zhu *et al.*, 1997; Cushman and Bohnert 2000). The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is represented in Figure1 (Wang *et al.*, 2003).

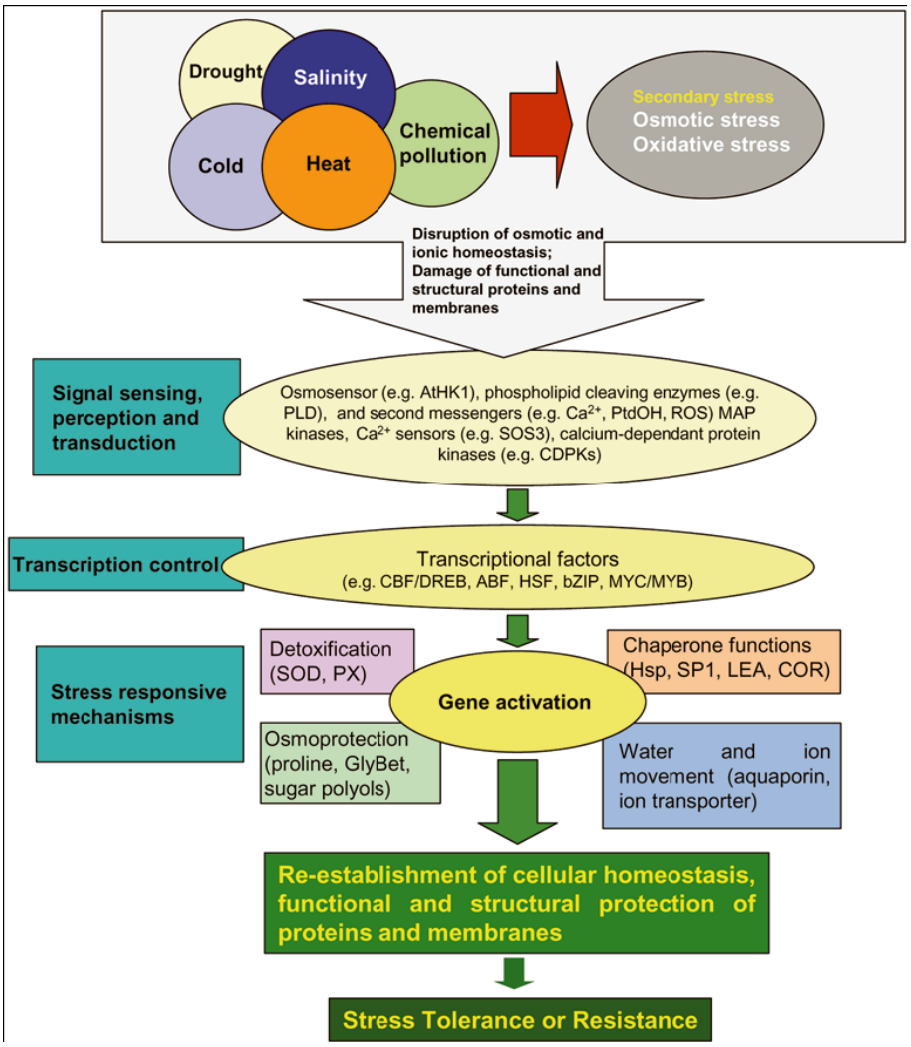


Figure.1: The complexity of the plant response to abiotic stress. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress (taken from Wang et al., 2003).

One strategy of salt tolerance of plants is to control the cytoplasmic Na^+ concentration via the maintenance of K^+/Na^+ and Ca^{2+}/Na^+ ratios in the cytoplasm (Yeo, 1998). This can be achieved by transportation of the cytoplasmic sodium out of the cell and /or deposition in the vacuoles or by active absorption of K^+ into the cytoplasm. Such tasks are carried out by Na^+/H^+ antiporters and K^+/Cl^- cotransporters helping the plant to maintain K^+/Na^+ homeostasis (Colmenero-Flores *et al.,* 2007). The transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter, driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes the H^+-ATPase and the H^+-PPiase (Blumwald 1987). The overexpression of a *AtNHX1,* a vacuolar Na^+/H^+ antiporter from *Arabidopsis,* resulted in an increased salt tolerance of the transgenic plants up to 200mM NaCl, a concentration that severely damages the corresponding wild-type plants (Apse *et al.,* 1999).

Another mechanism for salt detoxification is Na^+ extrusion from plant cells, which is powered by the operation of the plasma membrane H^+-ATPase generating an electrochemical H^+– gradient that allows plasma membrane Na^+/H^+ antiporters to couple the passive movement of H^+ inside the cells, along its electrochemical potential, to the active extrusion of Na^+ (Blumwald *et al.,* 2000). Recently, *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiporter with significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi *et al.,* 2000). The overexpression of *SOS1* improved the salt tolerance of *Arabidopsis*, demonstrating that improved salt tolerance can be attained by limiting Na^+ accumulation in plant cells (Shi *et al.,* 2003).

But plants respond to salinity also with another strategy: salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure through the synthesis of compatible solutes (Greenway and Munns 1980; Chinnusamy *et al.,* 2005). This salt tolerance strategy is the accumulation of low-molecular-mass compounds like proline, glycine betaine or soluble sugars like glucose, fructose and polyols like mannitol and pinitol in the cytoplasm to establish osmotic homeostasis. This supports continued water influx by lowering the internal water potential (Parida *et al.,* 2004 and Zhu, 2001). At this point it is almost impossible to distinguish between responses to salt and responses to osmotic stress.

Oxidative stress occurs as a consequence of almost all abiotic and biotic stresses in plants. Increased levels of reactive oxygen species are observed as a consequence of cold stress (Benson and Bremner 2004) as well as of salt and osmotic stress (Jithesh MN *et al.,* 2006). Gluthatione plays a general role in the defense against oxidative stress. The reduced form itself (GSH) can be oxidized (to GSSG) to detoxify injurious ROS species and it furthermore plays a central role in the regeneration of the ascorbate pool which also acts as a protectant against ROS. Although care has to be taken in the interpretation of data, glutathione can be considered as a marker compound for oxidative stress (Tausz *et al.,* 2004).

Another indication for a response of the plant against salt and osmotic stress is an increased level of free amino acids. Some of the amino acids are by themselves compatible solutes like proline, other are precursors of compatible solutes, like glycin or alanin (Hanson *et al.,* 1994). But also other amino acids turned out to be enhanced (Rizhsky *et al.,* 2004) which might be necessary for *de novo* synthesis of induced proteins.

Improving salt tolerance in plants is possible in different ways: by direct selection of tolerant varieties of a species in saline environments, by mapping quantitative trait loci and subsequent use of selection markers for plant breeding or by generation of transgenic plants introducing a novel gene or changing the expression level of an existing gene (Yamaguchi and Blumwald, 2005).

1.4. Response of potato and potato cell cultures to salinity and osmotic stress.

Among many plant species, potato is a drought sensitive plant (Harris 1978). Potato is very sensitive to water stress because of its shallow root system (Iwama and Yamaguchi 2006). Moreover the potato plant is very susceptible also to high NaCl levels, which is responsible for a reduction of plant growth of about 50% in the presence of 50 mM NaCl and nearly 100% in the presence of 150 mM NaCl, respectively. Furthermore, potato rooting capacity is severely inhibited by 150 mM NaCl and the resulting salt saturation of intracellular spaces in vegetative parts can cause necrosis and cell death (Sherraf *et al.,* 1994). Despite the economic importance of potato, salt and drought stress response has been poorly investigated in this plant (Sherraf *et al.,* 1994; Jeong *et al.,* 2001 and Donnelly *et al.,* 2003). Therefore one of the

main breeding aims include selection of salt and drought tolerant varieties. As a complementary approach to breeding activity, *in vitro* culture is a very useful tool to study the osmotic stress tolerance in potato. *In vitro* evaluations of salt and drought tolerance are proposed as alternatives to the costly, labor-intensive and sometimes problematic field-based evaluations (Dobranszki *et al.,* 2003). Selection for high proline, carbohydrate level and accumulation of sodium and potassium has also been used for potato cultivar screening against salt stress (Sasikala and Devi 1994). Recently, Gopal and Iwama (2007) reported that screening of potato genotypes under *in vitro* conditions reflect the *in vivo* situation and might provide a highly efficient screening method for a biotic stress like drought tolerance. Dobranszki and coworkers (2003) reported that the increment of callus mass was a useful parameter for osmotic tolerance determination of different potato genotypes at the cellular level. It is therefore very likely that even undifferentiated cell cultures provide good model systems for the investigation of cellular mechanisms of salt and drought tolerance. Overexpression of proteins playing a role in osmotic and salt tolerance can be a novel approach to investigate their role in stress tolerance on a physiological and biochemical level.

1.5. Identification of targets for genetic engineering

Approaches to analyse differential changes in metabolism are often based on the genome level. DNA microarray and different methods for gene expression profiling provide technologies to identify genes showing altered transcript levels induced by stress. Often these methods are costly, time consuming and need a high input of already known information, for example EST sequences for gene identification.

Another powerful approach to identify directly the proteins that were up- or downregulated as a consequence of abiotic stresses is proteomics. Proteomics often provides a more physiologically accurate snapshot of a biochemical process by revealing the actual protein constituents performing the regulatory enzymatic and structural functions encoded by the genome and transcriptome at a given point of time. Moreover proteomics provides additional information on gene regulation especially important for the cases where mRNAs may not be translated or that post-translational events lead to changes in protein level or enzyme activity without any detectable changes in transcript abundance due to translation or other level of control (Gygi *et al.,* 1999).

Today mainly progress that has been made in mass spectrometry in combination with information for amino acid- as well as DNA sequences available in data bases, allows the

easy identification of single protein spots (Canovas *et al.,* 2004). This led to a situation were post-genomic approaches that investigate changes in the protein pattern instead of constituents of the genome, i.e. the proteome are being increasingly used in various disciplines in order to investigate molecular changes that occur in response to stresses including pathogen challenge (Rampitsch *et al.,* 2006; Salekdeh *et al.,* 2002; Srivastava *et al.,* 2004; Subramanian *et al.,* 2005; Yajima *et al.,* 2004; Zhou *et al.,* 2005; Braun and Schmitz (2006).

Jain *et al.,* 2006 described the characterization of differences on the proteome level between a salinity-tolerant and –sensitive callus cell line of *Arachis hypogaea.* Proteomics analysis revealed elevated levels of PR10 proteins in saline-tolerant peanut (*Arachis hypogaea*) calli that may be responsible for the higher salt tolerance. A proteomics-based strategy using two dimensional electrophoresis and mass spectrometry (MS) was also employed to characterize proteome-level changes in the roots of pea (*Pisum sativum* L.) plants in response to salinity (Kav *et al.,* 2004). Based on the proteome-level information obtained through these studies it may be possible to rationally select gene(s) that may have utility in improving plant productivity under stress conditions (Srivastava *et al.,* 2004;Salekdeh *et al.,* 2002). Furthermore if the function of these proteins is still unknown, overexpression may be a way to investigate its putative mode of action.

1.6. Simple expression monitoring achieved by coexpression of target and reporter gene

The investigation of the mode of action of a certain protein by overexpression has to face several problems. One of these problems is gene silencing. Proteins can exert there action only in cells where they are produced. Gene silencing may occur and lead to a situation where the recombinant gene is not expressed any more in certain parts of the organisms. This problem may be even more severe in plant cell cultures where the level of expression over time may change and some cells are expressing and others are not. Apart from epigenetic changes (Kaeppler *et al.,* 2000) even genetic instabilities in cell cultures are well known (Bayliss 1980; Alves *et al.,* 2005). To proof a correlation between a recombinant gene and a

physiological effect of gene expression should be monitored. In most cases expression monitoring requires complicated and time consuming methods like Western blot analysis.
An often applied method for expression monitoring is the use of fusion proteins. The sequence of a target protein is fused to the sequence of a reporter gene. The approach is most often used to investigate the intracellular localization by the fusion of a target protein with a fluorescent protein like GFP. In an ideal case the resulting fusion protein shows the activities of both proteins. But very often the physiological activity of the target protein is negatively affected by the attached reporter protein. In any case the physiological activity of the target protein has to be demonstrated. This is often achieved by the reconstitution of the normal function in a deficiency mutant by expression of the fusion protein (for review see Dixit at al. 2006). In this study a different approach is realized. With novel dicistronic transformation vectors the coexpression of physically independent target and reporter genes is achieved by linking the DNA sequences through a viral IRES element (Ali 2007). Although expression of the target gene can now be monitored by measuring reporter gene expression, the target gene can exert its physiological function unaffected by any sequence- or structural differences to the naturally occurring protein.

Another advantage of this approach is that any physiological effect caused by the overexpression of the target gene can clearly be attributed to the recombinant gene and clearly be discriminated from the same effect caused by an endogenous gene.
It is therefore expected that the strategy followed in this study is able to create novel model systems on the cell culture as well as on the plant level for the investigation of the role a certain protein of unknown or not clearly defined function plays in the complicated biochemical networks activated for stress response of plants against biotic an abiotic stresses.

2. MATERIAL AND METHODS

2.1. Plant material and cultivation

2.1.1. *Solanum tuberosum L. in vitro* plants

In vitro propagated plants derived from three potato cultivars *Solanum tuberosum* L 'Desiree', 'Unicopa, 'Ijsselster' were used in this study. The plant material was obtained from the *in vitro* collection of the former Institute of Crop Plant Research of the Federal Agricultural Research Station (FAL), Braunschweig Völkenrode in 1991 and from that time on were propagated *in vitro* at DSMZ. For propagation the plants were divided into nodal cuttings each containing one auxiliary bud and grown in solid Murashige & Skoog (MS) medium (appendix I) under controlled condition at 23°C and 16h light /8 h dark cycle in Magenta boxes. The plants were propagated every 8 weeks. Plants of the cultivar ´Desiree´ were used to obtain the starting leaf material required for subsequent the potato transformation.

2.1.2. Callus and suspension cultures of *Solanum tuberosum* L.

Callus cultures were initiated from sterile *in vitro* plant material of the three cultivars (see 2.1.1.) on 4X medium solidified with 8 g/l agar (appendix II). Suspension cultures were established from calli in liquid 4X medium. 100 ml of the suspension cultures were cultivated in 300 ml Erlenmeyer flasks on a gyratory shaker at 100 rpm (TR-250, Infors AG, Basel, Switzerland). Suspension and callus cultures were grown at 23 °C. The suspensions of ´Desiree´ and ´Unicopa´ were subcultured weekly by transferring 40 ml of cells to fresh 60 ml 4X medium. For subculturing ´Ijsselster´ medium was discarded from settled cells, fresh medium was added up to 200 ml and distributed equally into 2 new 300 ml Erlenmeyer flasks.

2.1.3. *Nicotiana tabacum in vitro* plants

Sterile *Nicotiana tabacum* cultivar SR1 plants from *in vitro* culture were used to obtain the explants leaf material required for tobacco transformation. Plants were grown in 500 ml Weck glass jars (Weck GmbH, Wehr, Germany) in solid MS medium (appendix I) under controlled conditions at 23°C and 16h light /8 h dark cycle. Light intensity was 4000 lux**.** Plants were transferred to fresh medium at 4-weeks intervals.

2.2. Cell culture methods

2.2.1. Mini test for osmotic tolerance

For testing stress tolerance by growth and biochemical measurements, incubation of cell suspension cultures were carried out in a mini-test system according to (Heine-Dobbernack *et al.,* 2008). For testing, wells of a 24multiwell plate (Greiner Bio One, Frickenhausen, Germany) are filled with 0.9 ml either 4X medium or 4X medium supplemented with different osmotics. 24multiwell plates were shaken on a Heidolph shaker for titerplates at 300 rpm (Titramax 101, Heidolph, Kehlheim, Germany). Each well was inoculated with 200 mg fresh weight of cell material. Cells for inoculation of 24multiwell plates were harvested from the logarithmic growth phase three days after inoculation by filtering off the cells from the medium through Nylon net (100µm pore size, NeoLab, Heidelberg Germany) in a Buchner funnel. 0.9 ml of normal 4X medium (appendix II) or 4X medium supplemented with various concentrations of the stress agents tested (NaCl, sorbitol, sucrose) were filled into each well of the 24multiwell plate and 200 mg of cells were weighed into each well. The plates were sealed twice with Parafilm to minimize evaporation and incubated for 3 or 6 days on a shaker at 300 rpm (Heidoplh Titramax 101, Kehlheim, Germany). After the incubation period, the medium was sucked out of the 24multiwell plates with cigarette filter cartridges (Efka, Trossingen, Germany). At the end of the incubation period cell viability was tested using the TTC test.

2.2.2. 2,3,5-Triphenyltetrazolium chloride (TTC) viability test

The test was performed according to Steponkus and Lanphear (1967). As a modification, Tris-HCl buffer (50 mM,Tris-HCl, pH 7.5) was used instead of phosphate buffer. The test was either performed directly in the 24 multiwell plates or with an aliquot of the cells in Eppendorf tubes. When directly performed in the 24multiwell plates 1250 µl of Tris–HCl buffer and 300 µl of TTC solution (2 % (w/v) TTC dissolved in Tris-HCl buffer) were added to each well after the medium were removed from the wells. The plate was sealed twice with Parafilm (Omnilab, Bremen, Germany) and shaken briefly. Then the plates were incubated in the dark over night at room temperature. For the extraction of the red water insoluble formazan derivative, the supernatant was removed completely and 1.5 ml ethanol (95 %) was added to each well. The plates were then sealed tightly as described (Heine-Dobbernack *et al.,* 2008) and kept in the dark for 24 hours at room temperature. The red supernatant was

transferred into Eppendorf tubes and centrifuged at 14000 rpm for 5 minutes to remove the residual cells. The absorption of the clear supernatant was measured spectrophotometrically at 500 nm.

For the test performed in Eppendorf tubes 50 mg of each fresh cell material was transferred to 2 ml Eppendorf tubes. 1200 µl Tris-HCl buffer and 300 µl TTC solution was added and the samples were incubated over night. The next day the samples were centrifuged at 14 000 rpm for 5 minutes, the supernatant discarded and 1.5 ml ethanol (95 %) added to the cells. The red formazan was extracted over night. Samples were centrifuged the next day (14000 rpm, 5 minutes) and the absorption of the cell free supernatant measured at 500 nm.

2.2.3. Chlorophenol red assay

For measuring the response to osmotic and salt stress of wild type and transformed potato cell cultures, a chlorophenol red assay was used. Cells were harvested and grown in 24multiwell plates as described before. The transgenic and wild type cell suspensions were subjected to the following conditions: not treated with salt or osmotic (control medium), treated with 4X medium (appendix II) containing 0.5 M or 0.7 M sorbitol, treated with 4X medium containing 200 mM NaCl, 250 mM NaCl or 320 mM NaCl. In addition, all these media contained the pH indicator chlorophenol red at a concentration of 50 mg /l. Each well was filled with 0.9 ml of these media and inoculated with 35 mg cells. Plates were grown under conditions described above at 23 °C on a shaker. Color changes in the culture medium (from red at pH 6 to yellow at pH 5), indicating active cell growth were monitored optically over a period of 4 days. Digital photos were taken.

2.2.4. Fluoresceindiacetate (FDA) viability test

For the test a stock solution of Fluoresceindiacetate (FDA) (5 mg FDA in 1 ml acetone) was prepared. 50 µl of the stock solution was diluted with2.5 ml medium on ice (FDA working solution). 500 µl of suspension cells were mixed with 500 µl FDA working solution and incubated for 5 minutes at room temperature. Fluorescence was observed under a microscope (Ex 350 nm, Em 450 nm).

2.2.5. Fresh and dry weight determination of cell material

For the determination of dry weight of the cell material grown in suspension in 24multiwell plates the medium was removed after cells had settled down. Cells were then transferred into pre-weighed Petri dishes using a small spoon. The Petri dishes with the fresh cell material

were weighed again and cells in the Petri dishes dried at 60 °C for 72 hours. Petri dishes containing the dry cell matter were weighed again and fresh weight, dry weight calculated.

2.3. Biochemical Methods

2.3.1. Proline determination

The detection of the free L-proline content was carried out according to Bates *et al.,* (1973) with some modifications. A sample of 150 mg fresh material either of wild type or transgenic cells was grinded under liquid nitrogen in a precooled mortar with a pestle. The homogenate was resuspended in 10 ml of 3 % salicylic acid in 50ml flasks and shaken for 20 minutes. The suspension was filtered through filter paper (Whatman plc, Maidstone, UK) to remove cell debris. 300µl of acidic ninhydrin was added to the same volume of supernatant, followed by the addition of 300 µl of glacial acetic acid. The mixture was boiled for 60 minutes. For extraction of L-proline 600µl of toluene was added to the mixture and the mixture shaken vigorously for 30 seconds. The toluene phase was collected and its absorption measured spectrophotometrically at 520 nm. Free proline was quantified by a standard curve obtained with pure standard solutions of L-proline as reference substance in the same assay.

2.3.2. Glutathione determination using High Pressure Liquid Chromatography (HPLC)

Detection and quantification of glutathione and related compounds were carried out by revered phase high performance liquid chromatography (HPLC). For separation and quantification a derivatization of thiol groups (glutathione) with monobromobimane was carried out. To discriminate between the oxidized and the total glutathione, the reduced thiol groups are first blocked irreversibly using N-ethylmaleimide. Then all remaining oxidized thiol groups are reduced with DTT, and derivatized with monobromobimane and resulting compounds separated and detected. The result measures the oxidized form of glutathione present in the sample. In parallel samples, DDT reduction and monobromobimane derivatization was performed without previous N-ethylmaleimide blocking to determine the total glutathione content.

2.3.2.1. Extraction of thiol compounds

For extraction of thiol containing compounds 100 µl (100 pmol) of 1 mM glutamylcystein was added to 50 mg of fresh plant tissue. The homogenate was prepared in a preccoled mortar under liquid nitrogen. Afterwards 100 µl of thiol standard solution containing 1mM L-cystein

and 1mM glutathione and 600 µl bi distilled water were added. 1 ml 0.1 M HCl was added and the homogenate transferred into a 1.5 ml Eppendorf tube and mixed thoroughly. The homogenate was incubated on ice for 15 minutes and vortexed every 2 minutes. The sample was centrifuged for 5 minutes at 14000 rpm and the supernatant was transferred into a new Eppendorf tube and centrifuged again for 10 minutes at 14000 rpm. The supernatant was used freshly for the derivatization.

2.3.2.2. Derivatization of extracted compounds

For HPLC separation and detection thiol-compounds were derivatized to obtain fluorescing products. For the blocking, DDT reduction and derivatization of standard and sample solution the following mastermix solutions were prepared:

Master Mix composition	MM1 [µl]	MM2[µl]	MM3[µl]	MM4[µl]
Bidestil. Water	140	130	230	220
1 M Tris-HCl, pH 8.0	20	20	20	20
10 mM DTT	10	-	10	-
5 mM NEM	-	10	-	10

To prevent oxidation by light the derivatization was carried out in brown 2 ml Eppendorf tubes. Either 10 µl of each standard solution (1 mM L-cystein, 1 mM glutathione) or a mixture of 50µl of 90 mM sodium hydroxide and 50 µl plant extract was added. The derivatization was carried out as follow: For detection of the amount of oxidized SH groups in the standard and the sample solutions 250 µl MM4 mixture was added to the standard solutions and 160 µl MM2 mixture was added to the samples (50µl 90 mM sodium hydroxide and 50 µl plant extract). Samples and standards were mixed and incubated for 10 minutes at room temperature. Then 10 µl of 10 mM DDT was added to the tubes containing standard solution as well as to the tubes containing the plant extracts. Tubes were mixed for 10 seconds, centrifuged for 30 seconds at 14000 rpm, incubated for 60 minutes in the dark at room temperature and then stored on ice. For detection of the total SH groups in standard

solutions and the plant samples 260 µl of MM3 mixture was added to the standard solutions and 170 µl MM1 mixture was added to the tubes containing the plant samples (50µl 90 mM sodium hydroxide and 50 µl plant extract). Tubes were mixed for 10 seconds, centrifuged for 30 seconds at 14000 rpm, incubated for 60 minutes in darkness at room temperature and then stored on ice.

All samples (those for detecting oxidized SH groups as well as those for detecting total SH groups) were then subjected to monobromobimane derivatization. For the reaction 25 µl of monobromobimane reagent (10 mM) was added to each sample and samples were vortexed for 10 seconds. Samples were then centrifuged at 14000 rpm for 30 seconds and incubated for 15 minutes in the darkness at room temperature. The reaction was stopped by adding 705 µl of acetic acid (5%) to each sample. Finally samples were mixed for 10 seconds and centrifuged for 30 seconds again. The supernatant was subjected to HPLC analysis.

2.3.2.3. HPLC separation

The separation and quantification of compounds was carried out by a reversed phase HPLC system using gradient elution and fluorescence detection. An ´AccQ tag C_{18}´, column, particle size 4.6 µm, column size 3.9 x 150 mm used (Waters GmbH, Eschborn, Germany). For gradient elution 100 mM potassium acetate pH 5.5 (adjusted with acetic acid) was used as solvent A and pure methanol as solvent B. Separation was run at a flow rate of 1 ml/min. Gradient elution was carried out as follows: solvent composition at specific times after onset of analysis: at time zero: 91 % solvent A / 9 % solvent B; time 12.5 minutes: 91 % solvent A / 9 %, at time 12:55 solvent B 100 %, at time 15:50 solvent B 100 %, at time 15:55 minutes 91 % solvent A / 9 % solvent B; at time 24 minutes 91 % solvent A / 9 % solvent B. Fluorescence detection was used at an excitation wavelength of 380 nm and an emission wavelength of 480 nm. For calculation of the percentage of the reduced glutathione the equation was as follow: "percentage reduced GSH = [(total thiols-oxidized GSH) x (100 / total thiols)]" was used. An HPLC system of Waters Corp., Milford USA was used consisting of a gradient pump (600), a degassing module, a cooled autosampler ((717) and a fluorescence detector. Chromatograms were recorded and analysed using the software program Empower.

2.3.3. Quantification of soluble sugars and amino acids

For analysis of sugar and amino acid accumulation under control and stress conditions, potato suspension cells were harvested after three days of growth under standard conditions

in Erlenmeyer flasks The suspension was filtrated out of the medium through a Nylon net (100µm pore size, NeoLab, Heidelberg Germany) in a Buchner funnel. 200 mg of cells were weighed in each well of the 24multiwell plates. Cells were grown either for control in 4X medium (appendix II) or in 4X medium supplied with 500mM sorbitol or 320mM NaCl. The plates were incubated on Heidolph titerplate shaker at 300 rpm for three days. At the end of the experiment, cells were rapidly washed four times with the respective 4X medium without any sucrose. A fresh weight of 50 mg cell material was transferred into 1.5 ml Eppendorf tubes with safe lock, frozen immediately in liquid nitrogen and stored until analysis at -80 °C.

2.3.3.1. Extraction of sugars and amino acids

0.75 ml of 80% Ethanol was added to the cells and the Eppendorf tubes were closed tightly using plastic clips. The samples were incubated at 80°C for 60 min immediately. The incubator was switched off for 15 minutes to cool the samples. Susequently the cells were centrifuged for 5 min at 14000rpm and 4°C. The supernatants was transferred to new Eppendorf tubes and evaporated under vacuum at 50°C to dryness (app. 60-90 min) in a Savant SpeedVac RVT 400 (Bachofer, Reutlingen, Germany). The pellets were resolved in 250 µl Millipore water and either used directly for measurement or frozen at -20 °C up to analysis.

2.3.3.2. Measurement of the soluble sugars sucrose, glucose and fructose

Sugars were determined photometrically in a coupled enzymatic assay according to the biosynthetic scheme shown below (Figure 2). Final detection is based on the measurement of the absorption at 340 nm linked to the conversion of glucose-6-phosphate and NAD to 6-phosphogluconolacton and NADH by glucose-6-phosphate dehydrogenase. The sequential addition of first hexokinase leads to the conversion of all glucose in the sample to 6-phosphogluconolacton and an increase in the absorption values at 340 nm. The subsequent addition of phosphoglucoisomerase in the presence of hexokinase leads to the conversion of the fructose pool into 6-phosphogluconolacton and an increase in absorption at 340 nm corresponding to the fructose content and finally sucrose is converted to glucose and fructose by adding ß-fructosidase, which leads in the presence of hexokinase and phosphoglucoisomerase again to the final formation of 6-phosphogluconolacton detecting now the sucrose content of the sample.

Sugar measurement was carried out in 96 well ultra microtiter plate reader EL808 (Biotek, Bad Friedrichshall, Germany). Measuring buffer contained 100 mM imidazol-HCl, pH 6.9 and 5 mM $MgCl_2$, 15 mg NAD and 6 mg ATP were added to 10 ml measuring buffer prior to the measurement. 280-290 µl measuring buffer was added to each well of the microtiter plate followed by adding 10-20 µl of the extract. 1 µl glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (diluted 1:1 with the buffer without ATP and NAD) was pipetted onto the teeth of a comb. The enzyme was released into the wells of the microtiterplate by dipping the comb into the wells of the microtiter plate and gently shaking. The mixture was incubated for 5-10 minutes in the microtiter plate. The base line absorption was measured at 340 nm and observed for approximately 5-10 minutes. When the baseline was constant, then the measurement was started by adding successively either 1µl hexokinase HK (app. 1 unit) or 1 µl phosphoglucoisomerase PGI (diluted1:3) or 1 µl ß-fructosidase (10 mg/100 µl buffer without NAD and ATP) The absorption was measured at 340 nm until a plateau phase was reached. The absorption difference of the height between the base line and the plateau was used as the amount of glucose, between the height of glucose and fructose as amount of fructose and between the height of fructose and sucrose as amount of sucrose The calculated values were further used to estimate the final concentration of the appropriate sugar.

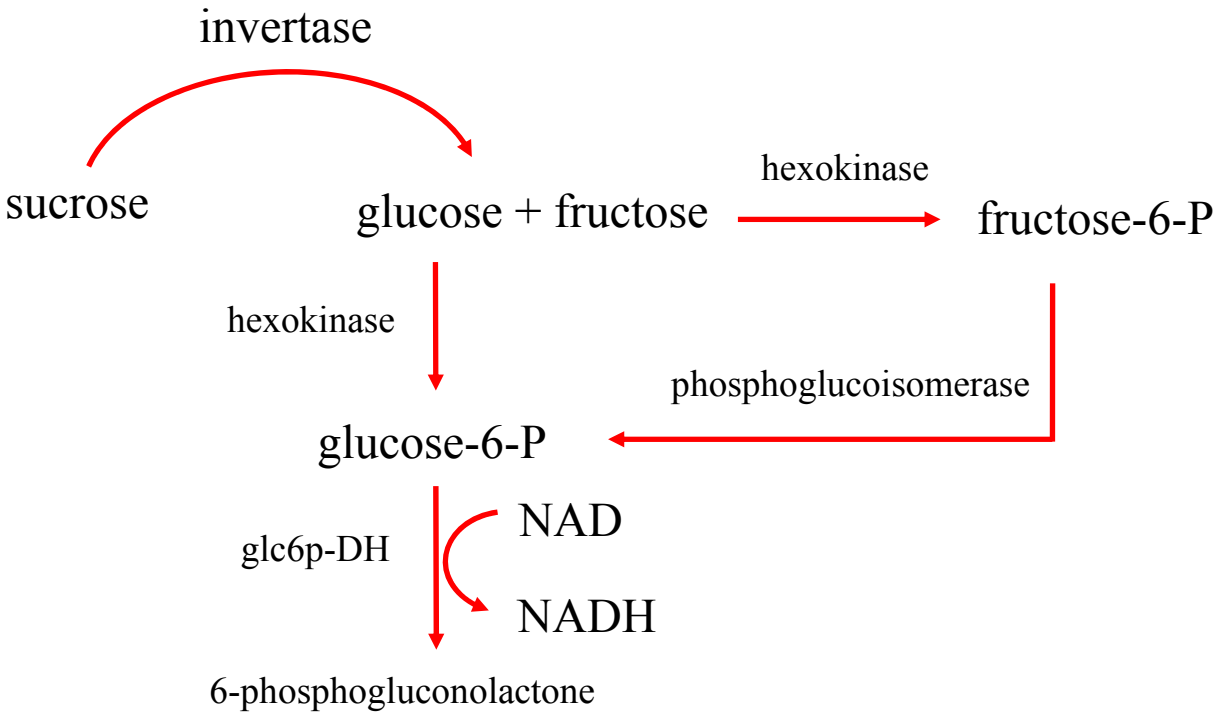


Figure 2 Biosynthetic scheme of sugar metabolism.

2.3.3.3. Derivatization of primary and secondary amino acids.

For separation and identification of amino acids a derivatization procedure was used by the fluorescent reagent ACQ according to Cohen *et al.,* 1993. The ACQ derivatives of primary and secondary amino acids show strong fluorescence at 400 nm. Identification and quantification was carried out by comparison with standard samples. Preparation of amino acid standards was done according to following procedure: For preparing the ACQ reagent 3mg of ACQ powder was dissolved in 1 ml pure acetonitrile and incubated at 55 °C for a maximum of 10 minutes. The mixture was shaken every 3 minutes. The ACQ reagent was used freshly or stored at -20 up to four weeks. Standard solution of amino acids was prepared according to the table given below:

Compound	Molecular weight	Dissolve in water or 0.1N HCl First try water then HCL if necessary	30% KOH	Stock solution 200mM mg /ml
Ala	89.1	yes	-	178
Arg	210.7	yes	-	42.1
Asn	132.1	-	yes	26.4
Asp	133.1	-	yes	26.6
Cys	121.2	yes	-	24.2
Gln	146.1	yes	-	29.2
Glu	147.1	-	yes	29.4
Gly	75.02	yes	-	15.0
His	209.6	-	yes	41.9
ILe	131.2	yes	-	26.2
Leu	131.2	-	yes	26.2
Lys	182.2	yes	-	36.4
Met	149.2	-	yes	29.8
Phe	165.2	-	yes	33.0
Pro	115.1	yes	-	23.0
Ser	105.1	yes	-	21.0
Thr	119.1	-	yes	32.8
Trp	204.1	Yes +heating at 37C	-	40.8
Tyr	181.2	-	yes	36.2
Val	117.1	yes	-	23.4
Tryptamin	160.2	yes	-	32.0

A standard mixture of amino acids with defined concentration was prepared by mixing 100 µl of each standard solution (200 mM) together. The following dilutions of this standard mixture were prepared: 1:5000 (25 pmol/10µl), 1:2000 (25 pmol/10µl), 1:1000 (25 pmol/10µl), 1:500 (25 pmol/10µl), 1:250 (25 pmol/10µl), and 1:100 (25 pmol/10µl).

2.3.3.4. Derivatization of standards and samples

80 µl borate buffer (0.2 M, pH 8.8) was filled into 1.5ml Eppendorf tube. 10 µl of the standard solutions or 10 µl of the samples were added. Finally 10 µl ACQ reagent was added. The mixtures were incubated for 10 minutes at 55°C (longer incubation leads to degradation). The mixtures were centrifuged for 1 minute at 8000 rpm. An aliquot of 10-20 µl was used for the HPLC separation.

2.3.3.5. HPLC separation and quantification

Separation and quantification of amino acids was done by reversed phase HPLC. An HPLC system of Waters Corporation, Milford USA was used consisting of a gradient pump (600), a degassing module, a cooled autosampler (717) and a fluorescence detector (474, excitation wavelength: 300 nm, emission wavelength: 400 nm). Chromatograms were recorded and analyzed using the software program Empower. For separation a reversed phase column (Symmetry, 3.9 x 150 mm) filled with C_{18}-group modified silica gel. An HPLC run was performed with a flow rate of 1 ml at 37 °C. For gradient elution a solution of 140 mM sodium acetate, pH 5.8 (Suprapur, Merck, Darmstadt, Germany), 7 mM triethanolamine 8Sigma, Munich, Germany) was used as solvent A, Acetonitrile (Roti C Solv HPLC, Roth, Karlsruhe, Germany) as solvent B and purest HPLC water as solvent C. The column was equilibrated with solvent A and gradient elution performed by increasing solvent B to 1 % at 0.5 minutes, 5 % at 27 minutes, 9 % at 28.5 minutes, 18 5 at 44,5 minutes, 60 % solvent B and 40 % solvent C at 47.5 minutes. Solvent B and C concentration was set back to 0 at 50.5 minutes and reconditioning of the column carried out for 10 minutes using solvent A.

2.3.4. Proteome analysis.

Two dimensional proteome analyses was carried out with equipment of GE Healthcare Bio-Sciences AB, Uppsala, Sweden, using a Multiphor II apparatus for isoelectric focusing and a Ettan Daltsix plates and chamber (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for second dimension SDS page analysis. Protein extraction was carried out according to Carpentier *et al.,* 2005 with modifications. Protein qquantification was done using the Amersham Quant kit according to the manufacturer's manual (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Isoelectric focusing and second dimension separation was carried out to standard procedures recommended by the manufacturer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Silver staining of the gels was performed by the procedure recommended

by (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For colloidal Coomassie staining the Brillant Blue G - Colloidal Concentrate reagent was used provided by Sigma Aldrich, USA according to the manufacturer's recommendation. Digital images of the gels were scanned by Image Scanner II and images were analyzed by Image Master 2D Platinum (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

2.3.4.1. Protein extraction.

For protein extraction a fresh weight of 100 mg cells was grinded in a cooled mortar with a pestle under liquid nitrogen and transferred immediately to sterilized 1.5 ml Eppendorf tube. 300 µl extraction buffer (50 mM Tris-HCl pH 8.3, 5 mM EDTA, 100 mM KCl, 50 mM DTT, 700 mM sucrose, 5 % PVPP, 1 tablet protease inhibitor cocktail (Roche) per 10 ml buffer) was added (Usuda and Shimogawara, 1995). The tube was briefly vortexed. Then 330 µl of Tris buffered phenol (10 mM Tris-HCl, pH 7.5) was added and the samples were vortexed for 10 minutes at 4 °C. Samples were centrifuged for 10 min at 13000 rpm and 4°C. The phenolic phase was collected and re-extracted by adding again 330µl of extraction buffer. Samples were centrifuged again for 10 minutes at 13000 rpm and 4 °C. The phenolic phase (ca-300ul) was collected and transferred into a new tube and the protein precipitated overnight with 5 volumes of 100 mM ammonium acetate in methanol at -20°C. On the next day samples were centrifuged for 60 minutes at 17000 g and 4 °C and the supernatant was discarded. The pellet was rinsed twice in 1.5 ml rinsing solutions (cold acetone, 0.2 % DTT) for one hour at -20 °C. After rinsing, the samples were centrifuged for 30 minutes at 17000g and 4 °C to collect. The pellet was dried briefly under vacuum in a SpeedVac for 20 minutes at room temperature and then suspended in 50 µl lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, prior to use 0.8 % IPG buffer pI 3-10 and 1 % DTT was added) and vortexed for 5 minutes at room temperature. To clear the samples they were centrifuged for 30 minutes at 17000g and 18 °C. The clear supernatant was stored in aliquots at -80°C until analyzed.

2.3.4.2. Protein quantification.

Prior to analysis protein content of the samples was quantified using the Amersham Quant kit according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). mmAbsorption measurement was done in Tecan Genios multiplate reader (Tecan GmbH, Crailsheim, Germany) at 492 nm using 250 µl sample volume. For the BSA standard curve and each sample duplicates were measured.

2.3.4.3. Isoelectric focusing.

For IEF focusing using 24 cm strips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) a sample volume containing a specific amount of protein was diluted to a final volume of 450 µl with rehydration buffer (7 M Urea, 2 M Thiourea, 30 mM Tris-HCl pH 8.5, 4 % CHAPS, 10 % glycerol, 0.002 % bromophenol blue, 2 % IPG buffer pI 3-10, 0.28 % DTT, IPG buffer and DTT was added prior to use). For silver staining 40 µg protein and for Coomassie staining and MALDI-TOF analysis 200 µg proteins were applied per strip. Reagents, Immobiline DryStrips and IPG buffer were obtained from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. The 24 cm Immobiline DryStrips were rehydrated in 450 µl protein sample dissolved in rehydration buffer over night at room temperature in the Amersham Reswelling Tray (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Rehydration was carried out according to the recommendation of GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Rehydrated Immobiline DryStrips were rinsed with deionized water for a few seconds and slightly blotted to remove excess water. Isoelectric focusing was carried out in the IEF unit of the Multiphor II apparatus according to the manufacturers' instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at a temperature of 20 °C. IEF focusing was run according to the program recommended by GE Healthcare Bio-Sciences AB, Uppsala, Sweden for 24cm IPG strips, pH 3-10 NL in gradient mode. The program is listed below:

1. 500 V, 0:01 h, 5 Watt, 0.5 mA per strip
2. 3500 V, 1:30 h, 5 Watt, 0.5 mA per strip
3. 3500 V, 12:00 -16:20 h, 5 Watt, 0.5 mA per strip

The total volt hours should be less than 60 kVh.

After IEF focusing the Immobiline DryStrips strips were either used directly for second dimension separation or stored in test tubes at -80 °C.

2.3.4.4. Second dimension separation (SDS-PAGE).

For the separation of proteins in the second dimension by SDS-Page electrophoresis the Immobiline DryStrips were equilibrated twice for 15 minutes in 2 x 100 ml equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 30 % glycerol, 2 % SDS) on a shaker. In the first equilibration step 1 % DTT and in the second equilibration step 4.5 % iodoacetamide was added to the equilibration buffer. The equilibration was carried out in equilibration tubes (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 15 ml of the equilibration solutions for each equilibration step per Immobilime DryStrip in each tube. The equilibrated IPG gel strips

were slightly rinsed and blotted to remove excess equilibration buffer and then positioned between the plates on the surface of the second dimension gel. The strips were fixed with 2 ml of 2 % warm agarose solution dissolved in running buffer (250 mM Tris base, 1.9 M glycine, 1% SDS). Electrophoresis was carried out in an Ettan DALTsix gel chamber (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 20 °C. Electrophoresis was started at 50 V and 400 mA for 30 min. Then the voltage and current were increased to 300 V and 400 mA. The electrophoresis was terminated when the Bromophenol Blue tracking dye has migrated off the lower end of the gel and then it was used for staining or blotting.

2.3.4.5. Silver staining.

Siver stainig was carried out according to the procedure recommended in the Amersham Silver Staining Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Gels were first incubated for 30 minutes in fixing solution (40 % ethanol, 10 % acetic acid) over night. They were then incubated for 30 min in sensitizing solution (75 ml ethanol 95 %, 1.25 ml 25 % glutaraldehyde, 10 ml 5 % sodium thiosulfate, 28.17 g sodium acetate, filled up to 250 ml with ddH2O per gel). Gels were washed 3 times for 5 minutes in distilled water and then incubated for 20 min in silver staining solution (0.625 g silver nitrate, 0.1 ml formaldehyde, filled up to 250 ml with H_2O per gel). Gels were again washed with distilled water 2 times for 1 minute. Gels were then incubated for 2-5 min in developing solution (6.25 g sodium carbonate, 0.05 ml formaldehyde in 250 ml H_2O per gel) until protein spots became visible. The developing solution was poured off and 250 ml stopping solution added to each gel 3.65 g Na-EDTA x 2 H_2O, filled up to 250 ml with H_2O). Developing time was kept constant for each batch of gels for comparative analysis. Gels were incubated in the stopping solution for 30 minutes. Gels were washed 3 times for 5 minutes in water. Finally the gels were incubated twice for 30 min in 250 ml distilled water containing 75 ml ethanol (95 %) and 11.5 ml glycerol (87 %) solution per gel and stored sealed in plastic foil at 4 °C.

2.3.4.6. Coomassie blue staining

Colloidal Coomassie stain was carried out using the Brilliant Blue g-colloidal concentrate (Sigma Aldrich, Munich, Germany, product number: B 2025) according to the manufacturer's instructions as follows: 1. After electrophoresis protein was fixed by incubation of the gel for 30 minutes in a solution of 7 % glacial acetic acid in 40 % (v/v) methanol. 2. 800 ml deionized water were added to the purchased reagent bottle containing the dye concentrate

and mixed to prepare the working solution. 3. The staining solution was prepared by mixing 4 parts of the working solution and 1 part of methanol. 4. The gel was stained in the staining solution for 2 hours. 5. Afterwards the gel was detained with 10% acetic acid in 25 % (v/v) methanol for 5 minutes with shaking. 6. Finally gels were rinsed with 25 % methanol for up to 24 hours. Stained gels were stored in 25 % ammoniumsulfate solution at 4 °C.

2.3.4.7. Analysis of the proteome pattern.

Digital images of the stained gels were scanned with an Image Scanner II and for analysis and comparison of different proteome patterns the Image Master 2D Platinum software was used (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.3.5. Analysis of protein spots by mass spectrometry

2.3.5.1. Sample preparation

For mass spectrometrical identification of single protein spots pieces of 1 - 3 mm^2 size were cut out of a Coomassie stained gel and transferred to a clean 1.5 ml Eppendorf tube. Fixation of the proteins in the gel pieces and washing out of Coomassie stain was carried out in a solution of 10 % acetic acid and 30 % ethanol over night under gentle shaking at 4 °C (cold room). Afterwards the acid was neutralized by adding 1 ml 50 mM NH_4HCO_3 for 1 hour. A reduction reaction was performed in the gel pieces for 30 minutes with 500 µl 20 mM DTT in 50 mM NH_4HCO_3 .Afterwards, alkylation of proteins in the gel pieces was done for 1 hour in 500 µl 50mM iodoacetamide in 50 mM NH_4HCO_3 in the dark. Gel pieces were then washed for 1 hour in 1 ml 50 mM NH_4HCO_3 and afterwards for 1 hour in 40 % acetonitrile in 50 mM NH_4HCO_3. Shrinking of gel pieces was achieved by incubation for 10 minutes in 20 gel volumes acetonitrile and samples dried from acetonitrile in SpeedVac. Samples were either analyzed directly or stored for further analysis at -20 °C.

2.3.5.2. Tryptic digest

The preparation of small peptides for mass spectrometrical analysis was done by trypric digest. A Trypsin solution (digestion solution) was prepared by suspending 25 µg enzyme (Trypsin "sequencing grade", Promega, Madison, USA) in 80 - 100 µl Promega buffer or 1 mM HCL and incubation for 30 minutes at 30°C. Aliquots of 5 µl were filled in 500 µl Eppendorf tubes and stored at - 20°C. For digestion Eppendorf tubes containing the aliquots of enzyme were filled with 250 µl 50 mM Bicarbonate, 200 µl water und 50 µl acetonitrile. Incubation in this solution for 10 - 30 minutes in the refrigerator leads to a swelling of the gel

pieces. Then the gels were sliced into app 1.5 mm^2 big cubes and suspended completely in the digestion solution. After 20 - 60 minutes more digestion solution was added if necessary. For the last hour of incubation, gel pieces were submerged completely in digestion solution. Then Eppendorf tubes were closed tightly and incubated over night at 37°C. The resulting digest can be stored for 1 - 2 weeks at 4 °C and even longer at -20 °C.

2.3.5.3. Peptide extraction

For extraction of resulting peptides after protein digestion, the 5 - 10 fold volume of water was added to the digest samples and tubes shaken gently for 30 - 60 minutes. The supernatant was transferred to a new Eppendorf tube and dried in a SpeedVac apparatus for 20 minutes. 10 gel volumes of 0.1 % TFA was added and samples and shaken gently for 30 - 60 minutes. The supernatant was transferred to the same Eppendorf tube and dried in a SpeedVac apparatus.10 gel volumes of 1 % TFA were added and samples shaken gently for 30 - 60 minutes. The supernatant was again transferred to the same Eppendorf tube and dried again. Finally 10 gel volumes of a solution of 0.1 % TFA and 40 % acetonitrile were added and samples shaken gently for 30 - 60 minutes. The supernatant again transferred to the same Eppendorf tube and dried for a third time. Shrinking of gels was achieved in 20 µl acetonitrile, supernatant transferred and dried. When the samples were prepared for Q-TOF ACN measurements 0.1% TFA was replaced by 0.5% formic acid.

2.3.5.4. Mass spectrometrical measurements

Re-solubilization of samples was carried out in 0.1% TFA. The 1 µl sample was mixed with 1µl matrix solution (20 mg/ml a cyanohydroxy ammoniac acid in 50% acetonitrile. Samples were measured using an Ultraflex II Tof/Tof mass spectrometer from Bruker Daltonics (Bruker Daltonik GmbH, Bremen, Germany). Processing of acquired spectra was done using flex analyses 2.0 and Biotools 2.1.

Matrix Science offered the Mascot search engine for rapid protein identification. Mass spectrometry data were used to search the spectra. Modifications and parameters used for protein identification were carbamidomethylation, oxidation of methionine, 1 missing cleavage, Trypsin digestion; measurements were carried out with 100 ppm mass error. Basis for protein identification were the SwissProt and NCBI databases and for taxonomy Viridiplantae. Mass spectrometric measurement and analysis has been performed by Dr. J. Wissing, HZI-Helmholtz Center for Infectious Disease Research, Braunschweig

2.3.6. Monitoring of luciferase activity

2.3.6.1. Quantitative luciferase assay

The functionality of the constructs used in this study was tested by monitoring expression of the luciferase gene by a quantitative assay for luciferase enzyme activity. Promega luciferase assay Kit (Promega, Madison, USA) was used. For measurement of luciferase activity, plant materials (leaves, cell suspension, callus) was harvested, frozen quickly in liquid nitrogen, ground to powder under liquid nitrogen using mortar and pestle. Powdered cell material was suspended in 300 µl of 1X lysis (CCLR) reagent (Promega) by mixing thoroughly (Vortex) at room temperature. The suspended plant material was incubated at 4°C for one hour. Pellet debris was removed by centrifugation at 14000 rpm for 10 minutes at room temperature and the supernatant was transferred to a new tube. Measurement was carried out in a Berthold Luminometer (Lumat LB 9501, Berthold Technologies, Wildbad, Germany). 20µl of cell lysate was mixed automatically by the Luminometer with 100 µl of Luciferase Assay Reagent (LAR)

2.3.6.2. Qualitative luciferase assay

Qualitative visual monitoring of luciferase activity was done using Luminescence image analyzer (LAS 3000, Fuji Deutschland, Düsseldorf, Germany). Tobacco and potato leaves were immersed in 5% Tween 20 for 3-5 minutes to stabilize emulsions and suspension, and then washed three times with water to remove the residual Tween, whereas cell suspension and calli were used directly for the analysis. A Luciferin (Promega) solution (1mM) in water was sprayed under sterile conditions over the leaves or the cells. The materials were incubated at room temperature for 5 minutes and chemiluminescence was measured after 1-5 minute exposure time depending on the plant material

2.4. Methods of Molecular Biology

2.4.1. Extraction of genomic DNA

For genomic DNA extraction, two different methods were used. To obtain genomic DNA of high quality for PCR based gene isolation the Charg Switch g DNA plant kit (Invitrogen Corporation, Carlsbad, USA) was applied. For routine applications a modified method based on the CTAB method of Doyle and Doyle 1990 was used. The methods were also used to isolate high quantities of gDNA for transgen identification by Southern blot analysis.

2.4.1.1. Isolation of genomic DNA with Charge Switch g DNA plant kit

Chemicals and specific equipment used in this method was provided by Invitrogen Corporation Carlsbad, USA. About 100 mg of fresh plant material was homogenized with pestle and mortar under liquid nitrogen and then transferred to a 1.5 ml Eppendorf tube. 1 ml of charge switch lyses buffer and 2 µl RNase (20 mg/ml) were added. The mixture was mixed vigorously and 100 µl of 10 % SDS was added and the mixture was incubated for 5 minutes at room temperature. During the incubation the lysate was mixed by inverting the tube inverted for several times. Then 400 µl charge switch precipitation buffer was added. The mixture was mixed by vortexing until a precipitate was formed, then centrifuged for 5 minutes at 14000 rpm at room temperature. The clear aqueous phase was transferred to a new reaction tube and 100 µl of re-suspended magnetic beads were added and mixed gently by pipetting. After 1 minute incubation time at room temperature, the tubes were placed in the respective Magna rack for about 1 minute to form a pellet. The supernatant was discarded carefully without removing the tubes from the rack and the pellet was washed three times with 1 ml washing buffer.150 µl of elution buffer was added and the pellet were re-suspended carefully by pipetting gently up and down. Then the tubes incubated at room temperature for 1 minute. The tubes were placed back in the Magna rack to collect the beads and the clear supernatant was transferred to a fresh tube. Finally, the DNA concentration was determined as described.

2.4.1.2. Isolation of gDNA by the Cetyltrimethylammoniumbromid (CTAB) method

The CTAB extraction buffer (Tris-HCl pH 8.0 (base) 100 mM, CTAB 3 %(w/v), NaCl 1.4 M, EDTA, 20 mM, ß-Mercaptoethanol, 0.2 % (v/v) (add fresh directly before use)) was heated up to 65 C before use. 100-150 mg plant material (leaves or cells) were harvested, placed immediately in liquid nitrogen and grinded to powder under liquid nitrogen using mortar and pestle. The ground material was transferred into 2 ml Eppendorf tubes. 800 µl of pre-heated (65°C) CTAB extraction buffer was added followed by vigorous vortexing under a fume hood. The tubes were incubated for 30 minutes at 60°C. After incubation 800 µl CI-mix (23 parts chloroform + 1 parts isoamylalcohol) were added and tubes were gently mixed by inverting the tube for 4-5 times to avoid shearing of genomic DNA. The mixture was centrifuged at room temperature for 10 minutes at 10000 g. The aqueous phase (app. 800 µl) was transferred into a fresh 1.5 ml Eppendorf tube. The centrifugation step was repeated to get a clear sample. 550 µl of pre-cooled (-20°C) isopropanol was added and gently mixed to

allow precipitation of DNA. The tubes were centrifuged for 10 minutes at 14000 rpm to precipitate the genomic DNA. The supernatant was discarded and the DNA pellet was washed with 200 µl washing buffer (76 % absolute ethanol, 10mM Na-acetate, 7.5 M NH_4-acetate, 0.5 M EDTA, pH 8) until the pellet floats. Washing buffer was carefully removed and the pellet was re-suspended in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with RNase A (10 µg/ml), (Fermentas, St. Leon-Rot, Germany) incubated for 30 minutes at 37°C, and then 100 µl 7.5 M NH_4-acetate and 750 µl absolute ethanol was added and gently mixed. The mixture was centrifuged at maximum speed for 10 minutes at room-temperature. The supernatant was discarded completely and the pellet was dried for 40-50 minutes at 37 °C. After drying, the pellet was re-suspended in 100 - 200 µl TE buffer and stored at 4°C over night. DNA quantification was carried out as described.

2.4.2. Isolation of RNA

For the isolation of RNA the NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany) was used. Freshly harvested plant tissues or samples frozen at -80°C (100 mg) were homogenized under liquid nitrogen using a mortar and pestle. Pulverized plant material was transferred to 1.5 ml Eppendorf tubes. 500µl of cooled Plant RNA Reagent was added and the sample mixed using vortex. (Genie2, Scientific Industries, Rockville, USA). Samples were incubated at room temperature for 5 minutes followed by centrifugation at 12000g at room temperature for 2 minutes. The clear supernatants were transferred to new Eppendorf tubes, 100µl of 5 M NaCl solution was added and mixed briefly. 300µl of chloroform was added and the solution was mixed by inversion. The samples were centrifuged for 10 minutes at 12.000 rpm and 4 °C. The aqueous phase (500µl) was collected in Eppendorf tubes and an equal volume of isopropanol added and briefly mixed by inversion. The samples were incubated for 10 minutes at room temperature and centrifuged for 10 min at 4 °C and 12.000 rpm. The pellet was washed by in 1 ml absolute ethanol and again centrifuged (1 minute at 12.000 and 4°C). After removing the supernatants the tubes were centrifuged again to collect the residual liquid which was removed carefully with a pipette. The pellets were dissolved in 30 µl of RNAse free water. RNA concentration was measured using a spectrophotometer (Eppendorf, Bio photometer-Hamburg, Germany).

2.4.3. Synthesis of first strand cDNA

Reverse transcriptase is an RNA dependent DNA polymerase and is used to catalyze the oligo-dT primed synthesis of first strand cDNA (DNA complementary to the appropriate mRNA) from total RNA. The reaction mix contained 1 µg total RNA, 5µl oligo dT primer (0.5µg/µl), 1 µl DMPC-H_2O and filled up to 7 µl. Denaturation of RNA at 70°C for 10 minutes was followed by cooling to 4°C and addition of the following reagent: 5 µl 5x 1st strand buffer (supplied with the enzyme), 2 µl 10 mM dNTPs and 1µl RNase inhibitor. The mixture was mixed and collected by centrifugation, then incubated at 37°C for 5 minutes followed by addition of 1 µl MMLV-RT (revertAid first strand cDNA synthesis kit, Fermentas). The reaction mixture was incubated at 42°C for 60 minutes, followed by heat inactivation of the enzyme at 70°C for 10 minutes. The cDNA sample was stored at -20°C until used.

2.4.4. Quantification of nucleic acids

The concentration of nucleic acids was determined by measuring the absorbance of the DNA or RNA samples at wavelengths of 260 and 280 nm. An absorbance of 1 at 260 nm corresponds to 50 µg/ml double-stranded DNA (Sambrook *et al.,* 1989). Care was taken that the ratio of absorbance 260nm/280nm was not less than 1.8 to ensure the appropriate quality. Contaminations with proteins or phenolics would reduce this value due to their absorbance at 280 nm. For RNA quantification, samples were diluted 1:200 (199 µl H_2O + 1 µl RNA). Then the RNA concentration was spectrophotometrically measured. The concentration of RNA was calculated as follows: RNA concentration in µg/ml = (absorbance at 260 nm x dilution factor x 40). The purity of the RNA was determined using the ratio of absorbance at 260nm / absorbance at 280 nm), which should be between 1.9 and 2.0 for pure RNA. Spectrophotometrical measurements were made using a Biophotometer (Eppendorf, Hamburg, Germany)

2.4.5. Electrophoresis of nucleic acids

Agarose gel electrophoresis is a method used in molecular biology to separate DNA and RNA molecules by size, and to determine the size of the separated strands by comparison to strands of known length. The concentration of the gel depended on the fragment lengths to be separated. 0.8-1 % (w/v) agarose gels were prepared in 1x TAE buffer (40 mM Tris-acetate, 20 mM glacial acetic acid, 1 mM EDTA, pH 7). The mixture was boiled in a microwave oven

to dissolve the agarose. After cooling down of the agarose solution to 60°C, ethidium bromide (0.5µg/ml) was added and the solution was poured into the gel chamber. For electrophoresis a horizontal electrophoresis apparatus (Polymehr GmbH, Paderborn, Germany) was used. Before loading on the gel, samples were mixed with 6x loading buffer. Electrophoresis was run at a voltage of 60-100 V for 30-40 min supplied by power supply (EPS600, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). To estimate the size of nucleic acids, a 1 kb or a 100 bp DNA marker was run parallel to the samples in the same gel DNA fragments were observed and photographed under UV-light.

2.4.6. Purification of DNA from agarose gels

Purification of DNA fragments was performed using QIAQuick kit (Qiagen, Hilden Germany). After gel electrophoresis DNA fragments had to be eluted from gels for further applications such as restriction digest or ligation reactions DNA fragments were eluted from agarose gel after gel electrophoresis. After ethidium bromide staining gel pieces containing DNA bands were cut out under UV light and the DNA eluted in TE buffer**.**

2.4.7. Digestion of DNA by restriction endonucleases

DNA was digested using different restriction endonucleases with respective buffers as recommended by the supplier. When two enzymes had to be used for digest, the buffer was selected to be suitable for both enzymes; otherwise it was done one after the other. Digestion was done usually at 37 °C for 2 hours or overnight, and then enzymes were heat-inactivated by heating the sample for 15 minutes at 65 °C, depending on the enzyme.

2.4.8. Purification of PCR product

For all of the PCR based cloning experiments, the amplified PCR products were purified by using the Qiagen MinElute PCR purification kit protocol as below:

5 volumes of Buffer PB were added to 1 volume of the PCR reaction mixture (before the amplified PCR product was confirmed by running on agarose gel) and mixed gently. Then the MinElute column, inside the 2 ml collection tube, was put in a suitable rack and the sample applied to the MinElute column. The tubes were entrifuged for 1 min, and then the filtrate was discarded and the MinElute column placed again into the same collection tube. For washing 750 µl of buffer PE was added to the MinElute column and the column again centrifuged for 1 min. The filtrate was again discarded and the column again put back into the tube. The column was centrifuged again for 1 min at maximum speed to remove the residual ethanol

completely. After washing the column was put into new 1.5 ml microcentrifuge tube and elution of the DNA was done by adding 10 µl buffer EB directly in the centre of the column. The column was kept standing for 1 min and centrifuged again for 1 min to collect the DNA in the microcentrifuge tube. The purified product was used for further cloning steps.

2.4.9. Primer lists

Primer name	Oligonucleotide sequence
STH2Xma1	5′-AATCCCGGGATGGGTGTCACTAGCTATACACATG-3′
STH2HindIII	5′-AAAAAGCTTTTAAGCGTAGACAGAAGGATTGGCG-3′
nptII NotI f	5′-AAAGCGGCCGCATGATTGAACAAGATGGATTGC-3′
t-nos Sac r	5′-AAAGAGCTCTATCAGCTTGCATGCCGG-3′
Luc 711 f1	5′-CGATTCGGTTGCAGCATT-3′
Luc 711 r1	5′-CGATCAAAGGACTCTGGTACAA-3′
Luc 837 f2	5′-CCTTCCGCATAGAACTGCCT-3′
Luc 837 r2	5′-TCCAAAACAACAACGGCG-3′

Table.1: specific primers used for the identification and cloning of gDNA and cDNA of the sth2 gene of potato, detection of the transgene and Southern blot analysis

2.4.10. Composition High fidelity (HF) PCR Mixture for cloning of target genes

Contents and Concentration	Volume
10x HF buffer + $MgCl_2$	5 µl
10 mM dNTPs	1 µl
10 pmol primer (F)	1 µ
10 pmol primer (R)	l µl
Taq DNA polymerase 5U/µl	0.3 µl
Template DNA	1 µl
Double dist H_2O	40.7 µl
Total	50 µl

2.4.11. PCR program for routine DNA amplification

PCR steps	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	5 min.	1
Denaturation	94	40 sec	35
Annealing	57	40 sec.	
Extension	72	2 min.	
Final extension	72	10 min	1
Cooling and storage	4	∞	1

2.4.12. Cloning of PCR product

2.4.12.1. Cloning in P Drive vector

High fidelity DNA polymerase *(Pflu Taq* DNA-polymerase, Fermentas) amplified PCR products were directly cloned into the P Drive vector. The PCR products that were amplified by using *Pflu* had to be incubated first with Taq DNA-polymerase at 72°C for 10 minutes in order to create a single dATP overhang at the 3`ends of the PCR products before cloning. The TA cloning reaction Mix contained 3 µl of PCR product, 5 µl buffer solution, 1 µl P Drive vector and 1 µl dest. H_2O (total volume 10 ml)

2.4.12.2. Selection of positive recombinants

The presence of the appropriate cloned DNA was determined by using the blue/white colony selection method. Blue/white cloning selection method was mainly used for cloning of DNA fragments into pDrive vector. To carry out the selection 40µl of x gal (20mg/ml) and 40 µl of IPTG (0,1M) were spread on the top of LB medium plates (appendix III) with a hockey stick spreader followed by spreading of 40 µl of bacterial suspension. The plates were incubated at 37°C overnight, and then incubated for 1 hour at 4°C to differentiate between white and blue colonies.

2.4.13. Isolation of plasmid DNA by minipreparation

Mini preparation of plasmid DNA was carried out by the alkaline lysis method developed by Birnboim and Doly (1979) with slight modifications. For Plasmid DNA extraction a growing bacterial colony was picked up from medium in a Petri dish using a sterile toothpick to

inoculate 2 ml liquid LB medium containing 50 mg/l kanamycin. The bacteria were allowed to grow in suspension over night with shaking at 225 rpm and at a temperature of 37°C. 2 ml of the bacterial suspensions were harvested by centrifugation at 14,500 rpm for 5 minutes. The bacterial pellet was then resuspended in 200 µl solution A **(**15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM Glucose, 2 mg/ml fresh lysozyme). By vigorous vortexing the bacterial pellet was dissolved completely and the suspension incubated for 15 minutes at room temperature.. For lysis of the bacteria, 400 µl solution B (0.2 M NaOH, 1 % SDS (Lysis buffer)) was added to the bacterial suspension and mixed by inverting the tube rapidly five times (no vortex). Then 300 µl solution C 3 M NaOAc, pH 4.8 (Neutrilizing buffer)) was added to the bacterial lysate and the lysate immediately mixed by gentle inverting the tube for 5-6 times. The tube was placed on ice for 15 minutes followed by centrifugation at room temperature at 14.500 rpm for10 minutes to precipitate protein and large fragments of chromosomal DNA.. The clear supernatant (800µl) was transferred to a fresh 1.5 ml Eppendorf tube followed by the addition of 600 µl of cold (-20 °C) isopropanol and mixed gently by inverting the capped tube to precipitate plasmid DNA. After 2 minutes incubation at room temperature, plasmid DNA was then pelleted by centrifugation at 14.500 rpm for 10 minutes followed by removal of the supernatant. The plasmid DNA pellet was re- dissolved in 200 µl of the solution D (0.1 M NaOAc, pH 7.0, 0.05 M Tris-HCl pH 8.0) and incubated for 5 minutes at room temperature 400 µl of absolute ethanol was added and mixed. Then the sample was centrifuged for 10 minutes at room temperature and the supernatant was discarded. The pellet was allowed to dry at room temperature and then dissolved in 50 µl TE buffer (Tris 10mM, EDTA 1.0 mM, pH 8.0, concentrated HCl) containing 0.5 µg/ml DNase free-RNase. The mixture was incubated at 37°C for 30 minutes to digest residual RNA. The isolated plasmid DNA was stored at -20°C until further use.

2.4.14. Preparation and transformation of competent cells

2.4.14.1. Preparation of competent E.coli

To prepare a suspension of competent *E. coli* bacteria for the admission of foreign DNA plasmids a modified method (Nakata *et al.,* 1997 and Tang *et al.,* 1994) was applied. A culture of *E. coli* strain (GM 2163) was grown overnight in 1-5ml of LB medium (appendix III) at 37 °C (without antibiotics) to the stationary phase. The culture was diluted in fresh LB medium 1:50 and grown at 37 °C until an optical density at 600 nm (OD_{600}) of app. 0.4 was

reached. The bacteria were harvested by centrifugation at 4 °C and 4400 rpm and re-suspended in 1/2 volume ice-cold 100 mM $CaCl_2$ and centrifuged again (4 °C, 4400 rpm**)**. The supernatant was discarded and the pellet was re-suspended once again in 1/2 volume ice-cold 100 mM $CaCl_2$. The bacteria were again centrifuged to collect the pellet which was re-suspended in 1/10 volume cold 100 mM $CaCl_2$ the bacterial suspension was kept at 4°C for 1 hour. The competent cells were either used immediately for heat shock transformation or stored. For preparation of a storage culture, 86 % sterile glycerol was added to reach a final concentration of 15 % glycerol and then aliquots of 100 µl of the suspension were transferred into 1.5 ml Eppendorf tubes which were filled with liquid nitrogen and stored at -80 °C for further use.

2.4.14.2. Preparation of competent *Agrobacterium tumefaciens* EHA105pSoup

The supervirulent strain EHA105 harbouring the plasmid pEHA105 (Hood *et al.,* 1993) was co-transformed with the pSoup helper plasmid according to the pGreenII system (pGreen website, Hellens *et al.,* 2000). An overnight *Agrobacterium* seed culture of 25 ml YEP (appendix IV) supplemented with 5 mg/l tetracycline was incubated with 250 µl of a glycerol stock of EHA105pSoup at 28 °C on a shaker. 2 ml of the bacterial suspension (overnight seed culture) were added to 50 ml YEP supplemented with antibiotic and grown for 2-5 hours until an OD600 of app. 0.4-0.5 was reached. Bacteria were twice pelleted by centrifugation at 4400 rpm and 4 °C for 10 minutes and re-suspended in 25 ml ice-cold 10 % glycerol. Then the pellet was re-suspended twice in 2.5 ml ice-cold 10 % glycerol after centrifugation at 4400 rpm at 4 °C for 10 minutes and finally re-suspended in 1 ml ice-cold 10 % glycerol. Aliquots of 200 µl were split in 2 ml Eppendorf tubes and transferred immediately into liquid nitrogen and stored at -80 °C.

2.4.14.3. Transformation of competent *E. coli* bacteria

For transformation a frozen suspension of competent *E. coli* bacteria was placed on ice (0°C) for thawing. 2-3 µl of ligation product or 1-2 µl plasmids were added to 100 µl of the competent bacteria cells was mixed by pipetting and incubated on ice for approximately 20 minutes. Heat shock was given by exposing the mixture to 42°C for 40-60 seconds and subsequent immediate cooling back to ice (o°C) for 2 minutes. Now 900µl of pre-cooled SOC medium(appendix V) without antibiotic was added to the bacterial mixture and the tube was incubated at 37°C with shaking at 225 rpm for 90 minutes 100 and 400ul of the growing cells

was spread independently on selective LB agar medium (appendixIII)containing antibiotic followed by incubation at 37°C overnight.

2.4.14.4. Agrobacterium transformation

A frozen suspension of competent *Agrobacterium* (EHA105-pSoup) was kept on ice to avoid melting. 50 ng (1-5 µl) of a plasmid solution was gently mixed with 50 µl suspension of the competent bacteria were filled in a 1.5 ml Eppendorf tube. The mix was transferred to a pre-cooled cuvette, gap 0.2 cm (PeQ lab,Biotechnologie,GmbH) and electroporated in a BioRad electroporator at: 25 µl capacitor, 200 Ω (ohm) resistance and 2.5 KV. The field strength was between 6.25 – 12 kV/cm for 4-8 sec. 500-1000 µl of pre-cooled SOC medium (with no antibiotic) were added immediately afterwards, then the mixture was transferred to a new 2 ml Eppendorf tube and incubated for 3 hours at 28 °C with shaking (250 rpm). 100 µl of the resulting culture was diluted (1:1, 1:10 ,1:100,1:1000) and aliquots of each dilution spread on LB agro plates(appendix VI) containing 50 mg/l kanamycin. The plates were incubated grown at 28 °C. Colonies were picked after 24-48 hours incubation time for plasmid isolation.

2.4.15. Leaf infiltration for transient studies

Nicotiana benthamiana, 5 weeks old (4-6 leaf stage) plants were selected for leaf infiltration. after Cazzonelli et al, 2006. *Agrobacterium* strain EHA-105 harboring the specific plasmid construct were grown overnight prior to leaf infiltration. Overnight grown cultures (OD_{600} 0.8-1.0) were independently centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was dissolved in the same volume of infiltration medium MMA (appendix VII). The suspended *Agrobacteria* pellet was incubated at room temperature for 2 hours. When all dead cells had settled down, the upper 10-15 ml of medium were collected; OD_{600} was adjusted to 0.9-1.0, and mechanically infused by pressing the tip of the syringe against the lower surface of the leaf and applying a gentle pressure on the plunger. The infusion of the bacteria was visually monitored by observing the bacterial suspension filling the air space of the leaf. In the controls only the infiltration medium was infused. Plants were watered after leaf infiltration equally. Plants were kept to grow at 20°C in the dark for 60-72 hours. Fully infused leaves were marked and selected and the crude protein was extracted for the quantitative luciferase assay. Furthermore the infiltrated leaves were monitored under a Fuji LS 3000 imager (Fuji Deutschland, Düsseldorf, Germany).

2.4.16. Transformation of tobacco plants

Nicotiana tabacum SR1 leaves obtained from 4 to 6 week old in vitro grown plants were used for the leaf disc transformation protocol. (Horsch *et al.,*1985). The midrib and the edges were removed from the leaves. Then leaves were cut into discs of 1 cm^2. Two days before the inoculation, a culture of app. 25 ml of *Agrobacterium tumefaciens* harboring the different T-DNA constructs in LB medium (appendix VI) containing 50 µg/ml kanamycin was set up. The culture was incubated overnight at 28°C under shaking at 150 rpm. 500 µl of the bacterial suspension was diluted with 50ml liquid MS medium (appendix VIII) tobacco leaf discs were incubated in *Agrobacterium* suspension for 20 min in a Petri dish (90 mm in diameter). The leaf discs were blotted dry and placed upside down in Petri dishes on solid medium containing MS salts supplemented with B5 vitamins (MS-1 medium, see appendix IX). Leaf discs were co-cultivated with the bacteria in the dark for 2 days at 25 °C. Then the infected leaf discs were washed with liquid MS liquid medium containing 300 mg/l ticarcillin and blotted dry. Dry leaf discs were transferred to fresh MS-1 medium containing 300 mg/l ticarcillin in order to eliminate the bacteria. To induce callus formation the medium contained also phytohormones (1 mg/l BAP and 0.5 mg/l NAA). The leaf discs were incubated at 25°C with a light cycle of 16/8h of light/dark, respectively. Petri dishes were checked daily for presence of *Agrobacteria* or possible contaminations. After one week leaf discs were transferred to fresh MS-1 medium containing 200 mg/l ticarcillin and 5 mg/l ppt. Further subculturing was carried out on the same MS-1 medium reducing ticarcillin to 100mg/l. After approximately four weeks resistant calli were obtained. These were further transferred to fresh MS-2 medium (appendix X) containing 0.2 mg/l BAP for the induction of shoot formation. Well developed shoots were transferred to MS-0 medium containing 100mg/l ticarcillin and 5 mg/l ppt (appendix XI) in glass jars. Once plantlets had developed roots and three to four pairs of leaves, they were transferred into pots with soil. For this purpose, the agar was carefully removed from the roots with warm water and plants were planted. In the first few days after planting the plantlets were well watered and covered with plastic bags to avoid desiccation due to an undeveloped cuticle. After three days, plastics bags were opened to allow a better air exchange. The bags were completely removed after 8 days. Plants were kept under optimal growth condition until flowering.

2.4.17. Transformation of suspension cultures

More efficients transformations can be achieved by activation of the *vir* gene on the Ti-plasmid of *Agrobacterium tumefasciens* applying acetosyringone (Hiei *et al.,* 1997; Trieu *et*

al., 2000; Mahmoudian *et al.,* 2002; Cardoza and Stewart JR, 2004; Moghaieb *et al.,* 2004; Zaragozá *et al.,* 2004). The *Agrobacteria* harboring the binary vector were grown overnight in LB agro liquid medium (appendix VII) of pH 7 at 25°C in the dark. The *Agrobacterium* culture was adjusted to an OD_{600} of 0.8-1.0 and then centrifuged at 5000 rpm at 4°C for 10 minutes. The *Agrobacterium* pellet was resuspended in the same amount of liquid 4X medium (appendix II) of pH 5.6 containing 100 µM acetosyringone and further incubated for up to three hours at 25°C.

50 ml of a one week old potato (cv. Desiree) cell suspension culture were sieved though a nylon filter of 100 µm pore size in a sterile Buchner funnel on a vacuum flask. The cells were washed three times with 4X medium (appendix II). Residual medium was carefully removed by applying a mild vacuum. 3 ml of the *Agrobacterium* suspension were poured over the cells in the Buchner funnel, which was covered with sterile aluminium foil. Plant cells and *Agrobacteria* were incubated in the Buchner funnel avoiding other microbial contaminations in dark for three days. After incubation cells were washed three times with the liquid 4X medium containing 300 mg/l ticarcillin. 200 mg fresh weight of cells was taken for a transient expression assay (B. Heidinger, pers. comm.). The remaining cells were spread on solid 4X medium containing 150 mg/l ticarcillin and 5 mg/l ppt in Petri dishes (90 mm in diameter). The plates were kept in the dark at 25°C for four weeks.

The resistant transgenic calli were propagated on the same medium to get enough material for initiation of transgenic suspensions cultures. Cell suspension cultures were established by inoculating 3 g fresh weight of friable callus into a 300 ml Erlenmeyer flask containing 100 ml of liquid 4X medium (Appendix1I). The flask was shaken at 100 rpm. As soon as a homogeneous cell suspension was obtained routine subculturing was done on a weekly basis.

2.4.18. Transformation of potato plants

Transgenic potato plants were recovered from *Agrobacterium* mediated leaf and internode transformation according to Rocha-Sosa 1989 with some modifications. Leaves obtained form *in vitro* grown 4 week old plants were used for transformation. Wounding of the explants was achieved by injuring the intact leaves with a scalpel horizontally across the midrib Two days before the inoculation, ca. 25 ml of a culture of *Agrobacterium tumefaciens* EHA105 harboring the dicistronic plasmid pGII 0029 TR Sth2 IRES Luc (Figure 24) in LB medium (appendix VII) containing 50 µg/ml kanamycin was prepared. The plasmid contained a neomycin phosphotransferase (NPT II) coding sequence as a selectable marker. The leaf

explants were placed upside down in Petri dishes plates containing 10 ml liquid MS medium (appendixVII). 30-50 µl of an *Agrobacterium* suspension of OD_{600} = 0.8 was added, the plates were shaken gently for 3-5 minutes and further incubated in the dark at 25°C for two days. For callus induction, the infected explants were transferred to solidified MS (appendix XII) supplemented with kanamycin and ticarcillin and kept in dim light for one week at 21°C. After one week the explants were transferred to MS plant regeneration medium (appendix XIII) and subcultured weekly. The regenerated plantlets were transferred to MS medium (Appendix I) supplemented with 200 mg/l ticarcillin and subcultured every 6 weeks.

2.4.19. Southern blot analysis

2.4.19.1. Isolation of gDNA for southern blot analysis

Southern blot analysis was used to determine the insert copy number in transgenic plants and cell lines. Isolation for genomic DNA for Southern blot analysis was done by the CTAB method of Doyle and Doyle 1990 as described before but scaled up as follows: About 2 g fresh plant material was homogenized with pestle and mortar under liquid nitrogen. 5 ml pre-warmed (65°C) CTAB buffer **(**see 2.4.1.2) was added to the ground plant material. The mixture was incubated in a water bath for 30 minutes at 65°C in a tube. After incubation the tube was cooled to room temperature and 1 volume of CI-MIX was added and mixed gently by inverting the tube to avoid shearing of DNA. Cell debris and other component were removed from the DNA solution by centrifugation (6400rpm, 15 min, 20°C,) and the clear supernatant was transferred by pipetting carefully to a new tube. Precooled isopropanol (2/3 volume) was added and the tube was gently inverted to precipitate the genomic DNA. Precipitated DNA was collected by centrifugation at RT for 10 min at 4000 rpm. The DNA pellet was washed with 1 ml washing buffer for 10 min until the pellet floats. The washing buffer was removed carefully and the pellet dissolved in 1ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with RNAse A (10mg/ml) and incubated at 37°C for 30 minutes followed by washing with 0,5 ml of 7.5M NH_2 –acetate and 2.5 ml of absolute ethanol. The mixture was centrifuged at room temperature for 10 minutes at 4600 rpm, the DNA pellet dried at room temperature for 20 minutes and then re-suspended in 100-300 µl 1x TE buffer. Depending on the amount of the precipitated DNA samples were kept at 4°C overnight to allow complete dissolving of the DNA.

2.4.19.2. Digestion of gDNA for southern blot analysis

For the digest of the genomic DNA isolated the restriction enzymes Sac1 and Xba1 were used either individually or together. Digestion was carried out using 1U enzyme/µg DNA and overnight incubation at 37°C followed by adding 1 µl more of the enzyme and a further incubation time of 1 hour to ensure a complete digest. The digested mixture was incubated for 15 min at 65°C to in activate the restriction enzymes. After the digestion 1 volume of 7.5 M NH_4-acetate and 7.5 volumes of absolute ethanol were added and mixed gently. The mixture was centrifuged at 14,500 rpm for 10 minutes at room temperature, the supernatant discarded completely and the pellet re-dissolved in 100 µl TE buffer followed by adding 100 µl absolute ethanol to remove salts. The mixture was centrifuged for 10 minutes and the pellet was dried at room temperature, then dissolved in 40 µl TE buffer and incubated overnight at 4°C. Completeness and quality of the restriction digest was tested by means of gel elctrophoresis. 5µl of DNA was mixed with 1 µl of 6x loading dye (Fermentas, St. Leon Rot, Germany) and loaded on a 0.8% (w/v) agarose gel. Electrophoresis was carried out at 60 V for 1.5 hours and the DNA pattern observed under UV light.

2.4.19.3. Gel electrophoresis

After the successful restriction digest, the digested DNA was incubated at 65°C for 10 minutes and resulting fragments were separated on 0.8% (w/v) agarose gel. Separation was done in 1x TAE buffer (40 mM Tris-acetate, 20 mM glacial acetic acid, 1 mM EDTA, pH 7) at a constant voltage of 15 V (0.6 V/cm) overnight. For determination of fragment length the standard DIG-labeled-DNA Molecular Weight Marker II (Roche) was loaded on the same gel. 1-2 µl (1: 10000 dilution) of the digested plasmid was loaded as a positive control.

2.4.19.4. Gel preparation for Southern-transfer

Southern transfer of the separated DNA fragments to the blotting membrane was carried out after electrophoresis. To prepare the gel for blotting it was incubated in depurinization solution (0.25 M HCl) for 10 minutes at room temperature followed by rinsing in distilled H_2O. For a neutral transfer of the restricted DNA fragments, the agarose gel was stored for 30 minutes (2x15) in a denaturation solution **(**0.5 N NaOH, 1.5 M NaCl) on a shaker (Rocking Platform, Biometra GmbH, Göttingen, Germany). The gel matrix was washed in distilled water and then incubated for 30 minutes (2x15) at room temperature in neutralization solution (0.5 M Tris-HCl, 3 M NaCl, pH 7.5).

2.4.19.5. Capillary Southern transfer

The capillary Southern transfer of the DNA fragments from the gel to the nylon blotting membrane was carried out as follows: A tray was filled with 20x SSC buffer (3 M NaCl, 0.3M sodium citrate, pH 7). A glass plate was placed as a gel support onto the tray and paper bridges of Whatman 3MM paper (Whatman plc, Maidstone, UK) over the support so that the edges were submerged in the transfer buffer (20x SSC). Three layers of Whatman 3MM paper of the same size as the gel were placed on top of the glass plate in contact with the paper bridges. The gel was placed on top of the paper (avoiding air bubbles between Whatman paper and gel). A plastic wrap was placed in between filter papers under and on top of the gel to prevent a bypass of transfer buffer. A positive charged nylon membrane was cut to the same size as the gel, wetted first with water and then with transfer buffer and placed on top of the gel (avoid air bubbles between gel and the nylon membrane). Three layers of Whatman 3MM paper of the size of the gel was first soaked with 20x SSC buffer and placed on top of the membrane. Tissue paper stacks were placed on top of the Whatman paper on top of the membrane. A 500 ml bottle with water was placed on top of the tissue stacks to distribute the weight evenly over the gel. Blotting took place by capillary forces overnight. The next day the membrane was removed, rinsed three times for 15 minutes with 100 ml 2x SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7) and air dried. For covalently cross-linking the DNA, the membrane was placed in the oven for 30 minutes at 120°C.

2.4.19.6. Preparation of DIG labeling probe by PCR

2.4.19.6.1. Amplification mixture

Reagent and concentrations (PCR DIG Probe Synthesis Kit)	Probe (µl)	Control (µl)
10x buffer	5	5
dNTPs	1	5
PCR DIG Probe synthesis	4	-
Plasmid DNA	1	1
10 pmol forward primer	5	5
10 pmol reverse primer	5	5
Taq polymerase	0.75	0.75
Double distilled water	28.25	28.25
Total	50	50

2.4.19.6.2. PCR program

The following PCR program was used for amplification

Steps	Temperature (°C)	Time	No. of cycles
Initial denaturation	95	2 min	1
Denaturation	95	10sec	2
Annealing	58	2 min	
Extension	72	2 min	
Denaturation	95	1 min	35
Annealing	58	1 min	
Extension	72	1 min	
Final extension	72	10 min	1
Cooling and storage	4	∞	1

2.4.19.7. Prehybridization and Hybridization

After cross linking the membrane was placed into a ´medium size hybridization bottle´ (15 cm x 3.5 cm, Biometra, Göttingen, Germany). 50 ml of prehybridization buffer (Dig Easy Hyb, Roche Diagnostics, Mannheim, Germany) supplemented with **(**preheated, 99°C, then cooled immediately) spermidine (5 µl) was added and the tube incubated for 30 min at 42 °C in a rolling hybridization oven. Then the prehybridization buffer was removed and preheated (65°C) hybridization buffer supplemented with the probe (45 µl probe + 50 ml Dig Easy Hyb.) was poured into the hybridization bottles and incubated over night at 42°C.

2.4.19.8. Detection and Striping

The membrane was washed 2 x 5 minutes at 42°C with pre-warmed 2 SSC buffer + 0,1% sodium dodecyl sulfate (SDS) solution, then washed again 2 times for 15 minutes at 65°C with pre warmed 0.5 SSC + 0,1% SDS solution. Finally the membrane was washed 2 times for 15 minutes at 65°C with 0.1 SSC + 0.1 % SDS solution. The membrane was incubated in

blocking solution (1 % blocking solution, Roche Diagnostics, Mannheim, Germany)for 30 minutes at room temperature followed by incubation in an antibody solution (Anti-Digoxigenin-alkaline phosphatase conjugate Fab Fragments diluted 1:20000 in blocking solution in maleic acid buffer, Roche Diagnostics, Mannheim, Germany) for 30 minutes. Finally the membrane was rinsed in washing buffer (1 %, Roche Diagnostics, Mannheim, Germany) 2 times 15 minutes at room temperature.After hybridization the membrane was incubated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) for 5 minutes at room temperature under shaking. The substrate for detection was prepared by mixing of 5 µl CDP star (Roche Diagnostics, Mannheim, Germany) with 495 µl detection buffer. Then the membrane was transferred upside down onto a plastic sheet containing drops of the substrate. Then it was covered with a second plastic sheet to distribute the substrate homogeneously over the whole area of the membrane. The excess of the substrate was removed and the membrane incubated for 10 minutes at room temperature. After usage the membrane can be stored and used for a second hybridization. For second use the membrane was rinsed two times in sterile H_2O and incubated twice for 10 min in stripping buffer (0.2 M NaOH, 0.1 % SDS) at 37°C followed by rinsing in H_2O. The membrane could be used after striping or stored in 2 x SSC buffer without SDS at 4°C.

2.5. Cryopreservation experiments

For cryopreservation experiments the classical approach of controlled rate freezing according to Withers and King 1980 was used (parameter for cell culture of ´Desiree´wild type previously worked out at DSMZ). Starting material were wild type and transgenic suspension cultures of *Solanum tuberosum* cv. ´Desiree´. Both cell cultures were routinely subcultured after 7 days of growth. Cells for cryopreservation experiments were taken from the logarithmic growth phase, three days after the last subculturing. For pretreatment cells were collected by filtration through nylon net of a pore size of 100 µm (Fa. Neolab, Heidelberg, Germany) in a Buchner funnel under sterile conditions. A fresh weight of 11.93 g of cells, corresponding to a packed cell volume (PCV) of 30.0% was transferred with a sterile spatula into a round sterile polystyrol jar (6.5 cm high and 6.5 cm in diameter, Fa.Greiner, Frickenhausen, Germany). A volume of 26 ml 4X medium (aappendix II) supplemented with 0.3 M or 1.2 M sorbitol was added to the cells and the suspension incubated for two days on a shaker at 100 rpm. For cryoprotection cells in the polystyrol jars were first cooled down to 3.6°C for 11 minutes on a shaker to 3.6 (Infors HT, Bottmingen, Switzerland) at 140 rpm

equipped with a cooling device (Julabo GmbH , Seelbach, Germany). Then 2 ml of the cryoprotectant DSMO (Duchefa, Haarlem, Netherlands) was added to the cells to reach a final concentration of 5%. The jars were incubated for 50 minutes again under cold conditions (4°C) at 140 rpm. Then the jar containing the cells was placed in an ice bath and aliquots of 1.5 ml of the cell material filled into 2 ml cryotubes (Nunc, Roskilde, Denmark). When a cryotube has been filled and placed on ice. The filling of the tubes lasted for 40 minutes so that the resulting incubation time in the cryoprotectant solution at 4 °C was 90 minutes. The cryotubes fixed to the aluminium holders were then inserted into the cooling chamber of a Planer Kryo10 automatical freezer (Planer PLC, Middlesex, UK). Viability test (TTC) and Luciferase assay were carried out using the residual cells in the box. Cooling started at 4°C with a cooling rate of -0.25 °C/min until a temperature of - 40 °C was reached. The cryotubes were kept at - 40 °C for 15 minutes in the freezer and then immersed rapidly in liquid nitrogen (-196 °C) in a cryotontainer. Rapid thawing was carried out at 40 °C by plunging the cryovials in a water bath for 3 minutes. The suspension from the cryovials was then poured onto 3 layers of filter paper (Rundfilter, Blauband, Fa. Schleicher und Schüll GmbH, Dassel, Germany) placed on top of solid 4X medium in Petri dishes (60 mm in diameter) and incubated in the dark for 2 hours. Viability test (TTC) and luciferase assay were carried out using the residual cells in the cryovials. The uppermost filter papers were transferred to fresh 4X medium (appendix II) and kept in dark at 23°C for four weeks. After 4 weeks of growth cells were harvested from the filter papers and fresh weight was measured. Dry weight of the samples was measured after 3 days of drying at 60 °C. Luciferase assay was carried out again for the regrown cells to monitor the expression of the respective gene.

2.6. Statistical analysis

All experiments were performed in triplicate; values represent means ± standard error. The data were analyzed by one way ANOVA using the software Sigma Stat® 9.0 and Sigma Plot® 9.0. The term significant has been used to indicate differences for which $P \leq 0.05$.

3. RESULTS

3.1. Growth and viability of plants and cell cultures under osmotic and salt stress

3.1.1. Preliminary tests

In 2003 Dobranszki, J. *et al.,* (2003) demonstrated that undifferentiated potato callus cultures showed the same degree of osmotic tolerance as intact plants of the corresponding cultivars. Callus and suspension cultures of different cultivars of *Solanum tuberosum* are present in the DSMZ plant cell culture collection. For this study cell lines of the cultivars ´Desiree´ (established in 2005, PC-1202), ´Ijsselster´ (PC-1203) and ´Unicopa´ (PC-1204, both established in 2006) have been selected because of their performance in cryopreservation experiments (H.M. Schumacher and J. Keller, pers. comm.). Since cryotolerance and osmotic tolerance are closely linked, differences in the behavior of the cell cultures against osmotic and salt stress seemed to be likely. In the first part of the study, growth and viability of these cultures under osmotic and salt stress were characterized. Tests were performed to find appropriate conditions like kind and concentration of the osmotic applied or duration of time of the respective osmotic treatments for physiological, proteome and molecular analysis.

To apply osmotic and salt stress, three different substances were chosen: NaCl as the most important naturally occurring salt stress component, sorbitol as substance exerting an osmotic effect but not (or only to a small extent) entering the cell and sucrose as a substance exerting osmotic stress but also entering the cell by inward transport and interfering with the intracellular metabolism.

Preliminary tests were performed using suspension cultures of the cultivar ´Desiree´. A cultivation system scaled down for small amounts of cell material used at DSMZ for the optimization of cryopreservation parameters was applied (Heine-Dobbernack *et al.,* 2008). An inoculum of 200 mg fresh weight of cells was grown in 0.9 ml of either 4X medium or 4X medium containing different osmotics at different concentrations in 24multiwell plates for three days. Viability of the cells was tested by the TTC assay (Figure 3). Based on these preliminary tests the concentrations of 0.25 M and 0.5 M sorbitol were chosen to exert stress conditions at a level where viability was decreased but growth and active protein synthesis can still be expected.

For subsequent experiments, the different osmotics were applied on the basis of the same osmolarity instead of the same stoechiometric concentrations.

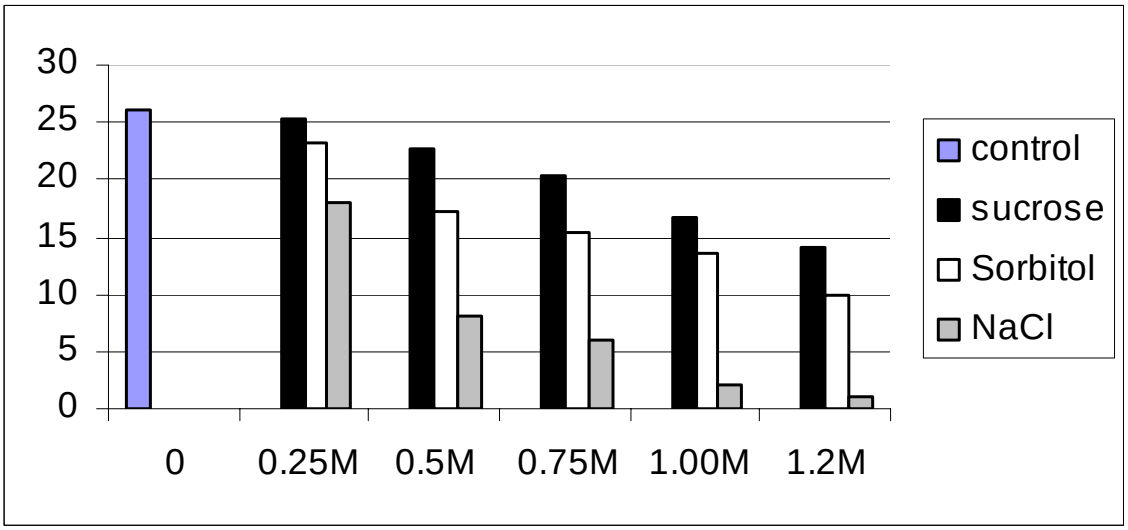


Figure 3: Viability of suspension cells of Solanum tubersoum cv. ´Desiree´ (PC-1202) after 3 days of growth under control and osmotic stress conditions. Viability was measured by the TTC test.

3.1.2. Growth parameters of *Solanum tubersoum* cv. Desiree suspension cultures under osmotic stress conditions

A series of physiological responses, i.e. fresh weight, dry weight and viability of the cells, osmolarity and pH of the medium were measured during the growth curve of a suspension culture of *Solanum tuberosum* cv. ´Desiree´ in control 4X medium and 4X medium supplemented with either 0.25 M or 0.5 M sorbitol. Cells were grown in 100 ml Erlenmeyer flasks containing 25 ml liquid medium and inoculated with 1 g fresh cell material. 2 flasks were harvested every second day for control and each concentration of sorbitol. Figure 4 shows that increased osmotic values of the medium lead to decreased accumulation of cell fresh weight during two weeks of growth. Nevertheless, accumulation of cell dry matter was higher in the medium containing the osmotics (Figure 5). The viability measured by TTC test dropped for the stressed cells after 2 days already. Data for viability and fresh weight accumulation (Figure. 4 and Fig. 6) showed a delay of the growth curve depending on the osmotic value of the medium. Increased dry matter accumulation (Figure. 5) may be due to a loss of water of the still growing cells under osmotic stress.

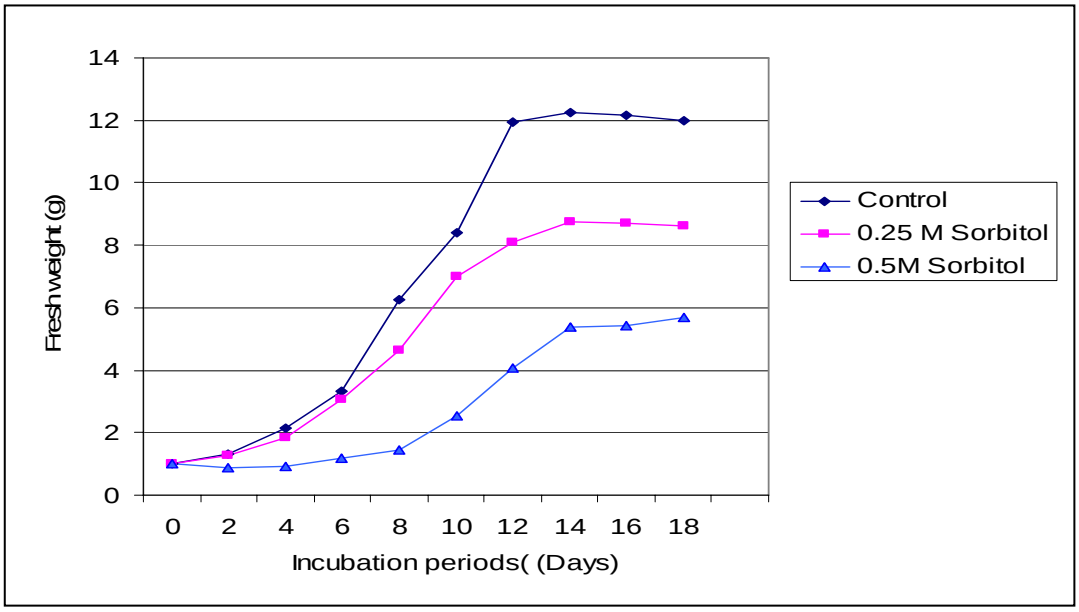


Figure 4: Growth curve (accumulation of cell fresh weight) of a suspension culture of Solanum tuberosum cv. ´Desiree´ in normal 4X medium and 4X medium supplemented with 0.25 M and 0.5 M sorbitol. Cells were grown in 100 ml Erlenmeyer flasks.

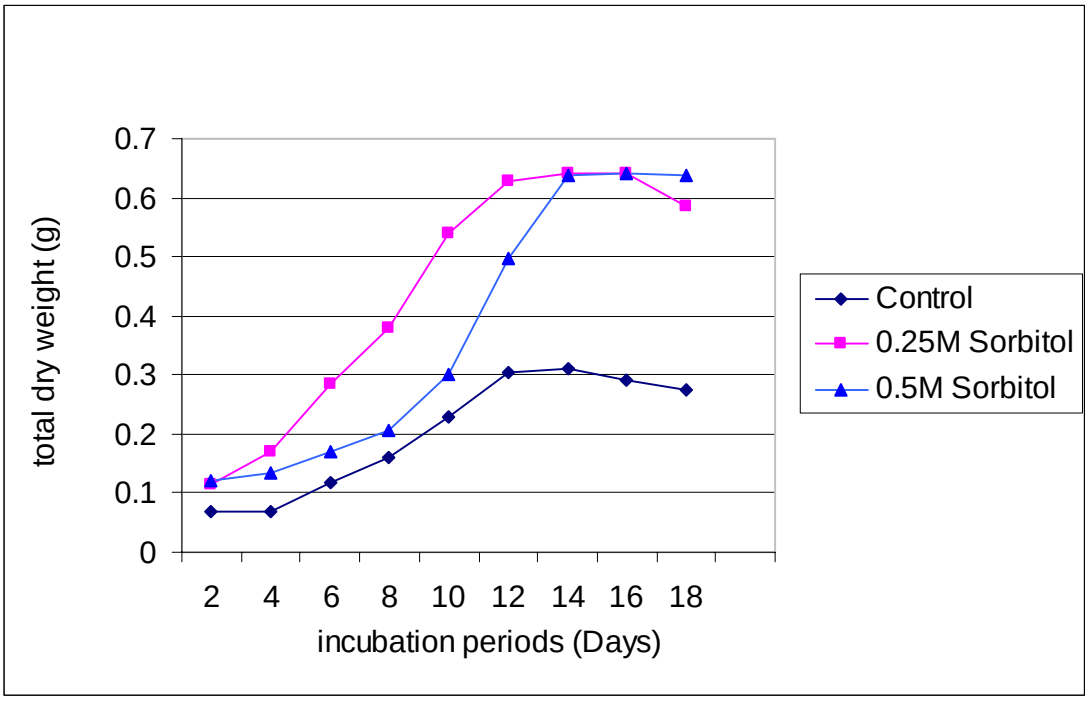


Figure 5: Growth curve (accumulation of cell dry weight) of a suspension culture of Solanum tuberosum cv. ´Desiree´ in normal 4X medium and 4X medium supplemented with 0.25 M and 0.5 M sorbitol. Cells were grown in 100 ml Erlenmeyer flasks.

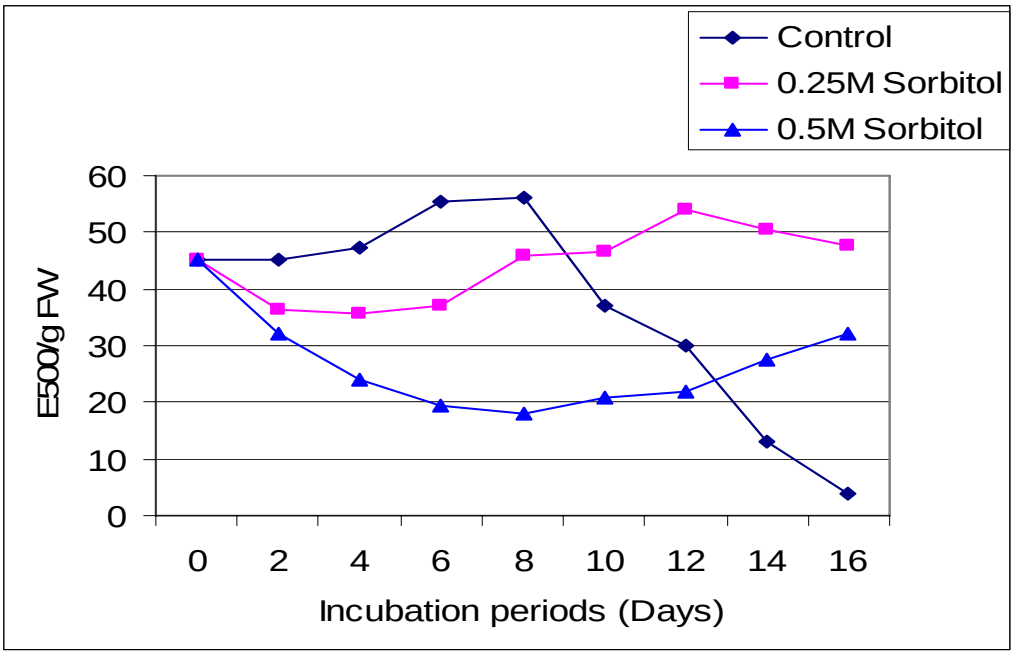


Figure 6: Viability of cells measured by TTC test during the growth curve of a suspension culture of Solanum tuberosum cv. ´Desiree´.

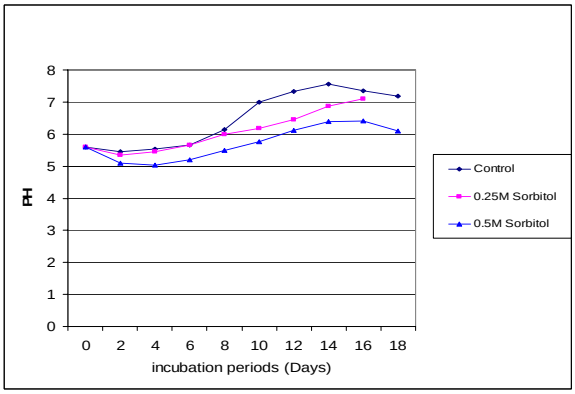


Figure 7 A

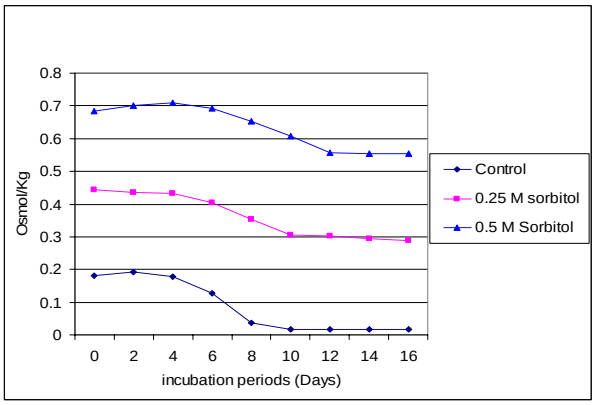


Figure 7 B

Figure 7: Changes of the pH (A) and the osmotic value (B) of the medium during growth of a suspension culture of Solanum tuberosum cv. ´Desiree´ in 4X control medium and 4X medium supplemented with 0.25 M and 0.5 M sorbitol.

Changes of the pH (Figure 7A) and the osmotic value (Figure 7 B) of the growth medium seemed to be similar in control cell lines and cell lines under osmotic stress exerted by sorbitol. Taking into account that the total osmotic value is higher in the medium supplemented with sorbitol the changes during growth in the different curves follow the same trend. An interesting aspect is that fresh weight accumulation depends on the sorbitol

concentration while dry matter accumulation is highest for medium with a sorbitol concentration of 0.25 M and lower for control medium as well as for medium with the higher sorbitol concentration (0.5 M).

3.1.3. Measurement of growth parameters of suspension cultures cultivated in 24 multiwell plates

In a next step, test conditions were optimized. Approximately 200 mg cell fresh weight was cultivated in 0.9 ml medium in the wells of a 24multiwell plate. The effects of salt - and osmotic stress on the osmolarity of the medium, viability and growth rates of the suspension culture of *Solanum tuberosum* cv. ´Desiree´ was investigated by cultivation of the cells in normal medium and medium with different concentrations of osmotics (sorbitol, NaCl, sucrose) in these plates for either 3 or 6 days (Figures, 8 and 9).

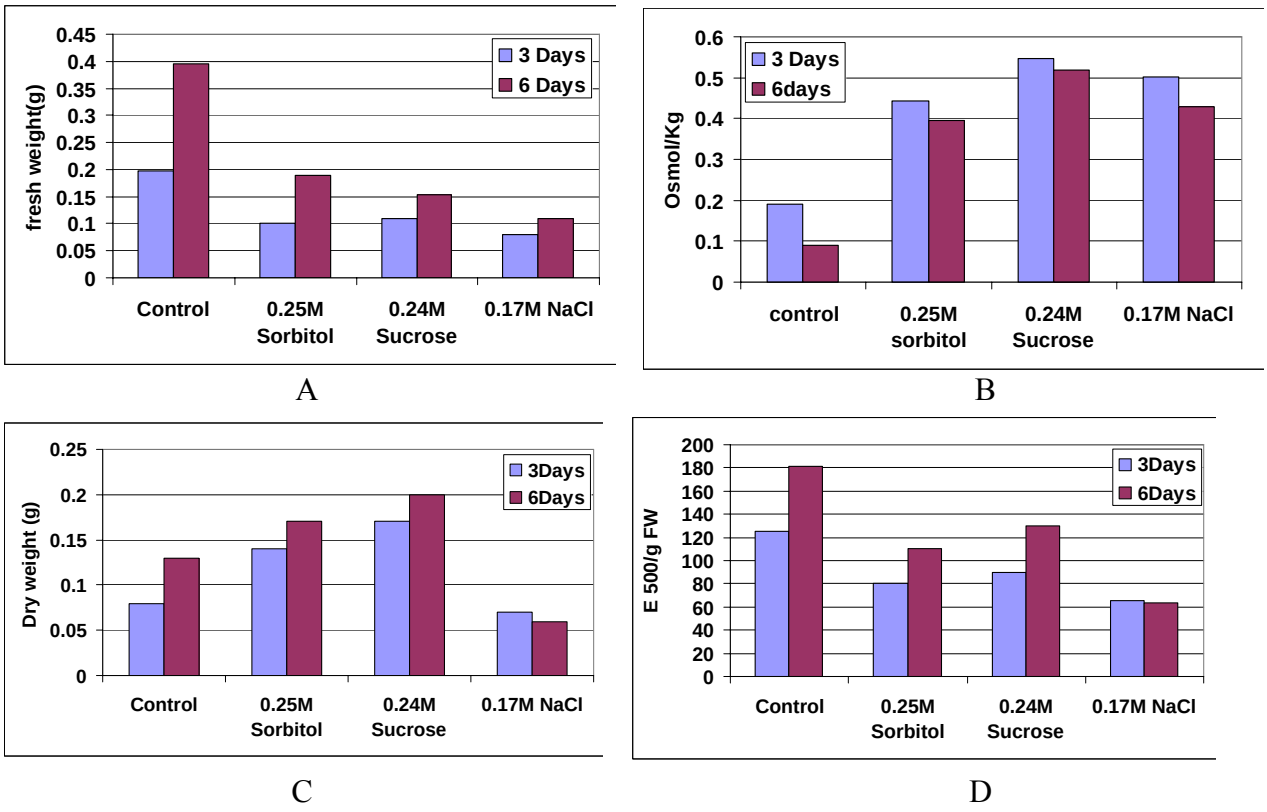


Figure.8: The different growth parameter (A) fresh weight accumulated, (B) osmolarity of the medium, (C) dry weight accumulated and (D) viability of a potato suspension culture (cv. Desiree) cultivated for 3 and 6 days with and without low osmotic stress (sorbitol, NaCl and sucrose). The different molar concentrations represent an osmolarity of 0.45 osmol/kg.

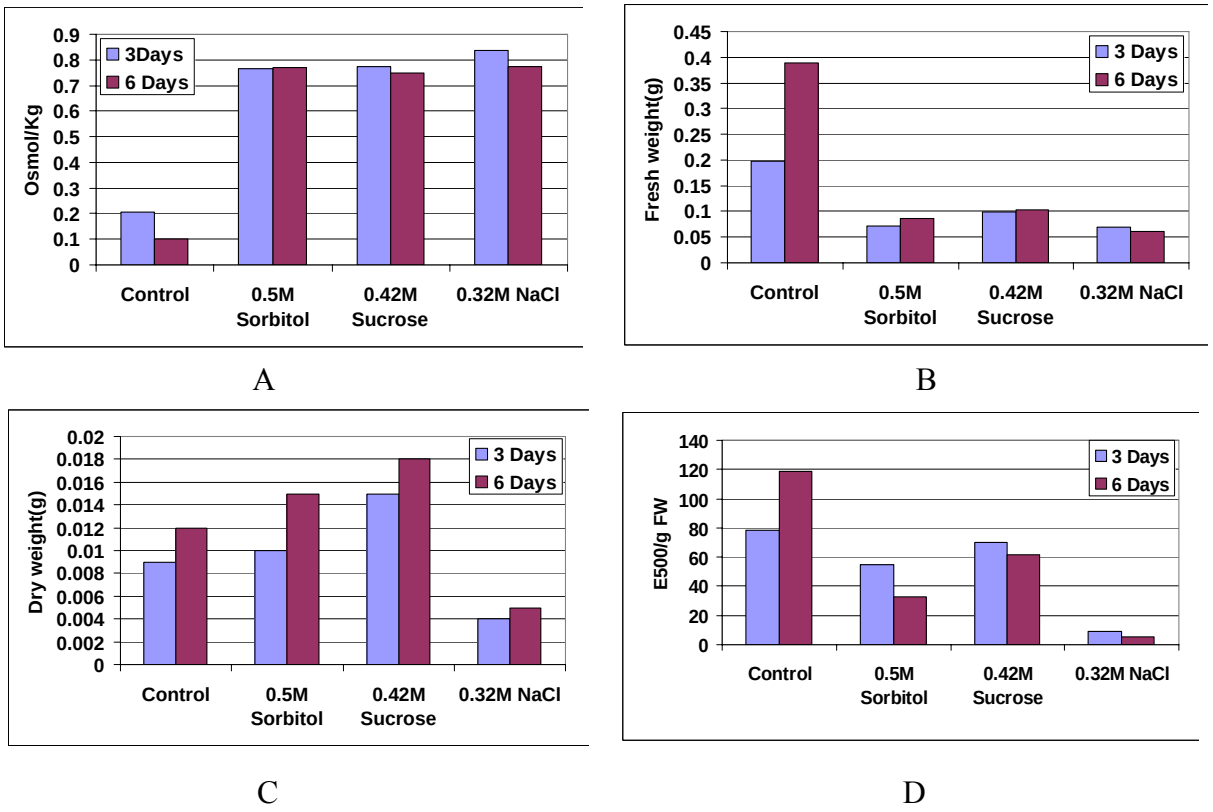


Figure 9: The different growth parameter (A) fresh weight accumulation, (B) osmolarity of the medium, (C) dry weight accumulation and (D) viability of potato suspension cultures (cv. ´Desiree´) cultivated for 3 and 6 days with and without high osmotics stress (sorbitol, NaCl and sucrose). The different molar concentrations represent an osmolarity of 0.74 osmol/kg.

The results demonstrate that the suspension culture grown in the 24multiwell plates showed similar reaction concerning viability, accumulation of fresh and dry weight cell material and osmolarity changes as compared to culture cultivated in the 100 ml Erlenmeyer flasks.

As expected, cells were affected more by the higher concentration of osmotics in the medium. But even after exposure to the higher concentration, fresh weight and dry weight accumulation as well as viability can be observed in the cells. Incubation of the cells at the higher concentration of osmotics and an incubation time of three days were chosen as standard conditions for further biochemical tests and proteome analysis.

3.1.4. Comparative salt and osmotolerance studies on three potato cultivar derived suspension cultures

To allow future comparative studies, similar tests as performed with the cell culture of the cultivar ´Desiree´ were also performed with cell lines of the potato cultivars ´Ijsselster´ and

´Unicopa´. The suspension cells were subjected to the same osmotic and salt stress conditions (together with the ´Desiree´ cell line) as a control as it was done before. It was remarkable that even at the higher concentrations of NaCl and sorbitol the cultivar ´Unicopa´ did not show any visible effect (Figure 10).

Apart from performance also viability and biomass production of the different suspension cultures under osmotic and salt stress were measured.

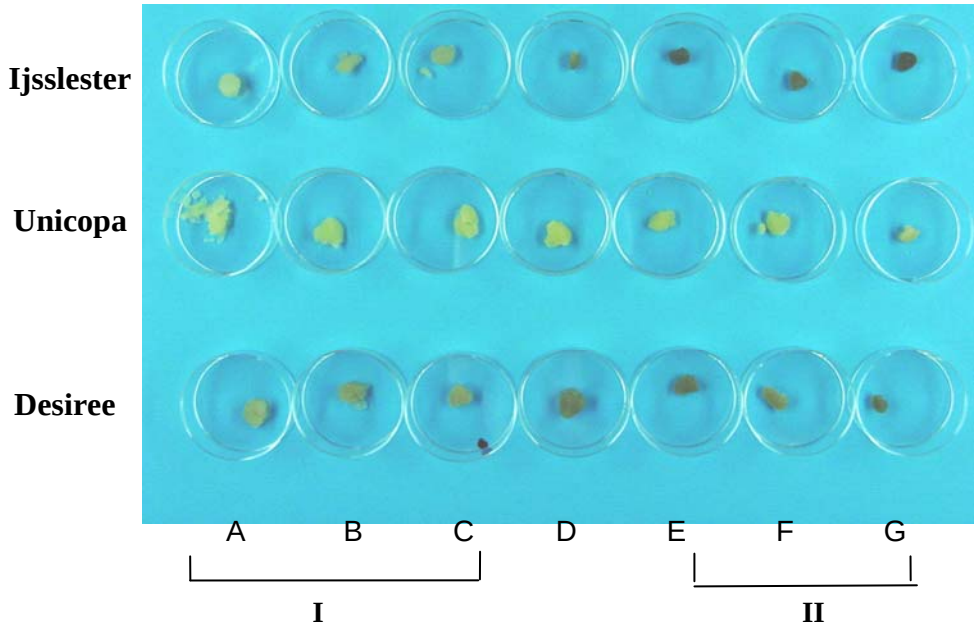


Figure.10: Morphological performance of potato cultivars growing under different salt and osmotic concentrations: (A) control, (B) 0.25M sorbitol, (C) 0.24M sucrose, (D) 0.17M NaCl, (E) 0.5M sorbitol, (F)0.42M sucrose, (G) 0.32M NaCl, (I) 6 days of incubation, (II) 3 days of incubation.

Figure 11 shows the viability measured by the TTC test for the different suspension cultures after growth for 3 days under osmotic stress exerted by the different osmotics tested. Under sorbitol and NaCl stress, the suspension culture of the cultivar ´Unicopa´ again seemed to be the most tolerant in comparison to suspension cultures of the cultivars ´Desiree´ and ´Ijsselster´. Between the latter two cultures no big difference were observed. When sucrose was applied as osmotic, the differences in viability between all three suspension cultures were negligable. Especially for salt treatment the suspension culture of the cultivar ´Unicopa´ showed a higher tolerance than the other cultivars.

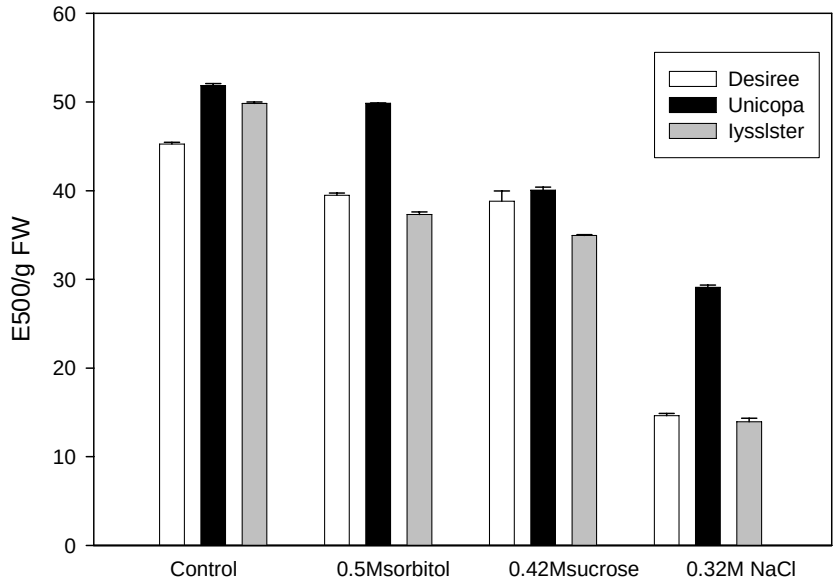


Figure 11: Viability of suspension cultures of the three different potato cultivars ´Desiree´, ´Unicopa´, and ´Ijsselster´ after cultivation for 3 days in 4X medium and 4X medium supplemented with different osmotics. Cultivation was performed in 24multiwell plates

For the biomass production, the surplus of sucrose in the medium is leading to much higher biomasses in comparison to the control in all cultivars. Differences in biomass production under osmotic stress conditions between the cell cultures of the three cultivars, as occurring under sorbitol and NaCl treatment, are not significant under sucrose stress. Under NaCl treatment the cell culture of the cultivar ´Unicopa´ showed significantly higher biomass production than the two others (Figure.12)

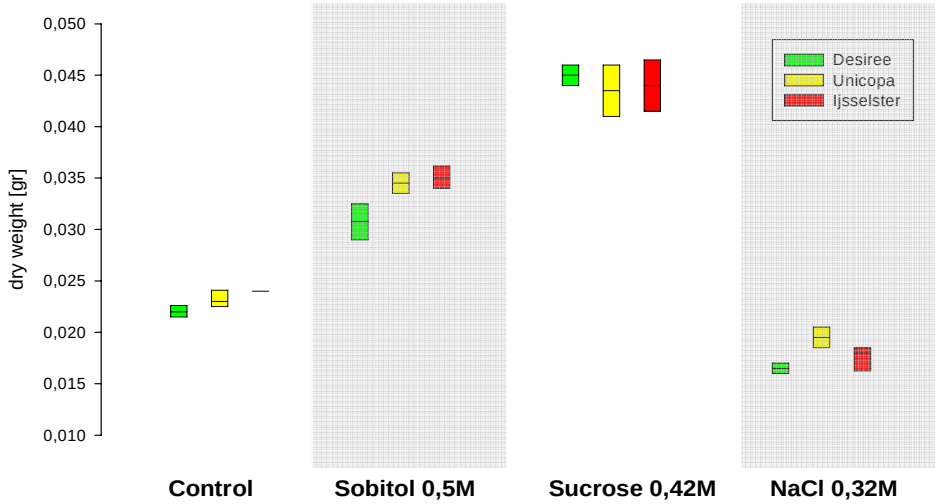


Figure 12: Box/Whiskers plot of dry weight accumulation of suspension cultures of the three different potato cultivars ´Desiree´, ´Unicopa´, ´Ijsselster´ after three days of growth in normal 4X medium and 4X medium supplemented with various osmotics. Cells were grown in 24multiwell plates.

3.1.5. Comparative salt and osmotolerance studies on plants of the three potato cultivar

To proof whether the relationship between osmotic tolerance of cell cultures and intact plants demonstrated by Dobranszki, J. *et al.,* (2003) for different potato cultivars is also true in our case, intact in-vitro plantlets of the three cultivars ´Desiree´, ´Unicopa´ and ´Ijsselster´ were subjected to similar osmotic and salt-stress treatments as the cell lines before. Plant cultivation was done in test tubes equipped with filter paper bridges (Mix-Wagner *et al.*,1999**)**, providing access of the plant roots to liquid medium. After an initial growth phase (21days) in normal MS medium, medium was changed and some plants exposed to the same sorbitol, sucrose and NaCl concentrations as the cell cultures in previous experiments. Plantlets were grown for 6 days under stress conditions. For the lower concentrations (0.25 M sorbitol. 0.24M sucrose and 0.17 M NaCl) only the cultivar ´Ijsselster´ showed slight leaf necrosis. (Figure 13, A). Under the higher stress conditions (0.5 M sorbitol, 0.42 M sucrose and 0.32 M NaCl) the cultivars ´Desiree´ and ´Ijsselster´ were severely affected, whereas the cultivar ´Unicopa´ showed only moderate damage on the leaves (Figure.13, B).

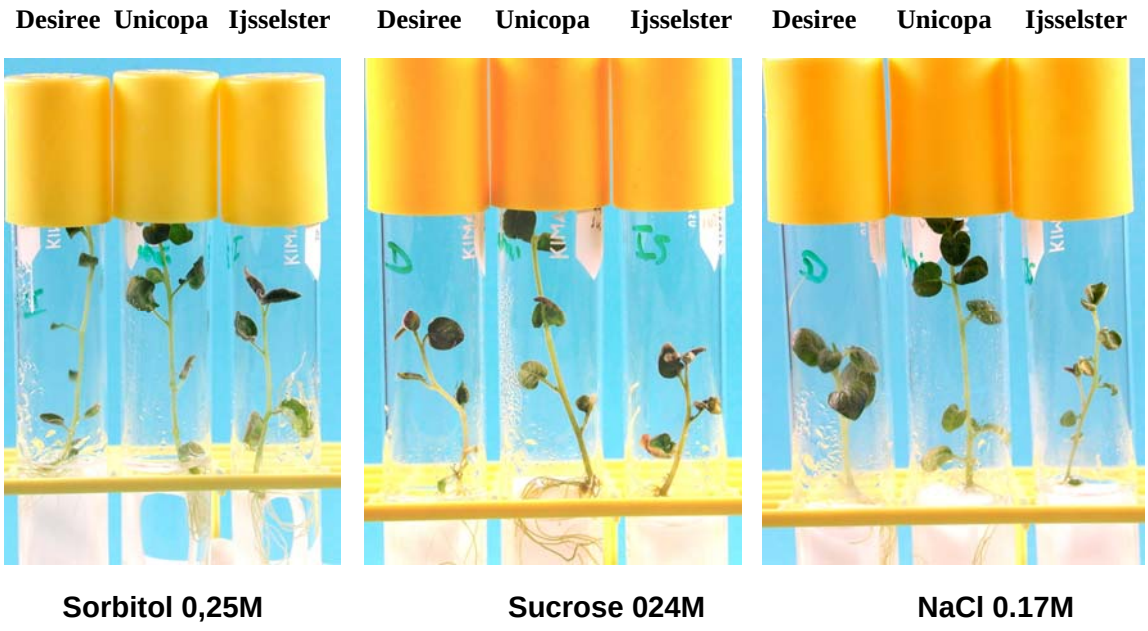


Fig.13 (A)

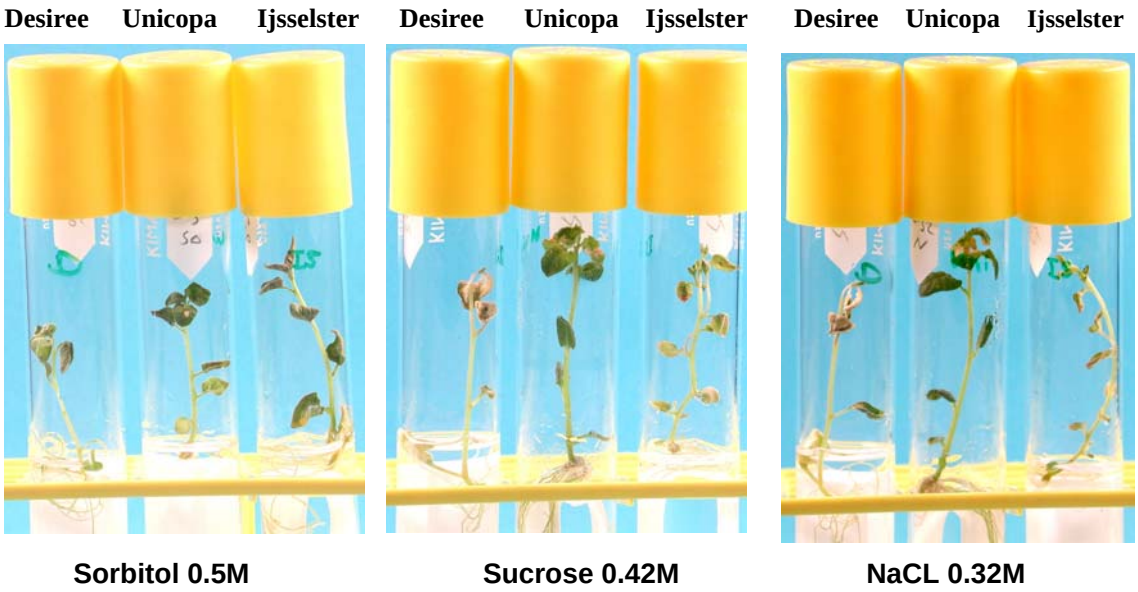


Fig.13 (B)

Figure 13: Representative examples of in-vitro potato plants after treatments with different concentrations of salt and osmotic stress. (A) Low concentrations (B) high concentrations

The three cultivars can be classified according to their salt and osmo tolerance level into: the more tolerant cultivar ´Unicopa´, the less tolerant cultivar ´Desiree´ and the cultivar ´Ijsselster´ showing the lowest level of tolerance of the cultivars tested.

3.2. Biochemical aspects of response to osmotic and salt stress for suspension cultures of different potato cultivars

3.2.1. Accumulation of proline

A well known reaction of plants to compensate for osmotic stress is the intracellular accumulation of free proline. Comparative measurements of proline accumulation in the different potato suspension cultures were carried out in this study. The same approach as for viability testing and biomass accumulation measurement described earlier (chapter 3.1.1.) was used. Although lowest in controls, the culture of cv. ´Unicopa´ accumulated the highest proline levels of all cell lines for all osmotics used. Comparing the two cultivars ´Desiree´ and

´Ijsselster´, the former showed a higher proline content under sorbitol treatment, whereas ´Ijsselster´ showed higher levels than ´Desiree´ under salt stress (Figure 14).

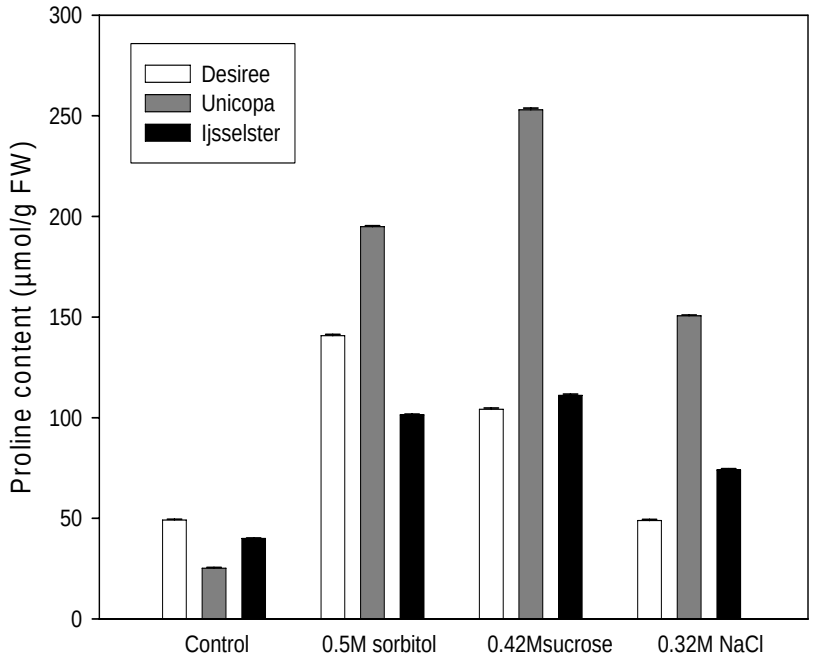


Figure 14: Proline content in cell suspension cultures of the three different potato cultivars ´Desiree´, ´Unicopa´, and ´Ijsselster´. Before proline extraction cell cultures were grown for 3 days in 24 muliwell plates in 4X medium and 4X medium supplemented with the different osmotics.

3.2.2. Glutathione and cystein content

Hyperosmolarity resulting from osmotic or salt stresses generates oxidative stress which is caused by excessive reactive oxygen species (ROS). ROS include, for example, hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS are usually generated by normal cellular activities but their levels increase when plants are exposed to biotic or abiotic stress conditions.

Normally the reduced form of glutathione (GSH) is the predominant form in tissues. Acting against oxidative stress it is oxidized to the disulfide form (GSSG). The enzyme glutathione reductase regenerates GSH from GSSG. In the case of massive stress the percentage of the oxidized from the total GSH may rise. The changes in the level of total glutathione, oxidized glutathione (GSSG) and the percentage of oxidized from total glutathione therefore reflects the degree of oxidative stress. For testing these parameters in the suspension cultures of the

different potato cultivars cells were again grown in 24multiwell plates for three days in normal medium and medium containing 0.5 M sorbitol or 0.42M sucrose or 0.32M NaCl.

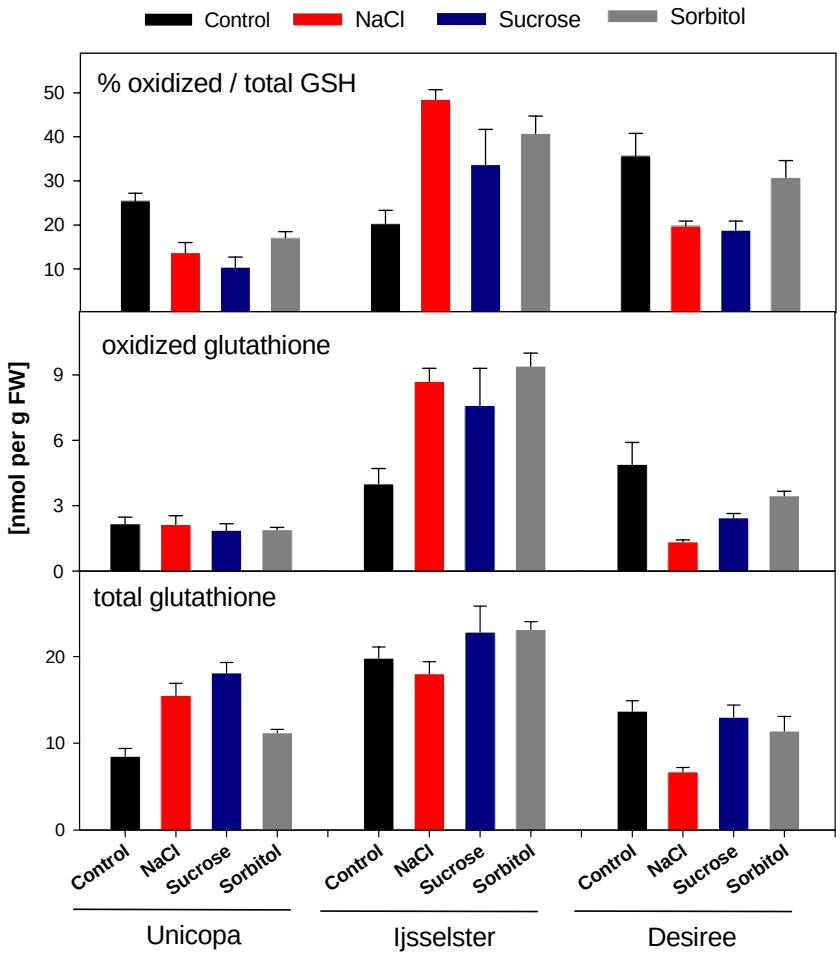


Figure 15: Levels of total glutathione, oxidized glutathione and the percentage of oxidized glutathione from total glutathione in suspension cultures of the potato cultivars ´Unicopa´, ´Ijsselster´, and ´Desiree´, after 3 days of growth under osmotic stress in 24 multiwell plates.

The results shown in Figure 15 demonstrate that especially in the cell culture of cultivar ´Ijsselster´ an increase of the level of total as well as oxidized glutathione and also of the percentage of oxidized from total glutathione occurred under stress conditions. For the cell line of cultivar ´Desiree´ all glutathione levels dropped after three days of osmotic treatment. The cultivar ´Unicopa´ showed a constant level of the oxidized form, an increase in the value for the total glutathione content and a decrease in the percentage of the oxidized form (GSSG) from total glutathione.

Glutathione is a tripeptide composed of L-cysteine, L-glutamate and glycine. Cystein is the key compound for the de-novo synthesis of glutathione. To proof whether the cystein pool

may be a limiting factor for glutathione biosynthesis, cystein levels where measured under the same conditions as the content of different forms of glutathione itself (Figure 16).For the cell culture of cultivar ´Unicopa´ the level of cystein increased even more than the content of total glutathione for all osmotics applied. Similar results occur for the suspensions of the cultivars ´Desiree´ and ´Ijsselster´ in case of treatment with sucrose and sorbitol.

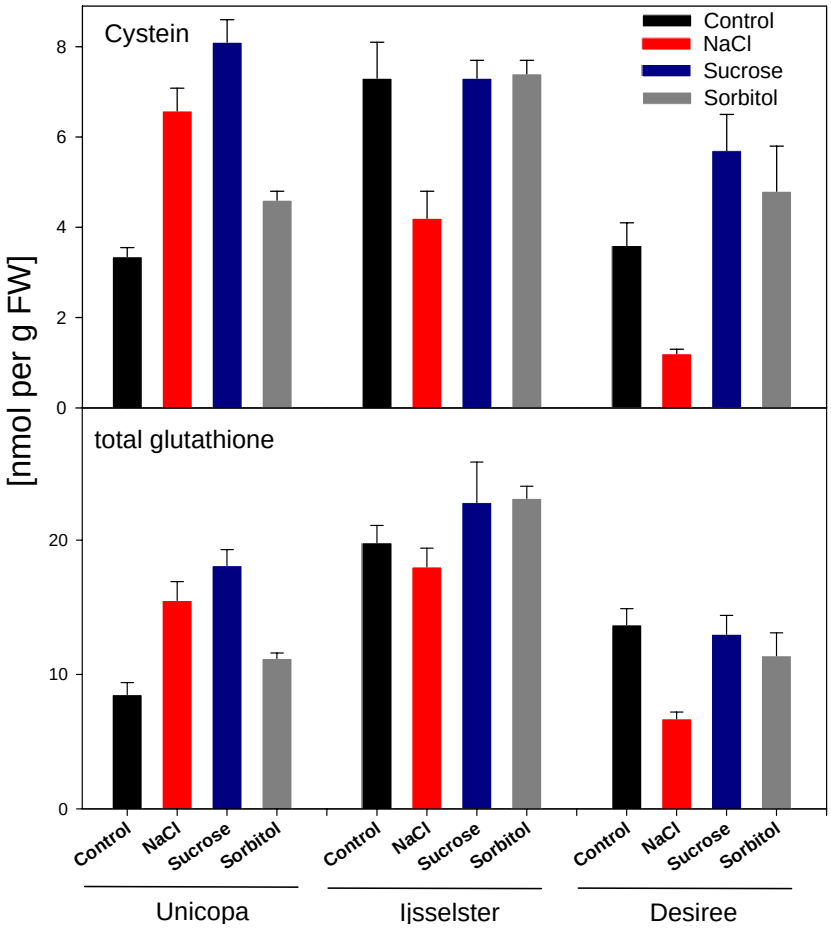


Figure 16: Content of total glutathione and cysteine in suspension cultures of different potato cultivars after growth for three days in 24multiwell plates in normal medium and in medium supplemented with different osmotically active substances.

A remarkable decrease of the content of total glutathione and even more in the content of cysteine occurred in these cultures after NaCl treatment.

3.2.3. Soluble sugar content

Intracellular soluble sugars, especially sucrose, may act as osmotic agent, but it has also an impact on oxidative stress. Apart from glutathione levels, the accumulation of intracellular sucrose, glucose and fructose in the three tested cultivars after sorbitol and NaCl treatment was measured (Figure 17). Growth and stress conditions were the same as for proline and

glutathione measurements. Under control condition, the sucrose content varied among the suspension cultures of the three cultivars. The cell lines of the cultivars ´Ijsselster´ and ´Desiree´ have a higher (9.52 and 7.9 µmol/g fresh weight) sucrose content as compared to those of ´Unicopa´ cells (5.17µmol/g fresh weight). For the cultivars ´Unicopa´ and ´Desiree´, sorbitol as well as NaCl treatment lead to a significant but moderate increase of sucrose, glucose and fructose concentration.

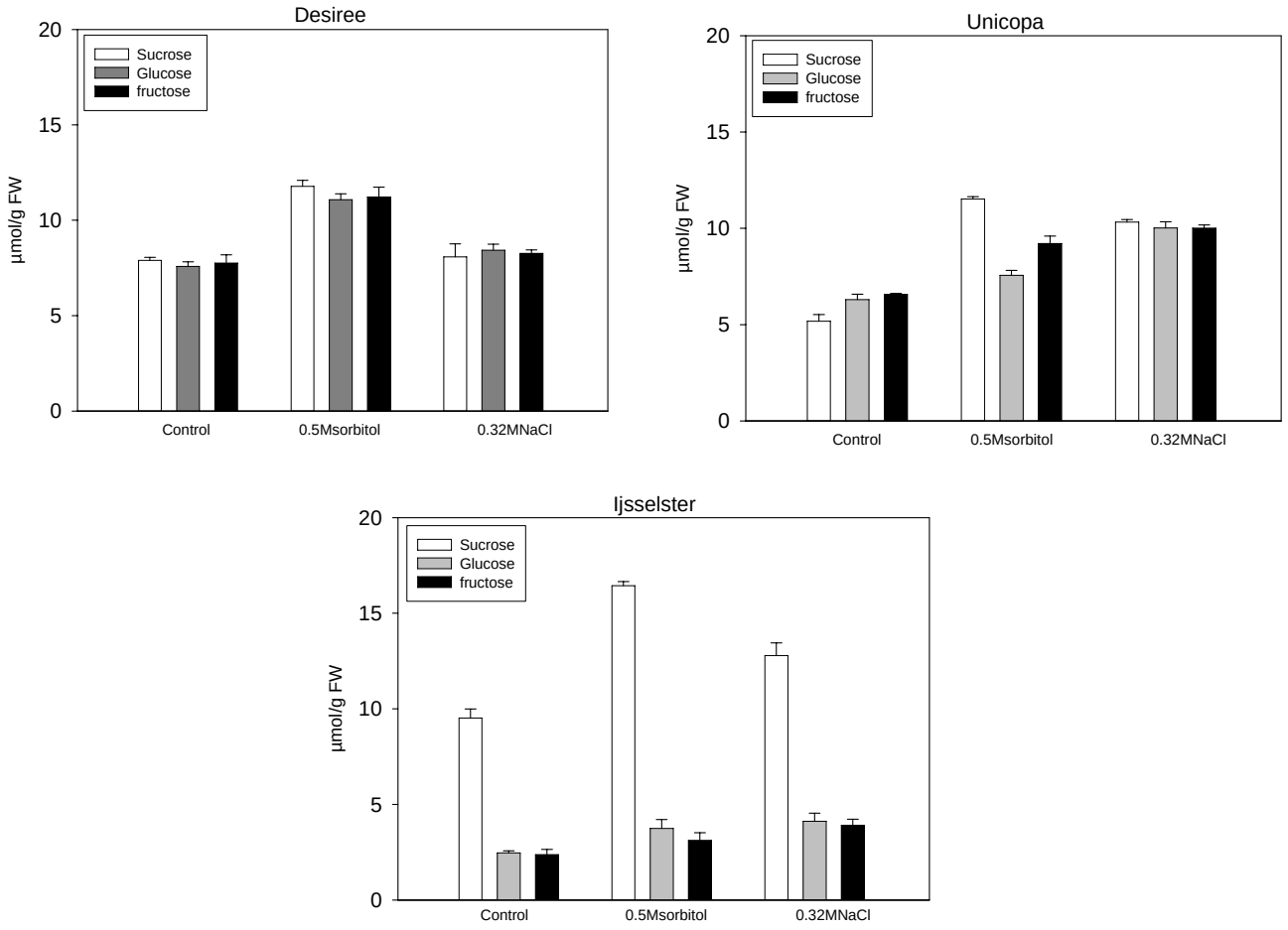


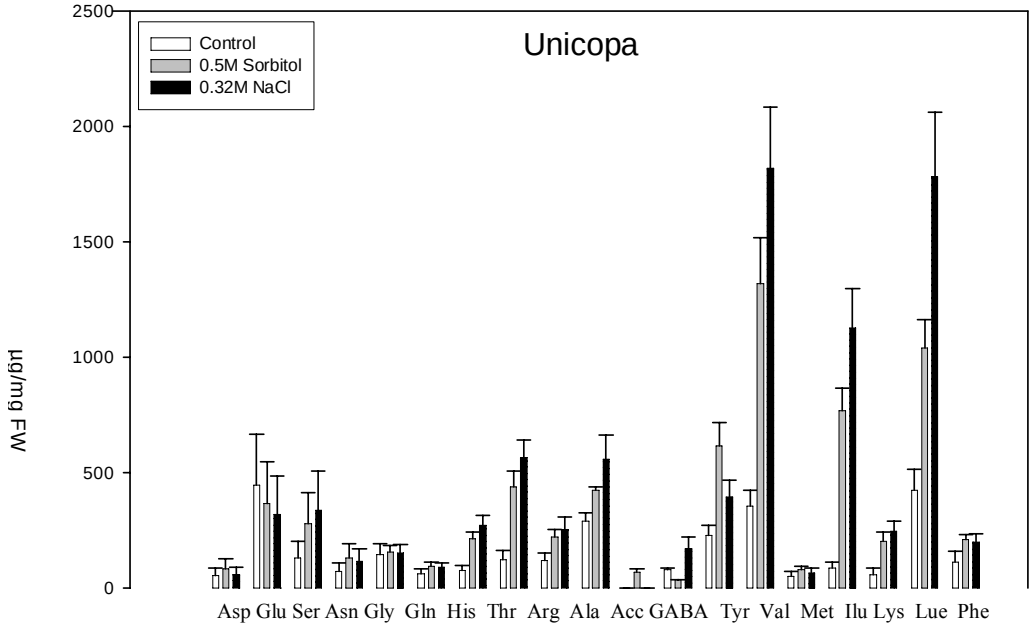
Figure 17: Intracellular content of the sugars sucrose, glucose and fructose. Cultures of the potato cultivars ´Unicopa ´Desiree´ and ´Ijsselster after three days of growth control medium and mediumsupplemente with different osmotics.

In these cultivars for all treatments the content of all three sugars was comparable. A completely different situation was detected in the cell cultures of the cultivar ´Ijsselster´. Compared to the other cultivars, already the control cultures showed a sucrose concentration much higher than those for glucose and fructose. After treatment with sorbitol or NaCl the increase in sucrose content was higher than for glucose and fructose but the general picture remained the same (Figure 17).

3.2.4. Osmotic Stress Effects on Amino Acid Pool

The content of 19 free amino acids in the cultured cells of the three cultivars were detected after three days of growth in the respective media exerting salt (NaCl) and osmotic (sorbitol) stress. In general, glutamate was the only amino acid which showed a decrease under salt and osmotic stress in all cultivars tested, although the decrease was only significant in the cultures of the cultivars ´Desiree´ and ´Ijsselster´. The content of most amino acids increased after salt and osmotic treatment compared to controls. Exceptions were aspartate (Asp) in the ´Unicopa´ under NaCl stress; glutamine (Gln), gamma amino butyric acid (GABA) in the ´Desiree´ cell culture under NaCl and lysine (Lys), Gln, Asp and glycine (Gly) in the ´Ijsselster´ cell line under sorbitol treatment.

Furthermore, the cell cultures of cultivar ´Unicopa´ showed an increase in the content of all amino acids under osmotic (sucrose, sorbitol) and salt (NaCl) treatment, except for glutamate, The ´Ijsselster´ cell cultures showed also a higher accumulation of all amino acids under salt stress, except for Glu, Gly, GABA. The cell culture of the cultivar ´Desiree´ showed a behavior resembling that of the ´Ijsselster´ cell culture concerning the amino acid content under NaCl: all amino acids (except Gln, GABA, Glu and ACC) increased under NaCl treatment compared to control. In comparison the result show that among the cell cultures of the three cultivars, ´Ijsselster´ showed the strongest increase in content of free amino acids under salt and osmotic stress. These amino acids showing increased content after osmotic treatment include Asn, Ser, Gly, Gln, His, Acc, Lys, Phe, Glu, Asp and Ala. Val, Ile and Lue were the most abundant amino acids in the cell cultures of the three cultivars (Figure18).



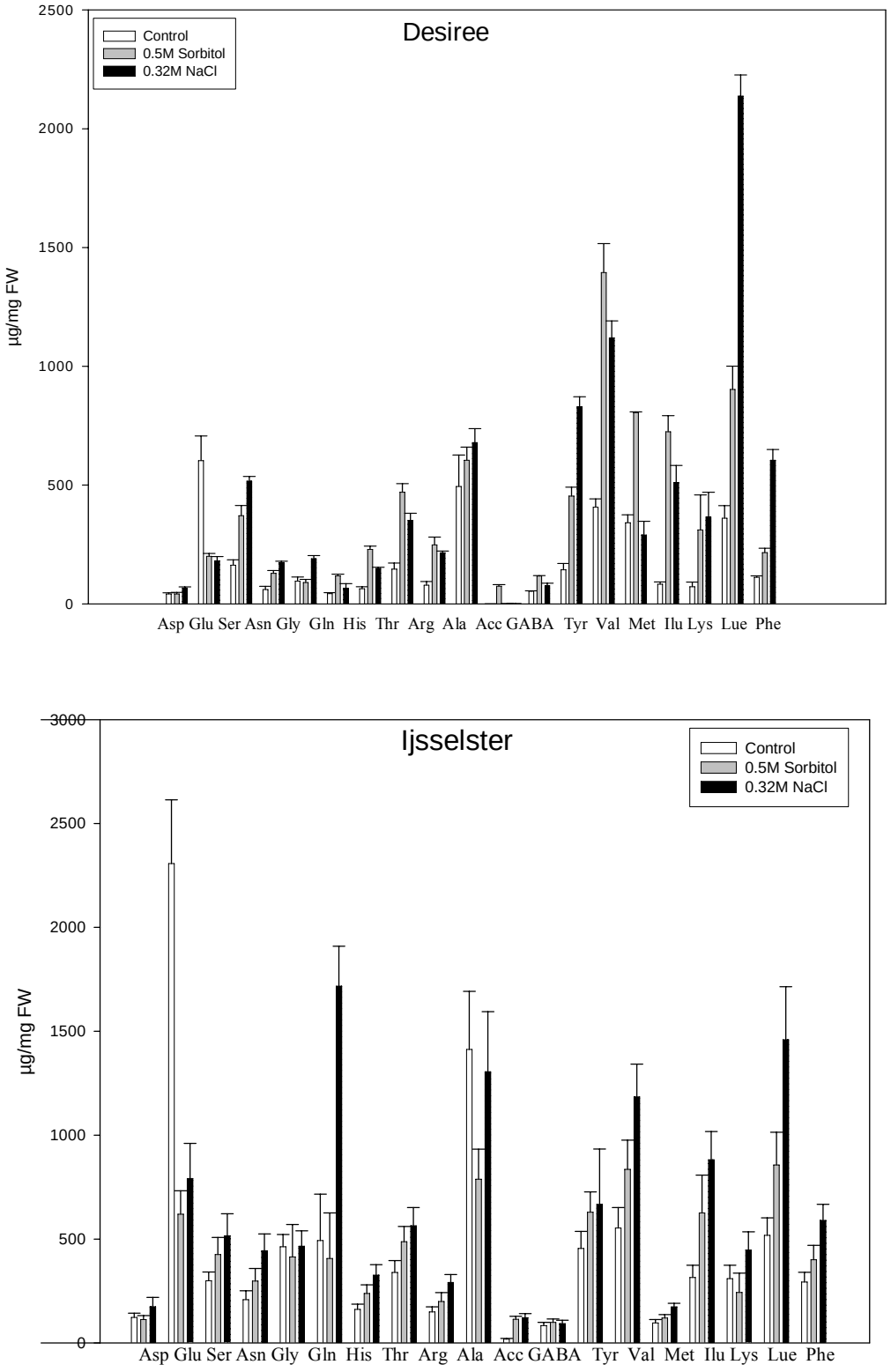


Figure 18: Content of free amino acids in suspension cultures of the different potato cultivars ´Unicopa´, ´Desiree´ and ´Ijsselster´ after three days of growth under control conditions (4X medium) and in medium supplemented with 0.5M sorbitol, 0.42M sucrose and 0.32M NaCl.

3.3. Analysis of proteome changes after growth under osmotic and salt stress

The analysis of changes of the proteome pattern induced by osmotic and salt stress was carried out to find possible targets for molecular analysis. The aim was to achieve an increased osmotic and salt tolerance by overexpression of a target protein. For the differential proteome analysis the suspension culture of cultivar ´Desiree´ was incubated at the low and high standard concentrations of sorbitol (0.25M, 0.5M), sucrose (0.24M, 0.42M) and NaCl (0.17M, 0.32M) in 24multiwell plates. After three and six days the total protein of the suspension cells was extracted. In order to establish a high level of accuracy and to exclude non reproducible biological sample variation, four replicates were performed and evaluated by using independent cell material for each treatment. For isolectric focusing, a pH range from pH 3 to pH 10 was selected and SDS page separation was done in a 12% acrylamide gel (MW 10-150 kDa). About 505-924 protein spots could be differentially separated using the selected parameters. The position of protein spots on the gel was analysed automatically by using the Image Master Platinum 2D software. Three gels of each treatment were used for comparison. As an example the image of a silver stained gel showing the protein pattern of cell suspension, treated with 0.5M sorbitol for 3 days are shown in Figure 19. In the present study mainly spots that appeared only after salt and osmotic stress and which were not visible under control conditions were considered as possible targets for isolation and subsequent transgenic overexpression. Therefore only 6 protein spots fulfilling this criterion were selected.

Unfortunately no prominent protein spot, which occurred only under one of the treatments, could be detected. In this case it would have been possible to use this protein to discriminate between salt and osmotic stress. The 6 most abundant protein spots (Figure 19) were manually picked from Commassie stained gels and undertaken a MALDI-TOF-MS analysis (Table 2).

Among the identified proteins, the pathogenesis related proteins STH2 and STH21 were considered to be involved in cellular salinity- and osmotic stress mediation. Another protein of interest with respect to cell detoxification was 1,4-benzoquinone reductase (spot no. 6). In addition, glutamine synthetase (GS), the key enzyme involved in the assimilation of inorganic nitrogen was also induced upon treatments with salt and osmotic compounds.

Spot	Accession no.	Functional category and protein name	kDa	sequence
1	P17641	pathogenesis - related protein STH-21	17189	NIEAEGDGSIKK
2	gi|169551	pathogenesis - related protein STH-2	17398	NIEAEGDGSIKK
3	gi|62751099	pathogenesis - related protein STH-21	17.339	VETEGDGSIKK
4	gi/226547	Glutamin Synthetase	16.257	HKEHISAYGEGNER
5	gi|2654440	Lemir [Lycopersicon esculentum]	22.931	TGVDYYILPVVR
6	gi|21539481	1,4-benzoquinone reductase-like protein	21.782	GAASVEGVEAK

Table 2: Proteins induced in suspension cells of the potato cultivar ´Desiree´ after osmotic and salt stress spotted and identified by MALDI-TOF-MS and data base search

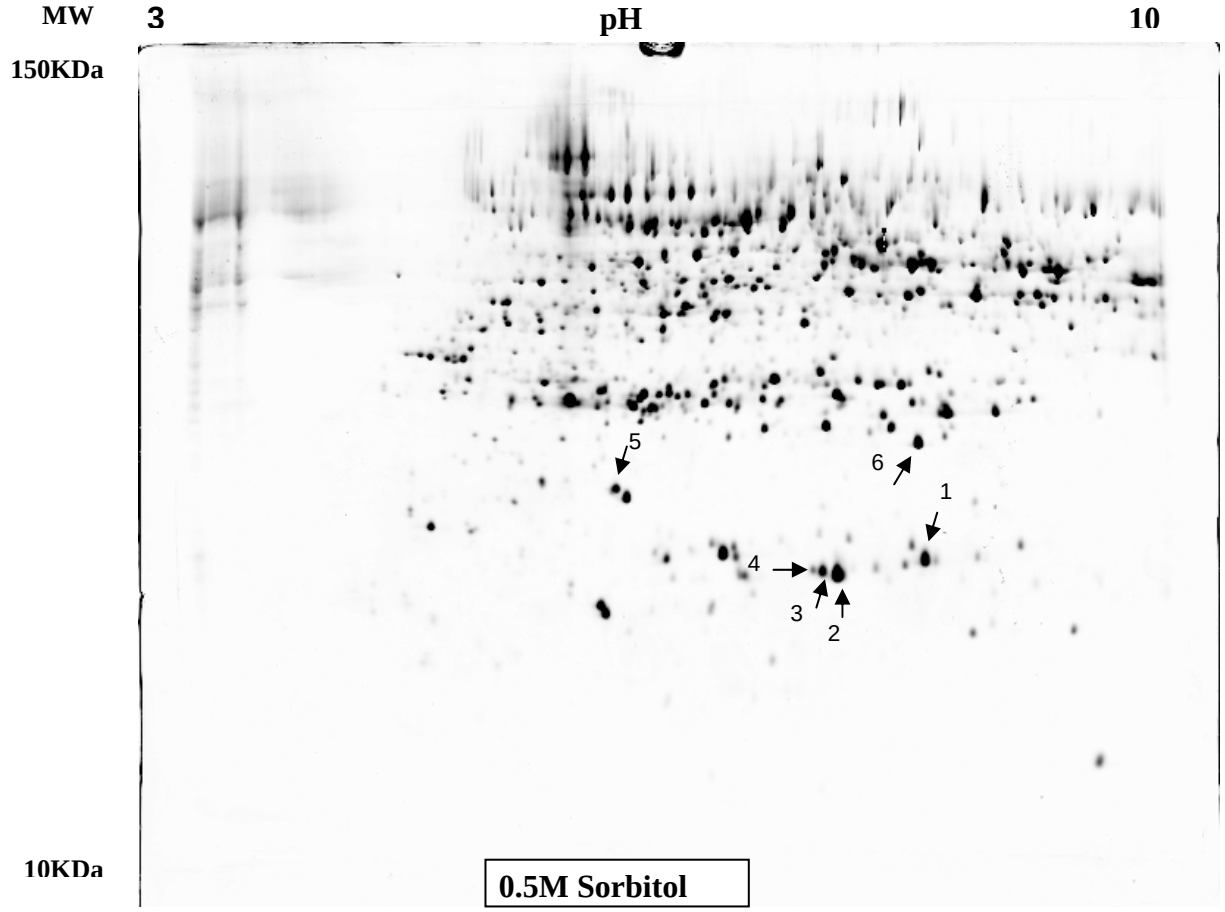


Figure 19: Differentially expressed proteins in a cell suspension culture of the cultivar ´Desiree´ exposed to 0.5 M sorbitol after IEF/SDS-PAGE and silver staining. The vertical dimension of the 2-D gels represents molecular weight scale (10-150kDa on 12.5% polyacrylamid gels), and the horizontal dimension represents the isoelectric point scale (pH 3-10 on non-linear 24 cm IPG strips).

This enzyme catalyzes the condensation of ammonium with glutamate to yield glutamine which provides nitrogen groups for the biosynthesis of all nitrogenous compounds such as proline in the plant. A 22 kDa protein (spot no. 5) was found to be induced only by higher concentrations of osmotically active compounds.

The STH2 protein was chosen for further investigation. To get a rough knowledge about the time scale of the induction of the STH2 protein, proteome analysis was down after 26 hours and 72 hours of salt treatment. It was demonstrated that already 26 hours after induction, the STH2 spot was visible. In the time between 26 and 72 hours the STH2 concentration still strongly increased (Figure 20).

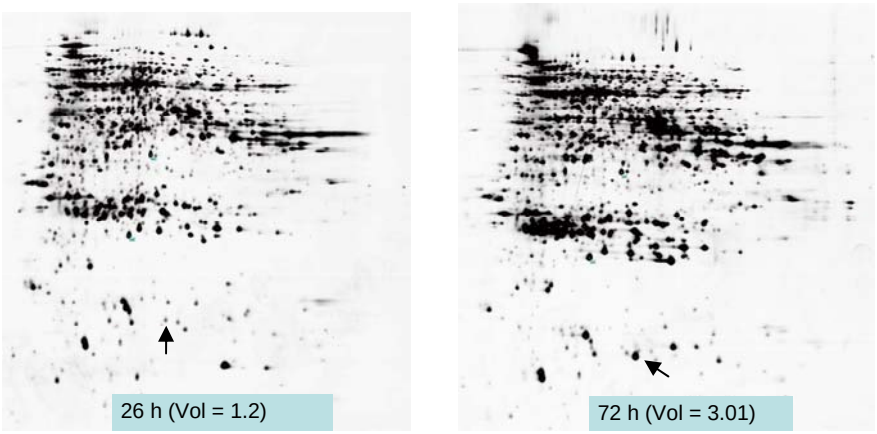


Figure 20: Induction of the STH2 protein in a suspension culture of the potato cultivar ´Desiree´ after 26 hours and 72 hours of exposure of the cells to 0.32 M NaCl. Quantification was done after silver staining by Image Master Platinum 2D software on spot volume basis.

3.4. Construction of transformation vectors for the over expression of the *sth2* gene

3.4.1. Amplification of the *sth2* gene from gDNA of potato

The first step was the amplification of the *sth2* gene from gDNA of potato. According to the nucleotide sequence information for the complete full length sequence *sth2* CDS obtained from the NCBI database (accession no. (gi|169551) (Figure.20), two primers ´sth2 XmaI´ and ´sth2 HindIII´ (Table 3) were designed. These primers were used for amplification and cloning of the genomic DNA encoding for *sth2* gene of potato. The XmaI restriction site is directly preceding the start codon ATG and the HindIII site is located behind the stop codon TAA of the amplified full length gDNA.

For the amplified PCR product the expected size of 828 bp could be confirmed (Figure 22).

Primer name	*Oligonucleotide sequence*
STH2Xma1	*5´-AATCCCGGGATGGGTGTCACTAGCTATACACATG-3´*
STH2HindIII	*5´-AAAAAGCTTTTAAGCGTAGACAGAAGGATTGGCG-3´*

.

Table 3: Sequence of the specific primers used for the amplification and cloning of gDNA and cDNA sth2 gene of potato.

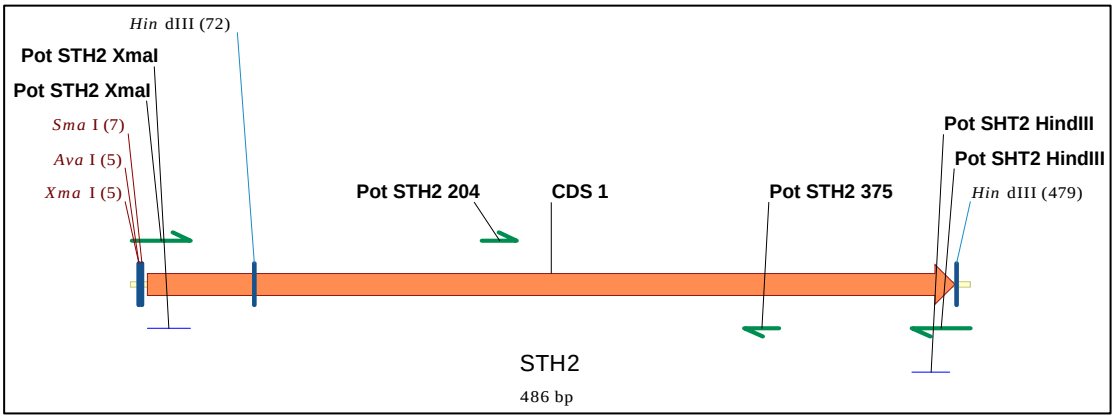


Figure 21: Solanum tuberosum pSTH-2 protein mRNA, complete cds

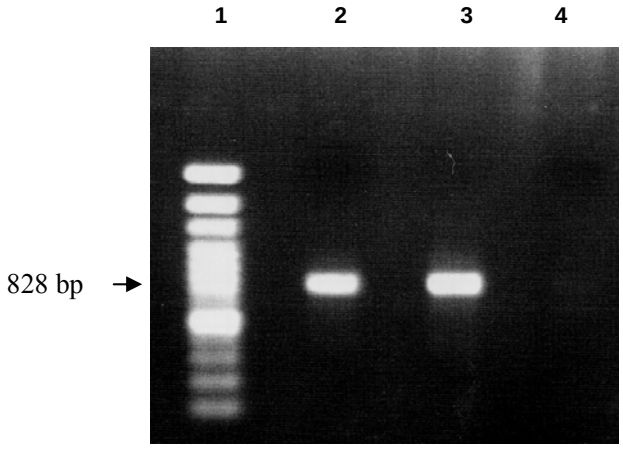


Figure 22: PCR products of gsth2 isolated from potato plants (Lane 1 molecular weight standard, Lane 2 and 3 PCR amplification products obtained with primer sequences, Lane 4 control.

3.4.2. Amplification of the *sth2* cDNA from potato

Potato cell suspension were incubated for three days in 24multiwell plates containing 4X medium without (control) and with 0.32M NaCl, 0.5 M sorbitol and 0.42M sucrose. Total RNA was extracted from 100 mg cells as described. 1 µg of the total RNA was used for reverse transcription and the obtained reverse transcription products were used as templates for PCR amplification. A cDNA fragment of 480bp (Figure 23) was amplified by using the cloning primer pair ´sth2XmaI´ and ´sth2 Hind III´ (see Table 3)

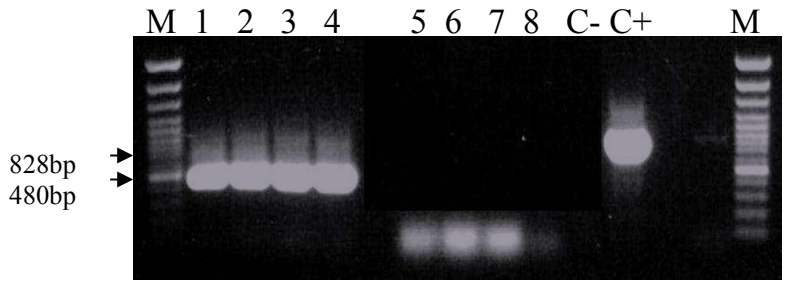


Figure 23: RT-PCR amplification of 480bp sth2 cDNA. M =1Kb DNA ladder, C+ = positive control, C- = water, Lane 1-4 = cDNA and Lane 5-8 =total RNA

3.4.3. Cloning of *sth2* g DNA and cDNA in P Drive vector

The amplified 828bp and 480bp fragments of *sth2* g DNA and cDNA respectively were cloned between the Eco RI restriction sites of the linearized pDrive vector (Figure 24) followed by transformation of the insert-vector construct into E. coli (GM2163). After confirmation of the cloned fragments, the plasmid integrity was checked by sequencing, revealing 100% homology to the *sth2* gene from potato (Figure 21).

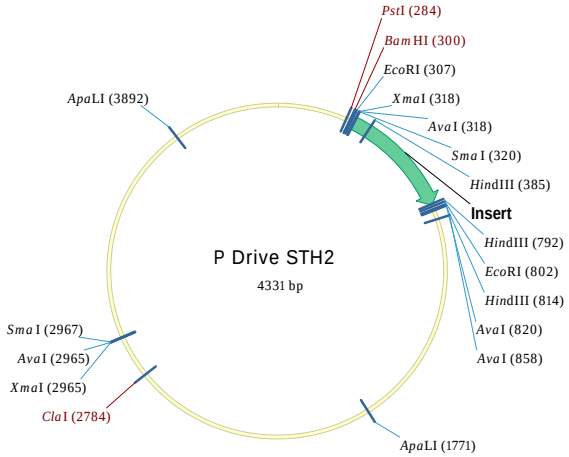


Figure 24: Restriction map of P Drive sth2 vector. The sth2 gene is inserted between Eco RI restriction sites of the original PDrive plasmid vector.

3.4.4. Construction of transformation vectors

After successful confirmation of the *sth2* integration into the P-Drive vector via PCR, restriction digestion and sequencing, the *sth2* gene was sub-cloned in several steps into pGreen II based dicistronic vector using the Xma I and Hind III restriction sites. The resulting T-DNAs are shown in Figue 25 A and B for PG0229 and pG0029, respectively. Resulting vectors were named pGreen II 0229 TR*sth2* cp148luc, pGreen II 0029 TR*sth2* cp148luc (Figure.26)

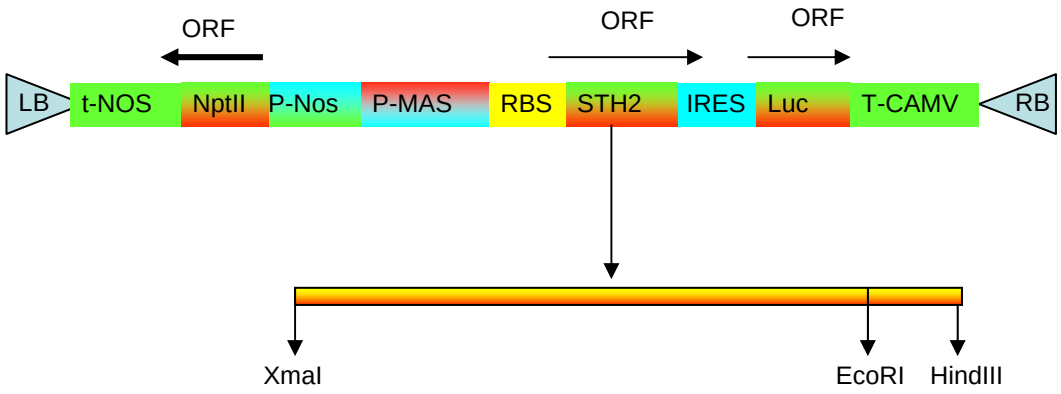


Figure 25 A

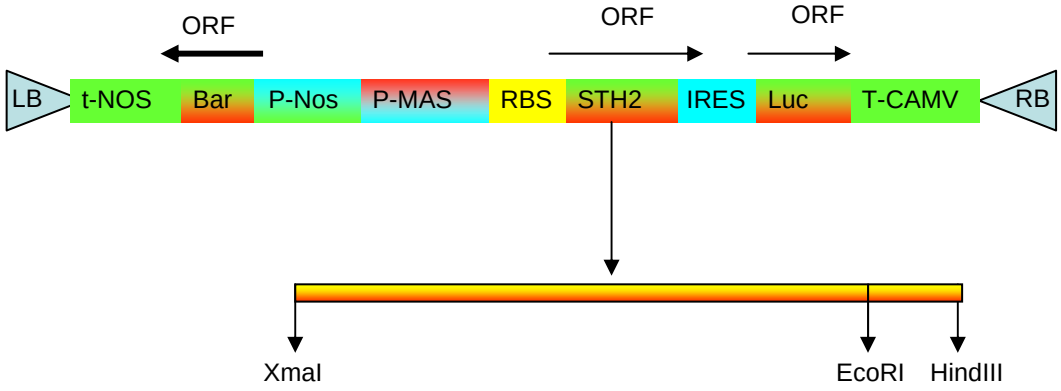


Figure 25 B

Figue 25: Schematic representation of the T-DNA region of the dicistronic binary vector pGreen II 0029 (A) and pGreen II 0229 (B) used in this study. LB = left border; Bar = phosphinothricin acetyle transferase; t-Nos = terminator signals derived from nopaline synthase; P-nos = promoter sequence derived from nopaline synthase; P-Mas = promoter sequence derived from manopine synthetase, RBS = ribosome binding site; IRES = internal ribosome entry site; Luc = Luciferase gene; t-CAMV = terminator signal of 35S Cauliflower mosaic virus; RB=right border.

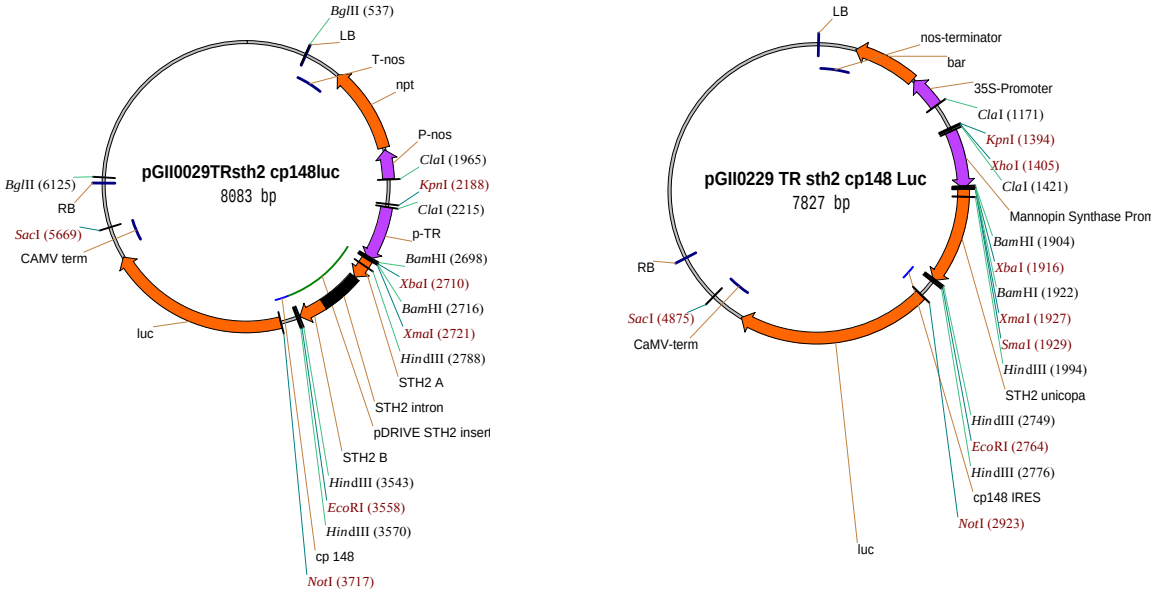


Figure 26: Restriction map of the transformation expression vectors pGreen II 0029 and pGreen II 0229.

After E. coli transformation, transformants were checked by means of PCR and restriction digesition (Figures 27, 28). Thereafter the plasmids were transferred into the hypervirulent *Agrobacterium tumefaciens* strain EHA105 (harboring the $_{p}$soup helper plasmid) by electroporation.

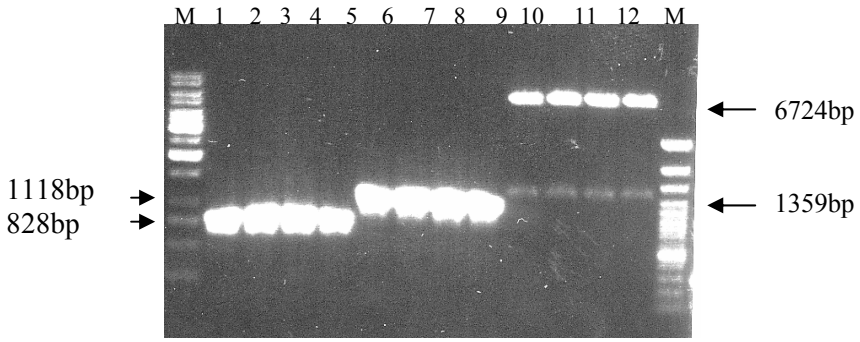


Figure 27: Confirmation of ´pGreen II 0029 MAS sth2 cp148 Luc´ vector (Lanes 2-5) PCR for sth2, (5-8) PCR for NPTII, (9-12) digestion using Eco R1 and Xho I.

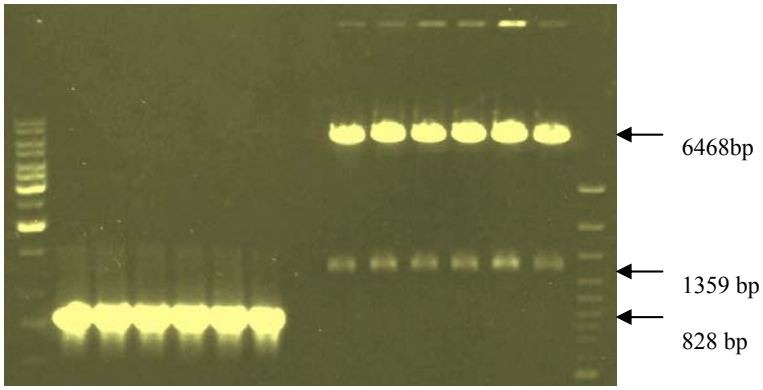


Figure 28 Confirmation of pGreen II 0229 MAS sth2 cp148 Luc vector (1-6) PCR for sth2(8-13) digestion using EcoR1 and XhoI.

3.4.5. Transient expression analysis of transformation vectors

The use of *Agrobacterium tumefaciens* infiltration (agroinfiltration) for transient assays has become well established for assigning gene function (Bendahmane *et al.,* 2000; Van der Hoorn *et al.,* 2000; Johansen and Carrington 2001; Shao *et al.,* 2003 and Wroblewski *et al.,* 2005), promoter element analysis (Hellens RP 2005) and inducible gene studies (Lee MW and Yang,Y., 2006). The majority of results have been obtained using *Nicotiana benthamiana*. Vector functionality was checked by measuring luciferase activity *in vivo* and *in vitro* by a transient expression approach in *N. benthamiana* leaves. Firefly Luciferase expression levels from the pGreenII 0029 and pGreen 0229 constructs following 3 days after infiltration were measured *in vitro* and *in vivo* to prove vector functionality (Figure 29 A and B)

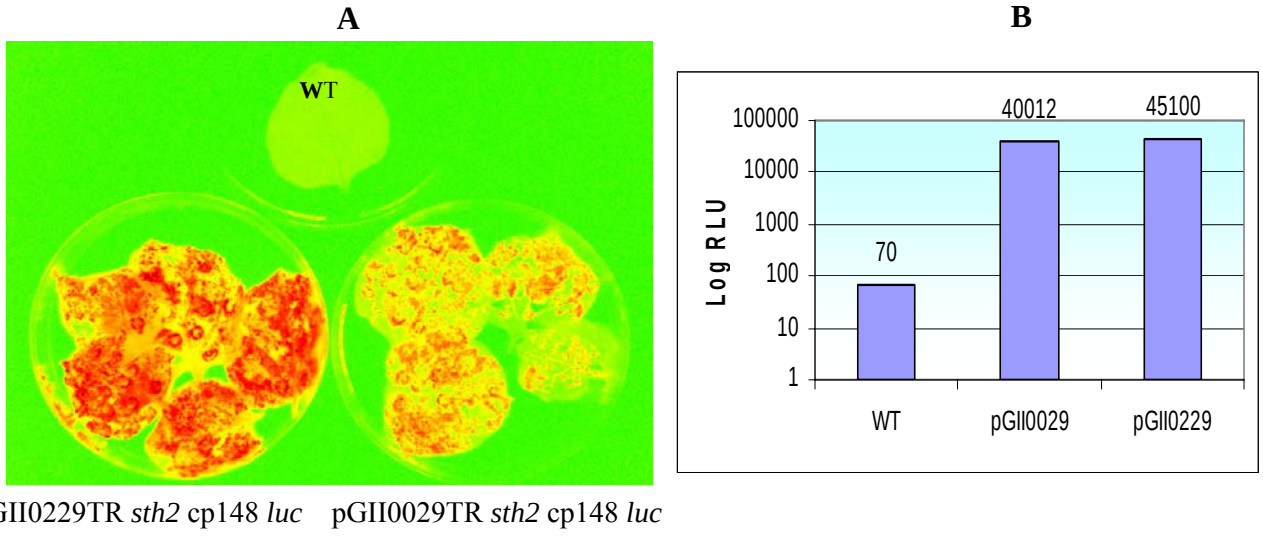


Figure 29: In vivo and in vtiro transient luciferase gene expression in tobacco (Nicotiana benthamiana) leaves A =Chemiluminescence in N. bentamiana leaves after spaying with Luciferin visualized by a Fuji LAS 3000; B=Chemiluminescense in extracts of N.benthamiana leaves measured in a Berthold Luminometer using the Promega kit.

3.5. Overexpression of *sth2* gene in tobacco

3.5.1 Transformation of *Nicotiana tabacum* cv. SR1

In a first approach tobacco plants of the cultivar SR1 have been used to investigate the functionality of the dicistronic vectors and the consequences of *sth2* overexpression. Using tobacco as a model plant, time and effort required to prove the functionality of the vector might be saved. The vectors containing the gDNA as well as the cDNA of the *sth2* gene were used for tobacco transformation experiments according to the leaf disc method (Horsch *et al.,* 1985)**.** 10 transgenic T0 plants were regenerated after transformation with the cDNA construct and 12 trangenic T0 plantlets from transformation with the vectors containing the gDNA sequence. Plantlets were regenerated under ppt selection. Successful integration of T-DNA into the tobacco genome was confirmed by means of PCR for the *sth2* gene (Figure 30). In addition RT-PCR and the monitoring of luciferase gene expression (Figure 31 A and B) by measurement of luciferase activity was performed. The presence of bands of a size of 828bp and 480bp confirmed the integration of the transgene. Furthermore, using RT-PCR the presence of bands at 480 bp in the Lanes 1-3 in Figure, 31 (A) confirmed the presence of *sth2* transcripts.

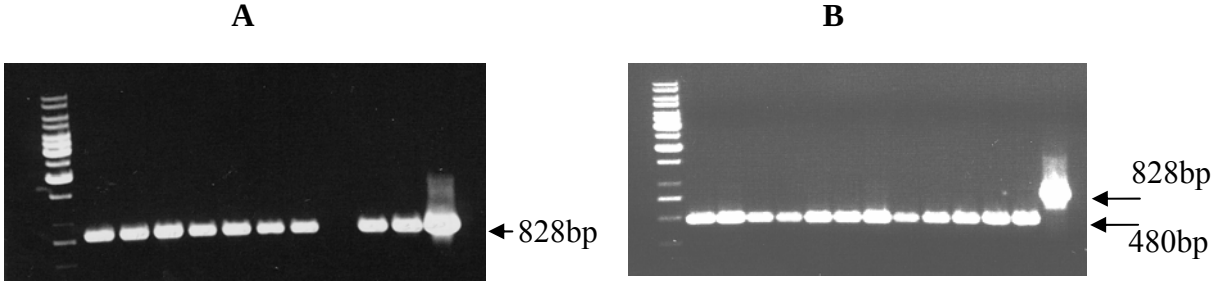


Figure 30: PCR analysis of different transgenic tobacco clones transformed with cDNA (A) and with gDNA (B)

3.5.2. Physiological assay with transgenic tobacco seedlings.

To examine the physiological effect of *sth2* overexpression, seeds of wild type and T0 plants from different clones that constitutively express the *sth2* gene were germinated on filter paper wetted with ½ strength of the MS macro- and microsalts and the same solution supplemented with different concentrations of salt (NaCl), ranging from 0 to 150mM.

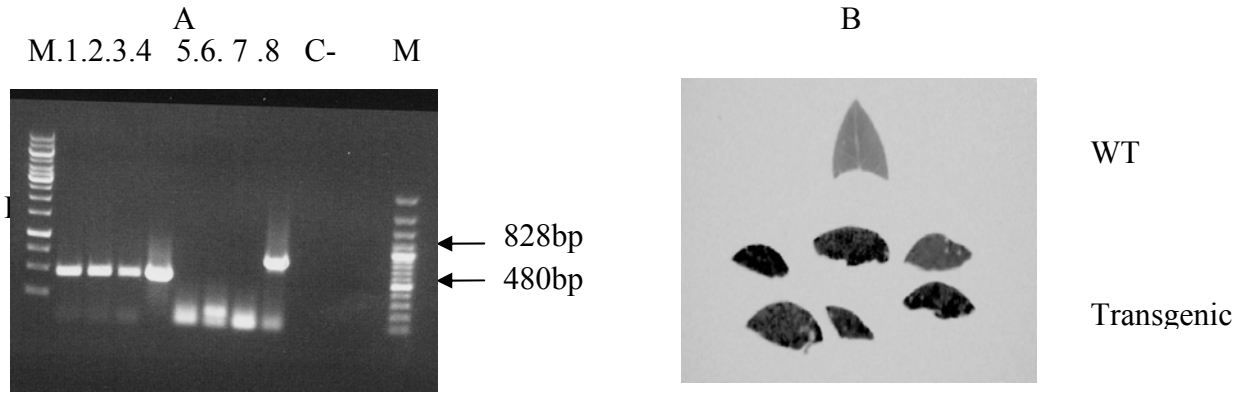


Figure 31: (A).PCR products of sth2 gene in T0 tobacco plants.Lane = 1-3 cDNA; Lane 4 = positive cDNA; Lane 5-7 = RNA; Lane 8 positive control (g DNA). (B) Luciferase activity detection by visualization of chemiluminescense in a Fuji LAS 3000 imager after spraying with Luciferin in transgenic T0 and wild type tobacco plants.

On the solution without salt supplementation, germination and growth of wild type and transgenic seeds were not noticeably different (Figure 32). Although both the wild type and transgenic seeds could germinate in the presence of 50 mM and 100mM NaCl, the transgenic seedlings grew better. On 150 mM NaCl, the growth of wild type seedlings was almost stopped and the leaves bleached. However, transgenic seedlings were not influenced and had longer roots and green and bigger leaves than the wild type seedlings. This indicates that overexpression of *sth2* increases the salt tolerance in tobacco plants.

Figure 32: Germination of wild type and transgenic tobacco seedlings (T1) overexpressing sth2 on ½ strength MS salts and ½ strength MS salts supplemented with 150 mM NaCl sth2 after 3 week.

To determine whether adult transgenic plants also had increased salt tolerance, some of the transgenic and wild type plantlets were transferred to pots, then subjected to gradually increased salt concentration (50-300mM NaCl).Before the stress treatments, the wild type and the transgenic plants were similar, but after treatments with salt (300mM NaCl), the growth of wild type plants was stopped and they flowered 13 days earlier than transgenic ones.

3.5.3. Physiological assay with a transgenic tobacco cell suspension culture.

To identify the physiological role of overexpression of the *sth2* gene on the cellular level, a callus culture and from the callus culture a suspension culture was established from a transgenic T0 tobacco plant. The effect of *sth2* overexpression on the suspension culture was investigated in comparison to a suspension culture overexpressing the At-nhx 1 gene. Equal amounts (400mg fresh weight) of cells from suspension cultures of wild type plants, from suspension cultures overexpressing the *sth2* gene and from At-nhx1 overexpressing tobacco cells were spread over solid 4X medium either without or with supplementation of NaCl and sorbitol at different concentrations in 60 mm Petri dishes. After 4 weeks of incubation, the expression of the luciferase gene (which is coexpressed with the *sth2* gene) was measured by the detection of luciferase enzyme activity using a Fuji LS 3000 imager (Figure 33). The results showed that luciferase activity reflected by chemiluminescence increased as NaCl and sorbitol concentration increased. Under osmotic stress, the highest level of luciferase expression was observed at 0.25M sorbitol. It was lower at 0.5M sorbitol or salt respectively. Suspension cultures were in parallel grown under ppt selection.

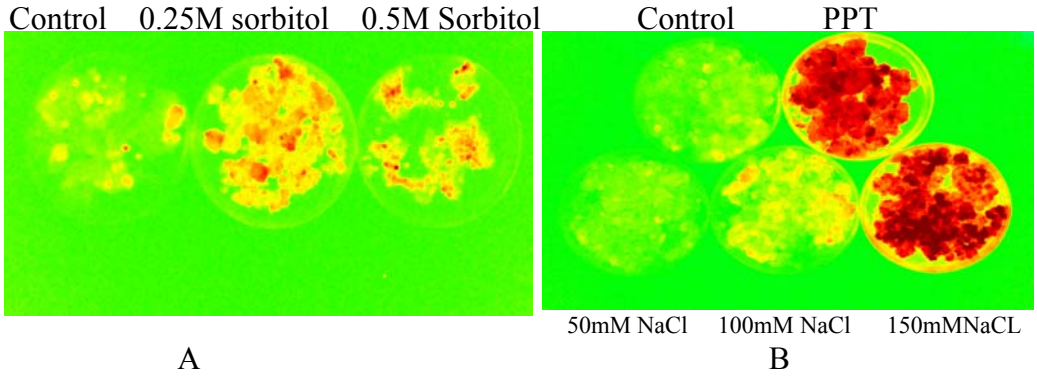


Figure 33: Luciferase activity in transgenic tobacco cells.(A) under osmotic challenge exerted by sorbitol and (B) under PPT and salt challenge visualized as chemiluminescence in a Fuji LS 3000 imager after spaying with Luciferin.

Furthermore both fresh and dry weight was recorded after 4 weeks of growth under osmotic and salt stress (Figure 34 and Figure 35). Under control conditions, no significant difference was observed between control and At-nhx1 expressing cells, whereas a significant difference occurred between *sth2* expressing cells and both control and At-nkx1 expressing cells.

Under salt stress, fresh weight and dry weight accumulation of the control cells declined already from 50 mM salt concentration on. Expression of the At-nhx1 gene provided an increased salt tolerance compared to wild type cells at concentration ranges from 50 mM to 100 mM NaCl but not at 150 mM salt. Overexpression of the *sth2* gene led to an almost unaffected fresh weight and dry weight accumulation of cells even at a salt concentration of 150 mM NaCl.

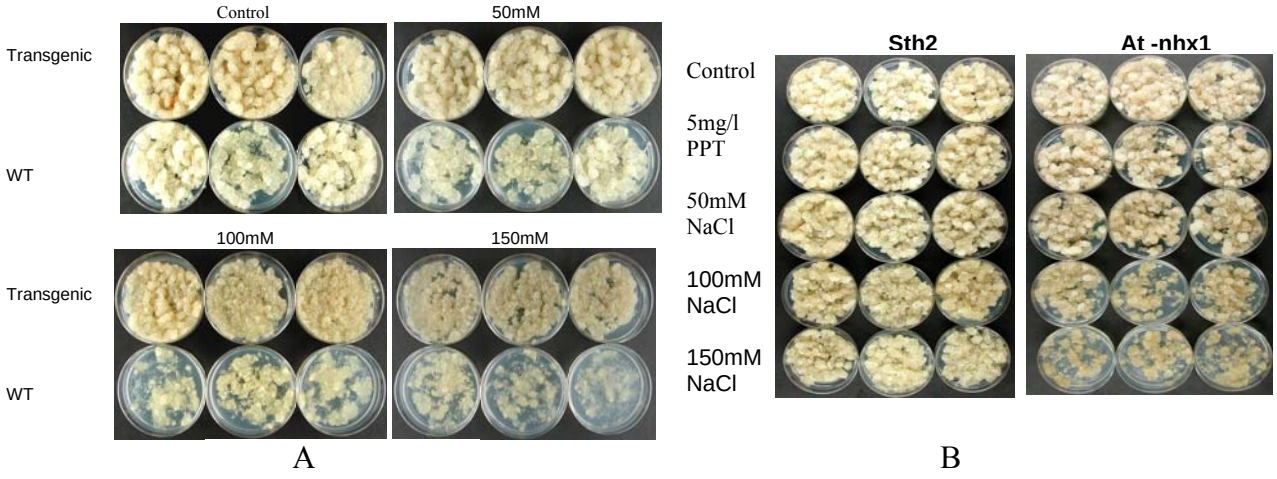


Figure 34: Growth of sth2 and At-nhx1 expressing tobacco cells under different salt concentrations. (A) Sth2 expressing and wild type cells. (B) Sth2 vs. Atnhx1 expressing cells.

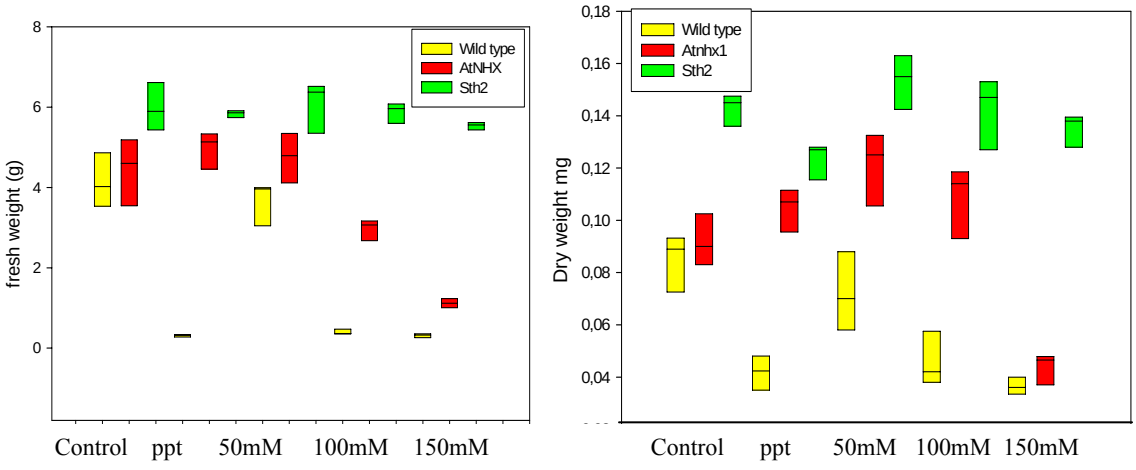


Figure 35: Box/Whiskers plots of dry weight and fresh weight accumulation of control cells and tobacco cells expressing the At-nhx1 gene and the sth2 gene.

3.6. Overexpression of *sth2* gene in cell cultures of *Solanum tuberosum* cv. Desiree

3.6.1 Transformation of *Solanum tuberosum* cv. Desiree cell cultures

A suspension culture of potato (*Solanum tuberosum* cv. ´Desiree´, PC-1202) was transformed by co-cultivation with *Agrobacterium* harboring the dicistronic binary vector ´PGII 0029TR *sth2* (cDNA) cp148 *luc´*. The putative transformed cells were selected by spreading the infected cells on medium containing ppt. The ppt resistant transformants formed calli of about 4 mm in diameter after three weeks of incubation on 4X medium containing 5 mg/l ppt. Luciferase activity was observed in most of the putative transgenic calli by Fuji LAS 3000 imager (Figure 36).

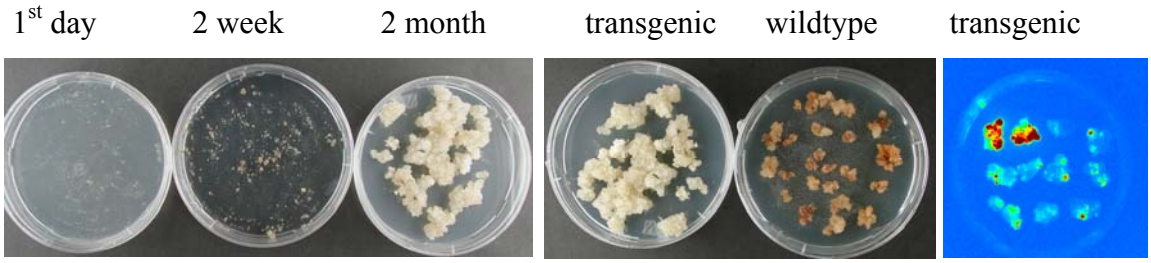


Figure 36: PPT and luciferase selection of callus after transformation.

3.6.2. PCR analysis of cell suspension-derived transgenic calli

PCR was used to demonstrate the presence of the recombinant *sth2* gene in the transformed cell cultures. The primers used (see Table 3) amplified not only the recombinant gene derived from cDNA but also the endogenous *sth2* gDNA sequence. Consequently two bands appeared among the amplification products. One band of a size of 828 bp for the endogenous gene and a band 480 bp for the recombinant gene appeared. The analysis was performed for the ppt resistant callus as well as for the suspension derived from the callus (Figure 37).

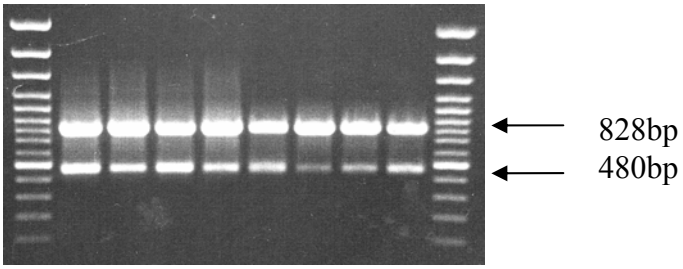


Figure 37: PCR analysis of transgenic potato cell cultures. The first 4 Lanes show band for calli, the second 4 Lanes those for analyzed suspensions.

3.6.3. Proteome analysis of *sth2* overexpressing cells

To confirm the overexpression of the *sth2* gene and the proper translation of the protein in the transgenic potato cell suspension, the proteome patterns of transgenic and non-transgenic cells were analyzed by two-dimensional protein electrophoresis using the same methodology as described in chapter 3. Proteins were extracted from transgenic and non-transgenic cells growing under control condition. The results showed that the spot representing the STH2 protein is present in the transgenic cells even without osmotic or salt stress, whereas the control gel did not show any spot at all in the same region of the gel (Figue 38). The analysis also showed that although the same amount of protein was used for analysis and the same developing time during silver staining for both gels some other proteins seemed to be up-regulated. A detailed analysis of the proteome pattern was so far not performed.

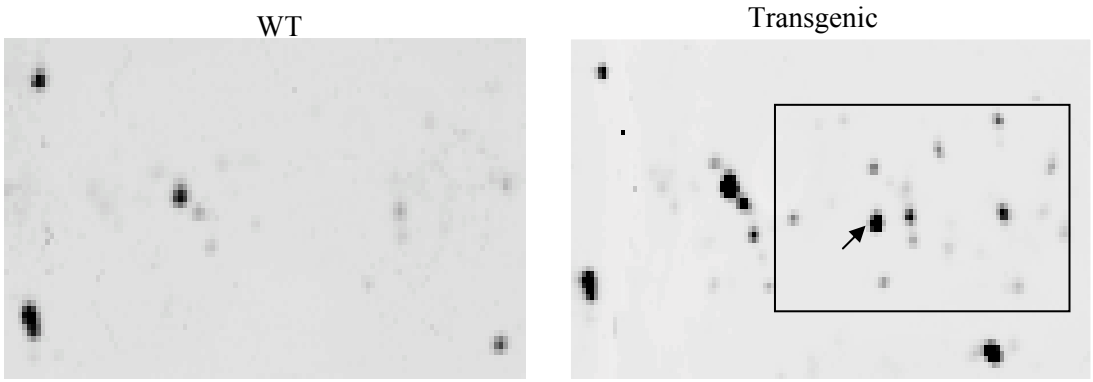


Figure 38: A zoomed region of a two-dimensional electrophoresis profile of proteins extracted from transgenic and wild type cells. The STH2 protein is indicated by the arrow and the proteins induced are indicated by the square (see for comparison Figure 19).

3.6.4. Characterization of osmotic and salt tolerance of transgenic cell lines

3.6.4.1. Experiments performed with cells growing on solid medium

For comparing the cell growth of wild type and transgenic cells, the *sth2* positive clones and control cells were grown on normal 4X medium and 4X medium supplemented with different salt and sorbitol concentrations. 400 mg of cells grown in suspension were harvested and spread on the different solid media in Petri dishes. Cells were grown on these dishes for 4 weeks. Apparent differences could be observed between wild type cell cultures and transgenic samples when growing under 0.5 M sorbitol, but no obvious difference was observed when

the medium contained only 0.25 M sorbitol.Under 0.75M sorbitol, no growth of transgenic and wild type cells could be seen and all cells probably died (Figure 39).

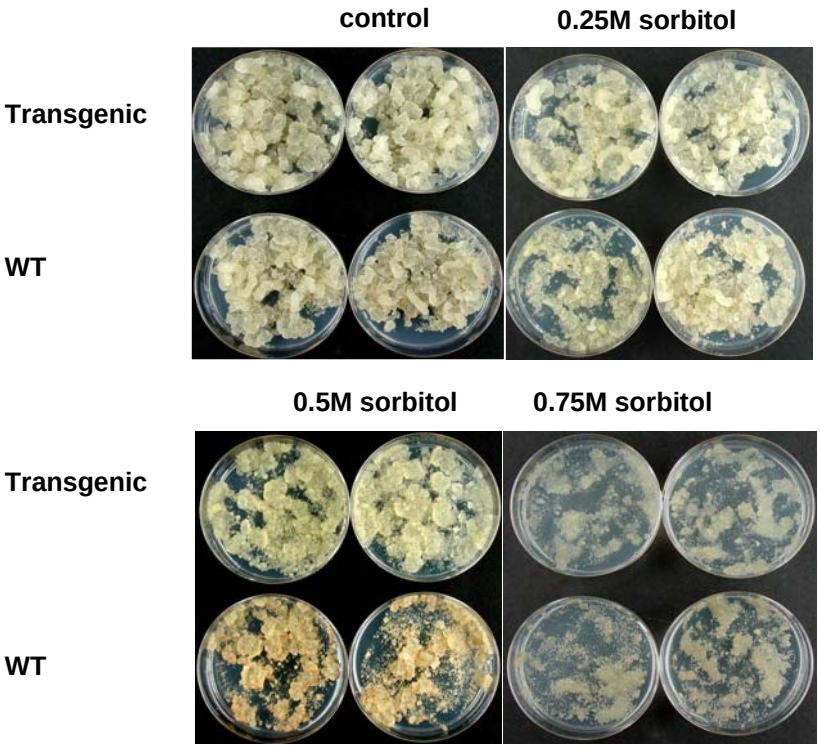


Figure 39: Wild type and transgenic potato cells grown for 4 weeks on solid 4X medium without and with sorbitol supplementation at different concentrations.

At the same time wild type and transgenic suspension cells were grown in the same way under salt stress. Salt concentrations used were 50 mM NaCl, 100 mM NaCl and 150 mM NaCl. In the same experiment cells were grown in parallel on 4X medium supplemented with 5mg/l phosphenothericin (PPT). When the NaCl concentration was increased from 100mM to 150 mM, growth of wild type cells was reduced dramatically and the cells turned brown. At 200 mM NaCl, the wild type cells did not grow at all. On the other hand the growth of the transgenic cells was vigorous even at 200mM salt. Under ppt selection the wild type cell culture showed reduced growth and strong browning (Figure 40 and 41).

3.6.4.2. Experiments performed with cells growing in liquid medium

Growth characteristics and viability of wild type and transgenic cells was also compared after growth in liquid cultures with and without salt and osmotic stress. Both suspension cultures, transgenic and wild type, were pre-grown in 300 ml Erlenmeyer flasks and were harvested in the logarithmic growth phase and used for inoculation of 24multiwell plates.

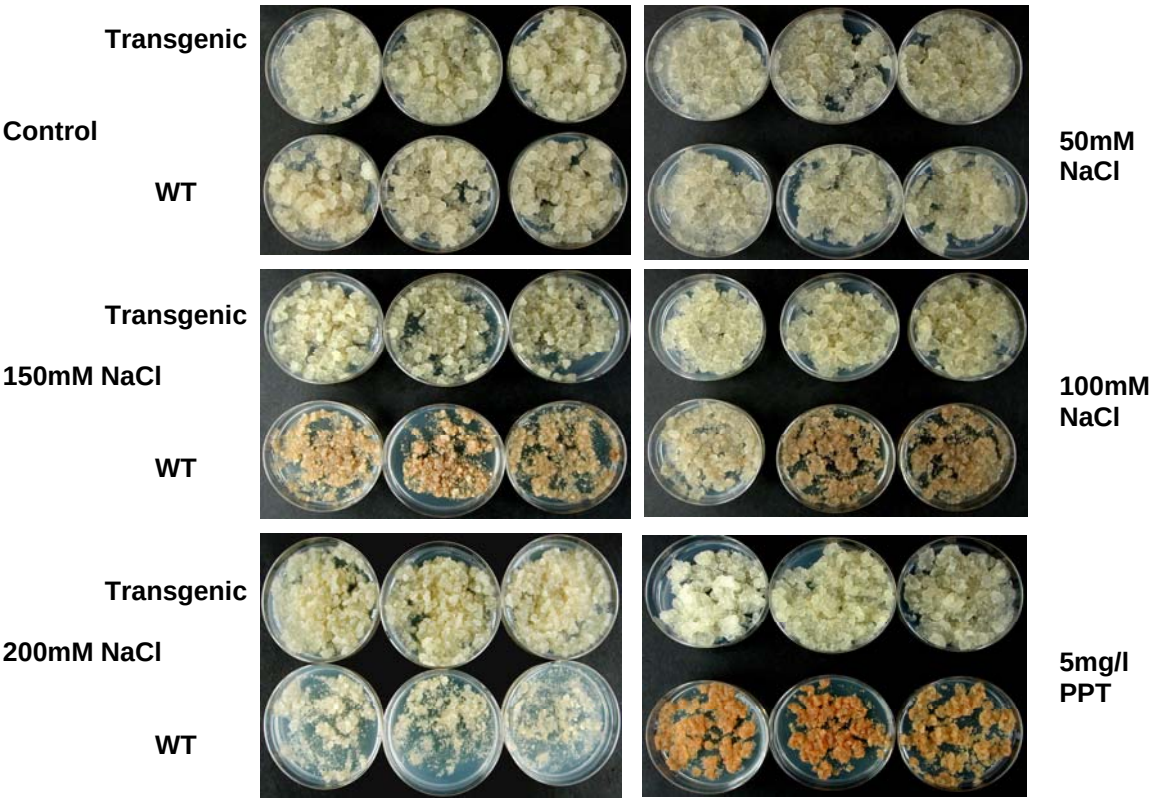


Figure 40: Wild type and transgenic potato cells grown for 4 weeks on solid 4X medium without and with NaCl supplementation at different concentrations. In addition cells transgenic and wild type cells were grown under ppt selection (5 mg/l).

Luciferase expression was monitored qualitatively after 4 weeks of cell growth as described. A Luciferin solution (1 mg/ml) was sprayed upon the calli and the resulting chemiluminescence was visualized under a Fuji LAS 3000 Imager. In the used color transformation algorithm a shift from yellow to red color indicates an increased chemiluminescence (Figure 41).

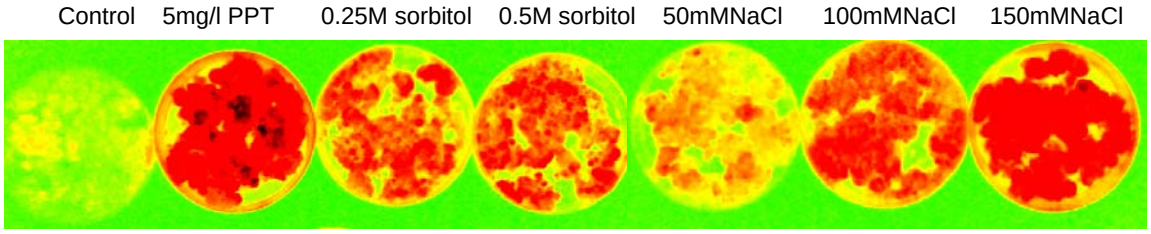


Figure 41: Chemiluminescence indicating Luciferase activity of potato cells overexpression sth2 gene after growth for 4 weeks under control, osmotic and salt stress conditionsVisualized after spraying with Luciferin solution in a Fuji LAS 3000 imager in color shift mode.

100 mg of cells were used for the inoculation of each well containing either 0.9 ml 4X control medium or medium supplemented with different concentrations of sorbitol or NaCl. The viability of the cells was monitored by adding the pH indicator chlorophenol red to the medium. After 5 days of growth the color shift from red to yellow, indicating cell viability, could be observed for the wild type cells clearly only under control conditions whereas the transgenic cells induced the color shift also under stress conditions (Figure 42).

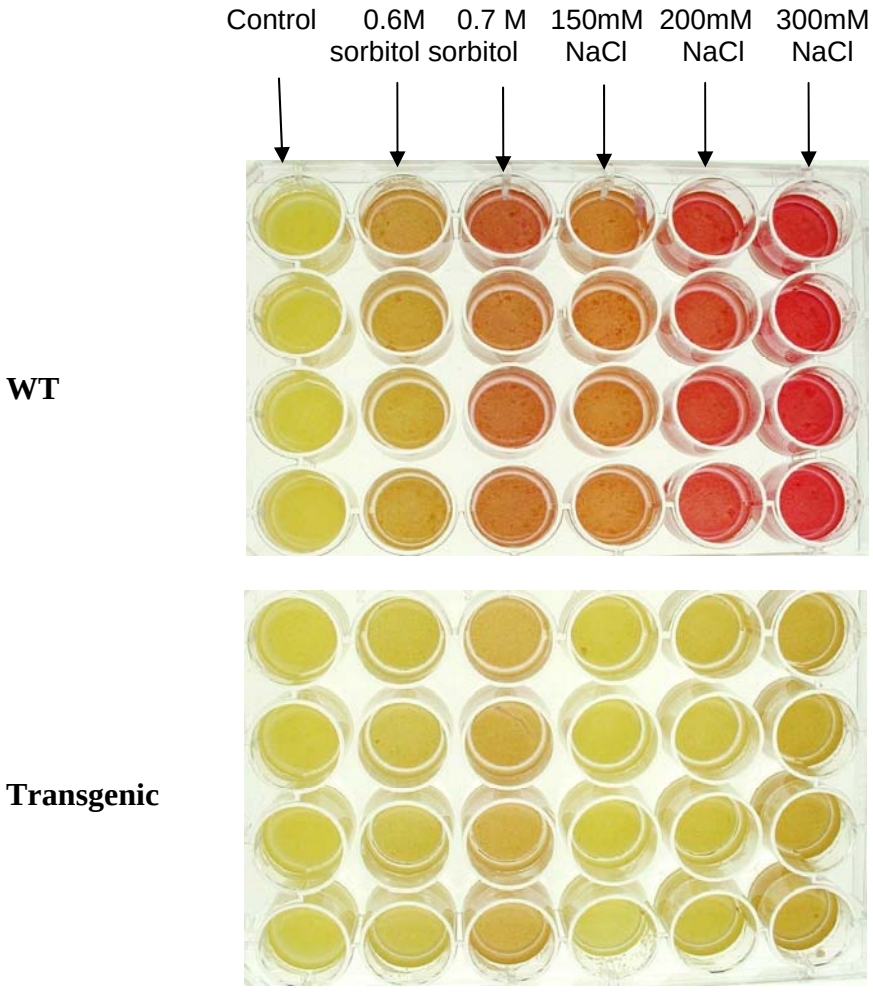


Figure 42: Cells of a wild type and a transgenic cell culture of the potato after 5 days of growth in normal medium and media supplemented wit sorbitol or NaCl in the presence of chlorophenol red.

Furthermore in parallel tests, cells were harvested from the wells of a 24multiwell plate and dry matter accumulation was determined and viability tested by TTC test. The dry weight of

the transgenic cell culture was significantly higher than that of the wild type culture under all osmotic and salt levels (Figure 43 A and B)

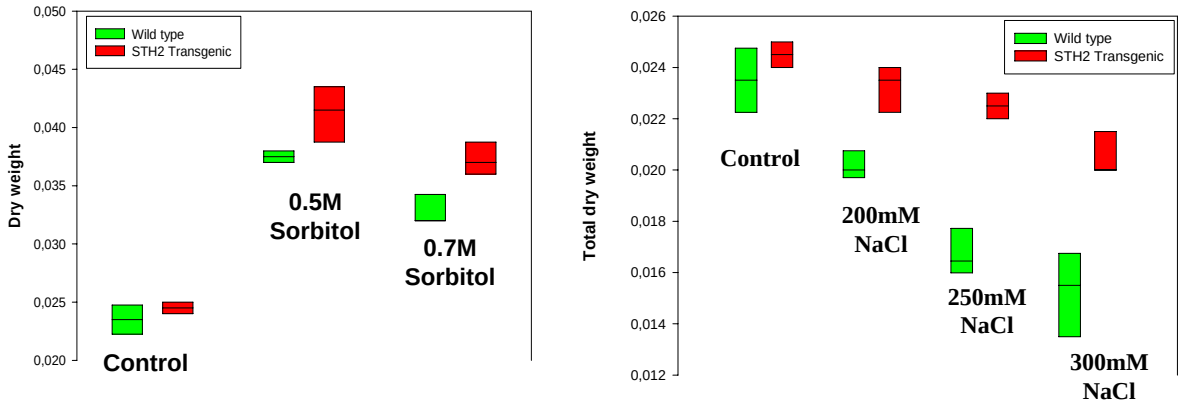
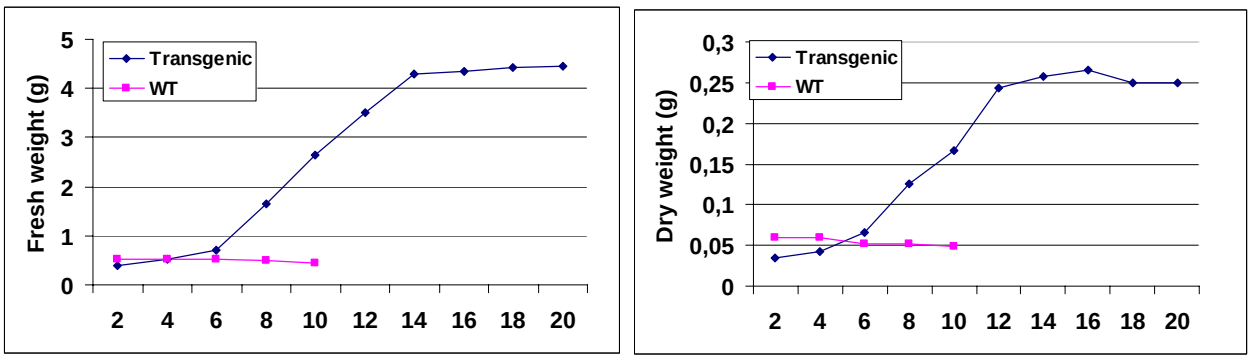


Figure 43: Accumulation of dry matter of a wild type and a transgenic suspension culture overexpressing the sth2 gene after growth for 7 days in 24multiwell plates in normal medium and under osmotic stress (A) (0.25 M, 0.5M sorbitol) and salt stress (B) (50 mM, 100 mM and 150 mM NaCl).

In a subsequent experiment it was tested whether osmotic and salt tolerance of a suspension culture acquired by *sth2* overexpression sustains over the whole growth period of the culture. Wild type and transgenic cells were grown in 100ml Erlenmeyer flasks in 4X medium supplemented with 200 mM NaCl over 20 days. Two Erlenmeyer flasks were harvested every day and fresh weight and dry weight were measured. Figure 44 A and B show the growth curve of the wild type and the transgenic cell culture.



A B

Figure 44: Fresh weight and dry weight accumulation of a wild type and a suspension culture expressing the sth2 gene of potato cultivar ´Desiree´. Cells were grown over 10 days in 100ml Erlenmeyer flask in 4X medium supplemented with 200 mM NaCl.

For cells harvested after 7 days of growth a FDA viability tests was performed and picture of the harvested cell material were taken. The transgenic cells looked healthy and the FDA test showed fluorescent cells whereas the wild type cells appeared to be black and showed only light fluorescence (Figure 45).

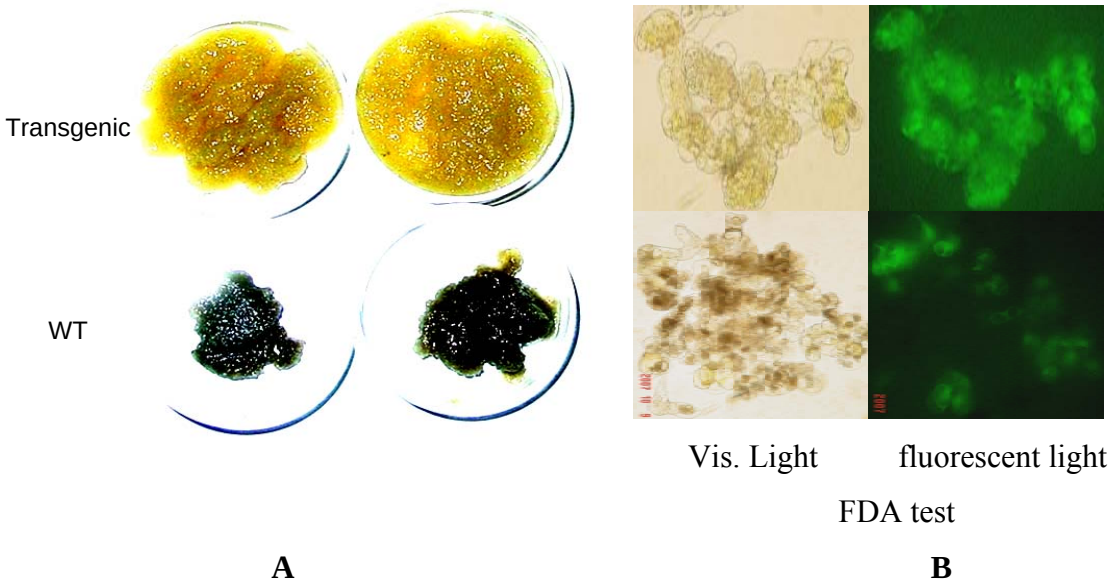


Figure 45: (A) Image of harvested wild type and transgenic cells of potato cv. ´Desiree´ after 10 days of growth in medium supplemented with 200 mM salt. (B) Microscopic image (Magn.. 100x) of wild type and transgenic cell suspension of potato cv. ´Desiree´ after 7 days of growth in medium supplemented with 200 mM salt in visible light and fluorescent light after incubation in FDA solution (Ex. = 345, Em. = 450)

3.6.5. Biochemical characterization of transgenic cells under osmotic and salt stress

The metabolic analysis clearly indicates that osmotic and salt stress leads to large changes in the metabolic network. Some biochemical stress reactions have been investigated in a comparative study with cell cultures of three different potato cultivars (see Chapter 3.2). To determine whether these changes are influenced by *sth2* overexpression, transgenic and non-transgenic cell cultures were grown in 24multiwell plates under control conditions (4X medium) or under osmotic and salt stress (0.5M sorbitol and 0.32M NaCl) for three days. The cells were harvested and soluble carbohydrate content, amino acid content and glutathione content were determined.

3.6.5.1. Soluble sugar content

Changes in the content of the soluble sugars sucrose, glucose and fructose were measured in the transgenic and non-transgenic cell cultures grown under control conditions (4X medium) and under salt and osmotic stress exerted by 0.5M sorbitol or 0.32M NaCl in the medium. In the wild type cell cultures, salt as well as sorbitol treatment leads to a slight increase (sorbitol) or a slight decrease (salt) of the soluble sugars but the ratio of the sugars does not change significantly. The same was also true for the transgenic cell cultures. The remarkable difference is in the ratio of the soluble sugars. The wild type cell culture contains more or less equal amounts of sucrose, glucose and fructose whereas the transgenic cell culture contains approximately 2 – 3 times more sucrose than fructose or glucose. The two latter are present in equal amounts in the transgenic cells. Especially after salt treatment the high amount of sucrose even increases in the transgenic cell culture (Figure 46).

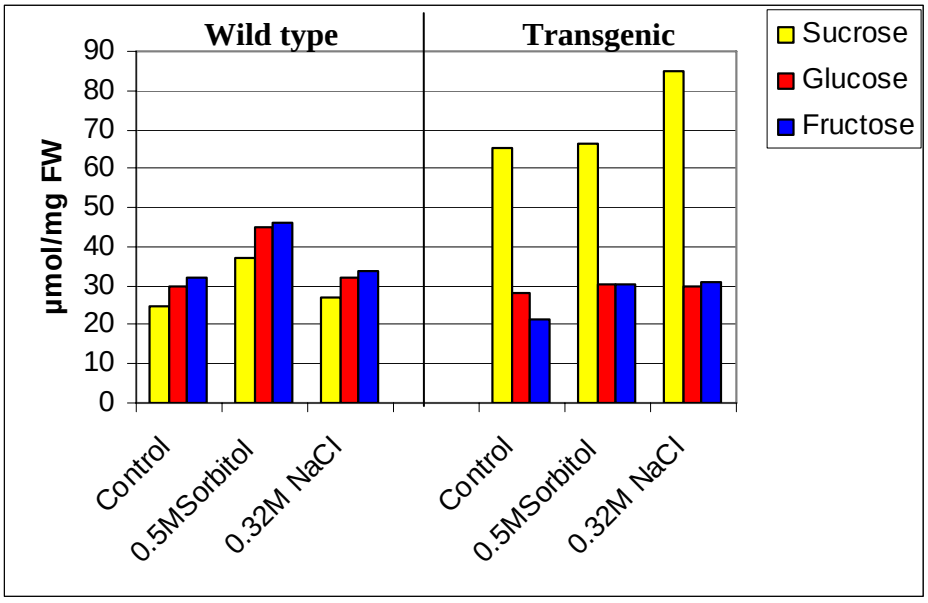


Figure 46: Content of sucrose, glucose and fructose of wild type and transgenic potato cell cultures cv. ´Desiree´ after three days of growth under control and osmotic and salt challenge conditions.

3.6.5.2. Glutathione content

The change in the percentage of oxidized glutathione (GSSH) from the total glutathione pool (GSH + GSSG) in response to osmotic and salt stress was measured in the transgenic and non-transgenic potato cell cultures (Figure 47) under standard test conditions (3 days of growth in 24multiwell plates, control = 4X, stress conditions = 4X medium + 0.5M sorbitol or

0.32M NaCl). The percentage of the oxidized form was equal in transgenic and wild type cultures under control conditions. Under all stresses there was a decrease of this percentage in both cell lines. Nevertheless this decrease was much more pronounced in the transgenic cell cultures. That means on the other hand that the transgenic cell culture showed 70%, 67% and 64% higher GSH concentration than the wild type under NaCl, sorbitol and sucrose respectively.

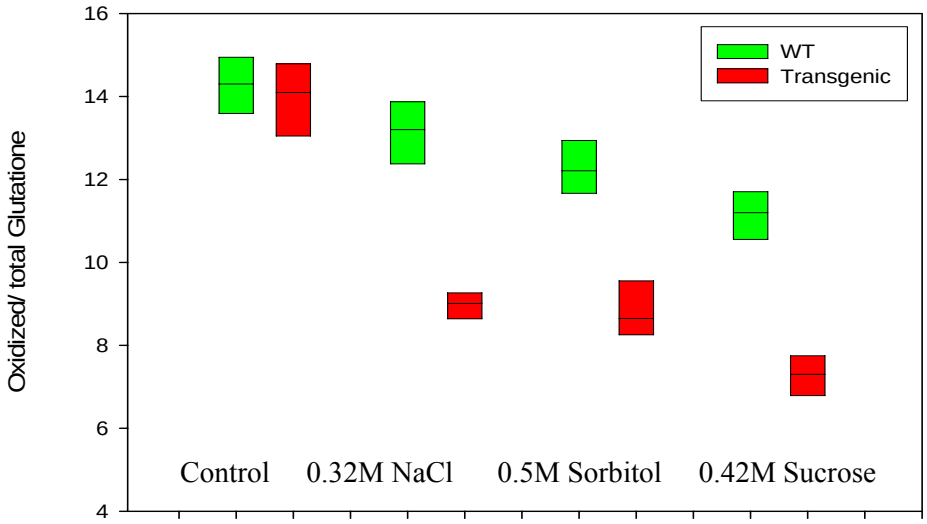


Figure 47: Effects of salt and osmotic stress on percentage of oxidized glutathione from total glutathione in transgenic and non-transgenic cell cultures grown for three days under control and osmotic stress conditions.

3.7. Transformation of *Solanum tuberosum* cv. Desiree plants

To confirm the physiological influence of overexpression of the *sth2* gene on salt and osmotic tolerance also in intact plants, attempts were made for the transformation and regeneration of potato plants. *Agrobacterium* mediated transformation was carried out by a modified leaf disc method (Horsch *et al.,* 1985) using internodes in addition to leaf discs.

Regenerants were obtained after cocultivation and kanamycin selection (Figure 48). Selection of transformants was done also by luciferase activity monitoring of one month old regenerated plantlets (Figure 49 A and B). Furthermore, the transgenic character of mature greenhouse plants representing normal morphology was also confirmed by means of PCR and luciferase activity measurement.

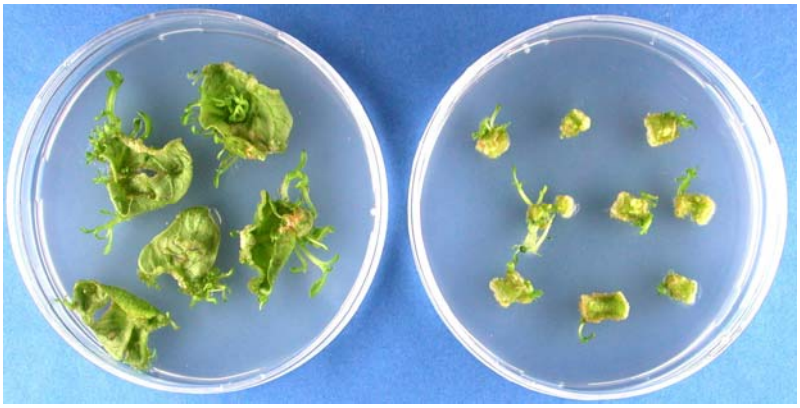


Figure 48: Plant regeneration after cocultivation of leaf discs and internodes.

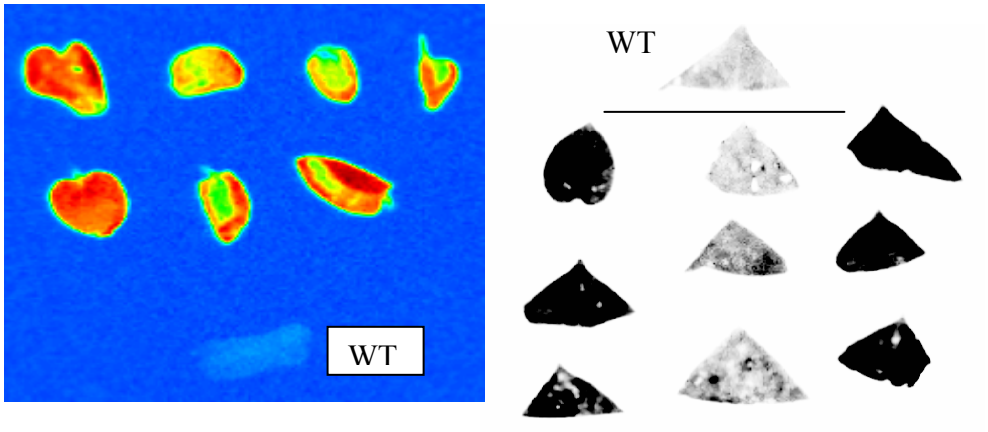


Figure 49: Transformed leaf discs after spraying with Luciferin solution. Chemiluminescence visualized with Fuji LAS 3000 imager (A) color transformation mode (B) normal mode

The regenerated mature transformed plants overexpressing the *sth2* gene were tested for increased salt tolerance. Three weeks old wild type and transgenic plants grown in solid medium, removed from solid medium, washed and soaked in a solution containing half strength MS salts supplemented with 300mM NaCl. Then they were grown on filter paper bridges in test tubes (see Chapter 3.1.4.) for four days in liquid medium containing 300 mM NaCl. The growth of wild type plants was totally suppressed and the leaves exhibited severe necrosis all over the surface whereas salt stress did not affect the growth of transgenic plants

and no necrotic symptoms appeared on the leave surface. Furthermore, cuttings from transgenic and non-transgenic plants were planted in medium containing half strength MS salts supplemented with 200mM NaCl solidified with agar. The reduction of root length as a result of salinity stress was lower in the case of transgenic cuttings whereas no roots emerged from the wild type cuttings. (Figure 50, A and B). These results were consistent with those of potato and tobacco cell suspension and seedlings and strongly suggested that the *sth2* gene plays an important role in salt and osmotic tolerance.

3.8. Molecular characterization of transgenic plants and cell cultures by Southern blot hybridization

The presence of the recombinant *sth2* gene in transformed cell cultures and plants has been confirmed by PCR previously. The degree of transgene expression may be influenced by the number of inserts that have been integrated into the plant genome. Stable integration and copy number of the T-DNA was therefore characterized by Southern blot analysis. An equal amount of XbaI digested genomic DNA from transgenic and non transgenic samples was separated, blotted and finally probed with *sth2* and *luc* amplified and DIG labeled genes.

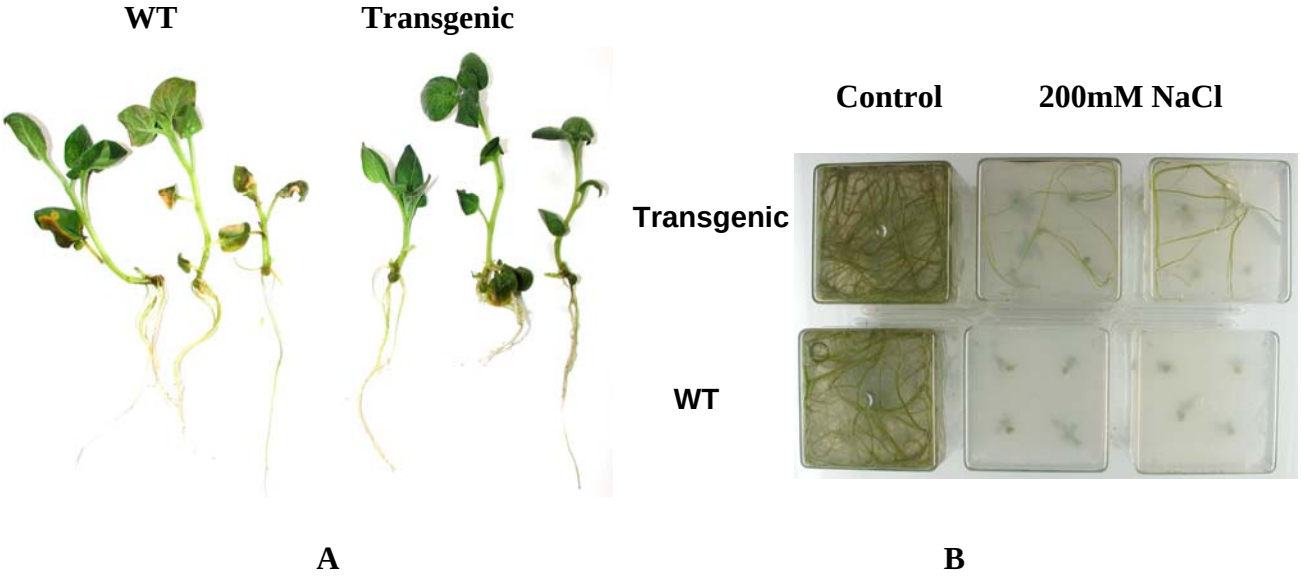


Figure 50: (A) Wild type and transgenic plantlets of Solanum tuberosum cv. Desiree grown in half MSmedium supplemented with 300 mM NaCl. (B) Wild type and transgenic plantlets grown in solid medium supplemented with 200 mM NaCl. Photos were taken from Magenta boxes upside down to demonstrate root development.

Southern hybridization analysis confirmed the integration of T–DNA in the selected cells and plants (Figure 51). Plasmid DNA as positive control generated a hybridization signal at the expected size. The *sth2* and *luc* integration was verified in both putative clones. Two insertions, corresponding to the *sth2* gene and *luc* genes were observed. Suspension cultures have been transformed with a cDNA containing vector pGII 0029 and the plants with the *sth2* gDNA containing vector pGII 0229. For this reason the control plasmid signal appears at different sizes in the gels. Hybridization signals are probably obtained from the sequence of the recombinant as well as from the sequence of the endogenous genes. To discriminate between recombinant and endogenous genes, Southern blot hybridization with a probe from the coexpressed *luc* gene was done in parallel. When bands occur in the blot after hybridization with the *sth2* and the *luc* probe the signals originate from the recombinant genes. As shown in Figure 51, there are hybridization signals in the wild type genomic DNA probed with *sth2* sequence, which are generated from the endogenous *sth2* gene. As a result, hybridization signals that showed variant integration profiles were probably caused by the recombinant gene.

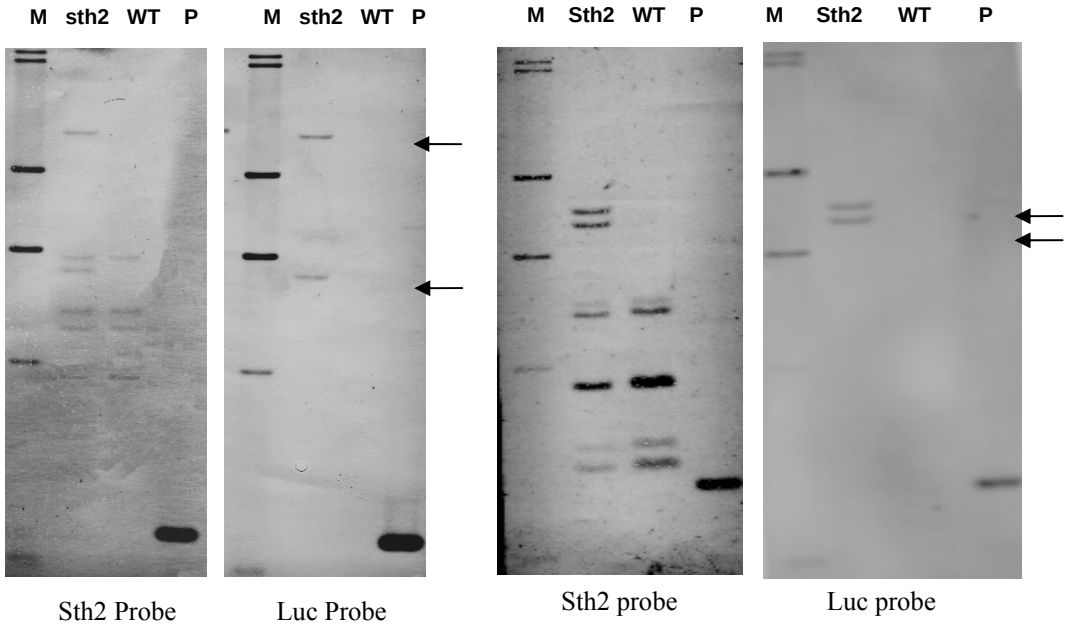


Figure 51: Southern blot analysis of DNA from the transgenic potato suspension culture (A) and transgenic potato plants (B). For hybridization probes for sth2 as well as for luc gene have been used M = DIG-labeled DNA Molecular Weight MarkerII (Roche); sth2 = transgenic clones (suspension or plant) of Solanum tuberosum cv Desiree; WT = non-transformed clones (suspension or plant, p = plasmid DNA.

3.9. Preliminary studies on the cryotolerance of wild type and *sth2* expressing cells

The previous results pointed out that there is strong evidence of the involvement of the *sth2* gene in enhancement of osmotic tolerance, carbohydrate accumulation, proline accumulation and glutathione content in the transgenic cells.

During cryopreservation intracellular ice formation exposes cells to severe osmotic stress by intracellular removal of water. Crystallization includes only water molecules and leads to concentration of intracellular molecules and withdrawal of water bound to cellular components like membranes or molecules like proteins or DNA. An avoidance of ice crystal formation can only be achieved by artificial dehydration of cells leading to the vitrification (solidification without crystallization) of the protoplast. In summary either ice crystal formation or artificial measures to achieve vitrification expose cells to osmotic stress possibly also salt stress. To investigate whether the transgenic cells characterized by an increased osmotic tolerance show also a higher level of cryotolerance, cryopreservation of wild type and transgenic cell cultures was performed according to a cryopreservation method previously worked out at DSMZ for the wild type cell line of potato cultivar ´Desiree´

Cells were subjected to a sorbitol concentration of 0.9 or 1.2 M for dehydration for 2 days, then incubated in a cryoprotector solution and finally cooled down with a programmed cooling rate (-0.25 °C/minute) to -40°C before quick exposure to liquid nitrogen (temperature - 169°C). After thawing, the cells were cultivated on solid 4X medium for recovery after 4 weeks of regrowth photos of recovered wild type and transgenic cells were taken (Figure 52).

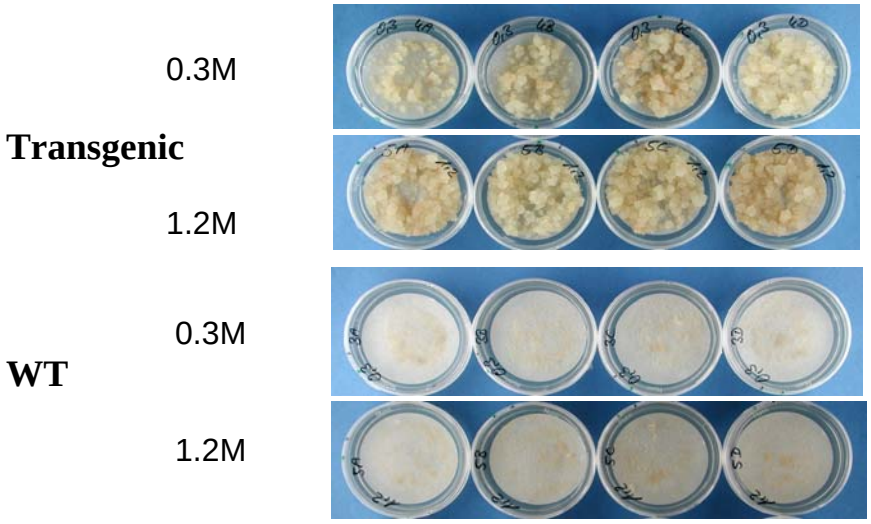


Figure 52: Wild type and transgenic cells of potato cv. ´Desiree´ after 4 weeks of recovery growth from cryopreservation after thawing.

The result clearly shows that the transgenic cells regrow much faster from cryopreservation than corresponding wild type cells. Also luciferase activity in re-growing cells could be measured (Figure 53 A and B).

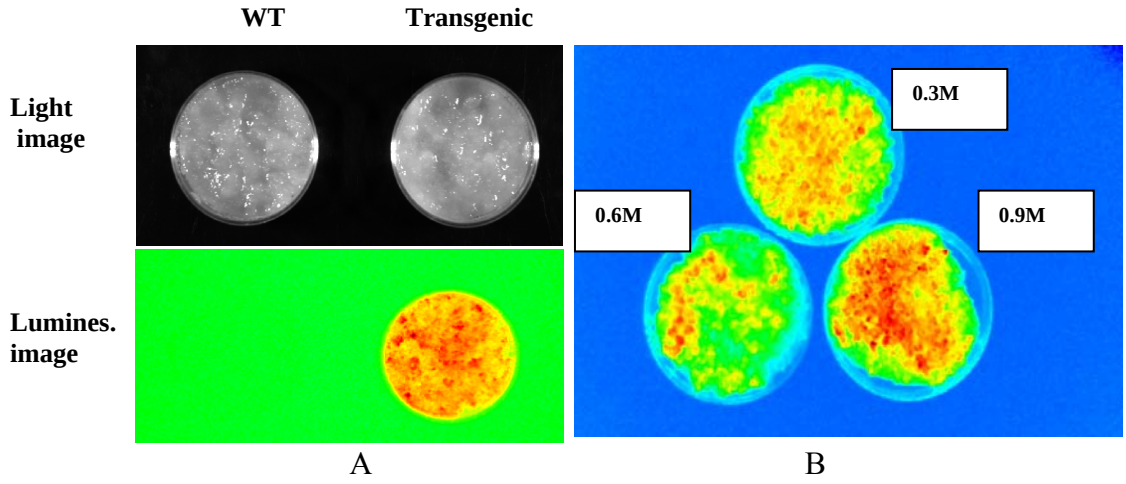


Figure 53: (A) Regrowing cells of a suspension culture of potato cv. ´Desiree´ during regrowth from cryopreservation. (B) Qualitative measurement of luciferase activity. Chemiluminescence was visualized by a Fuji LAS 3000 imager in color shift mode.

Since quatitative luciferase activity can be measured unter sterile conditions, it will be probably possible to analyse whether cells expressing *sth2* at a high level will regrow more efficient than low expressing cells from the same suspension cell line.

4. DISCUSSION

Dehydration stress induced by drought, salinity and /or freezing influences plant productivity and plays a major role in regulating plant species distribution in different types of environments. Furthermore, it causes the most fatal economic losses in agriculture. Nevertheless, plants do not only passively accept the injurious and damaging consequences of salt and drought stress but also developed various biochemical and physiological mechanisms to act against abiotic stresses like salt and drought. The understanding of the physiological and biochemical changes induced by salt and drought stress is essential to develop crop plants with enhanced tolerance to dehydration stress. The aim of our study was to achieve an increase of salt and/or osmotic tolerance in potato based on the overexpression of proteins with a presumed influence on salt and/or osmotic tolerance. Based on the experience of previous studies, cell cultures and *in vitro* plantlets (D.P.P. Sasikala and P.V.D. Prasad 1994, Dobrzanszki *et al.,* 2003) were used to characterize stress mechanism in different potato cultivars and to find suitable targets for overexpression. Finally the use of novel transformation vectors (Dorokhov *et al.,* 2002) should allow a simple monitoring of gene expression for future studies on the mode of action of the overxpressed protein.

In the initial part of this study the influence of various osmotically active substances on the growth characterization of a cell culture of *Solanum tuberosum* cv. ´Desiree´ has been investigated. The rational was that also in the investigations of Sasikala and Prasad (1994) as well as in those of Dobranszki (*et al.,* 2003) the growth characterization under osmotic and salt stress has been used to define the degree of osmotic tolerance. In contrast to the previous work, here suspension cultures were used. In the present study also 3 different osmotically active substances were used instead of only one. These included sucrose as an osmoticum which could be taken up into the cells, the non-penetrating osmoticum sorbitol and the salt NaCl. The major reason for including all three substances was to find - or at least not to miss - any mechanism or effect which may act only on salt but not on osmotic stress or *vice versa*. Another reason to choose sorbitol and sucrose was their use as osmotically active substances in the preculture step for osmotic dehydration prior to the cryopreservation of plant cells.

4.1. Measurement of growth parameter under osmotic and salt stress

Preliminary results obtained with a cell culture of the cultivar ´Desiree´ grown for three days under osmotic stress ranging from 0.5 to 1.2 M medium supplementation with the respective osmotically active substances (Figure 3) showed that the viability of this culture started to decrease under salt and osmotic stress. The fact that salt had the strongest effect at this stage of the study is probably to the use of molar concentrations, which result in a higher osmolarity for the salt. For further studies the different osmotics were applied on the basis of an identical osmolarity. An initial growth curve was measured for the cell culture of the potato cultivar ´Desiree´ under different levels of osmotic stress exerted by sorbitol. Judged from a fresh weight basis increased sorbitol led to delayed growth and decreased viability measured by TTC. On the other hand, under mild osmotic stress (0.25 M sorbitol) dry weight accumulation was even higher than under control conditions. The reason is probably that under mild stress, where cells are still growing the increased osmotic value of the medium leads to a water loss increasing dry weight which compensates for slightly decreased viability. Only at the higher concentration of the osmotics a reduced growth dominates. For further tests a cultivation system was used that has been developed at DSMZ to determine the parameters for controlled rate freezing cryopreservation. The system allows the cultivation of cells in 24multiwell plates. The advantages are the reduction of cell material necessary for testing, the increase of the number of replicate samples and a simple standardization. Before starting physiological tests, the practicability of this system for our study was tested. At first the cell culture of the cultivar ´Desiree´ was used again and stressed with all three substances selected. Two different concentrations resp. osmolarities were chosen. The concentrations applied were much lower than those recommended by Dobranzski *et al.,* (2003) to discriminate between different levels of osmotic tolerance. The reason to use the lower concentrations was to apply test conditions which still assure an active response of the cells to the stress including de novo synthesis of proteins rather than inactivating them. The results obtained for sorbitol resembled that obtained for the classical growth curve measurement. Again measurements obtained for viability and fresh weight accumulation showed convincing results whereas dry weight accumulation especially for the higher stress conditions needs further explanation. For all conditions, the negative influence on fresh weight accumulation, dry weight accumulation and viability was strongest when NaCl was used as osmotic. The mildest effect seems to result from sucrose application.

In this study, the potato cultivars ´Unicopa´, ´Desiree´ and ´Ijsselster´ have been selected for their different cryotolerance. Cryopreservation experiments at DSMZ (H.M. Schumacher, pers. comm.) and at IPK, Gatersleben (J. Keller, pers. comm.) had both shown that ´Unicopa´ had the highest degree of cryotolerance (80 % plant regeneration), ´Desiree´ a medium degree of cryotolerance (55% plant regenertion) and ´Ijsselstr´ the lowest (app. 20 % plant regeneration). Different authors stressed already the close links of osmotic and freezing stress (Martinez *et al.,* 1994, Verslues *et al.,* 2006) since both abiotic stresses decrease the availability of water for the plants respectively cells. It could therefore be expected that the different degrees of cryotolerance were also reflected by different degrees towards salt and/or osmotic stress.

Similar tests as before with the cell culture of cultivar ´Desiree´ was now performed with cell cultures of the three different selected cultivars. The cell culture of the cultivar ´Unicopa´ showed the lowest decrease of viability after exposure to sorbitol as well as salt and thereby the highest tolerance. (Figure 11) This was in accordance with the optical appearance of the cells at harvest (Figure 10). A clear and significant difference of the reaction between the cell lines of the other cultivars could not be demonstrated. This is even truer concerning the measurements of dry weight accumulation. Only the application of salt stress led to a significant difference between cultivars. Nevertheless, from these measurements it can be concluded that the selected parameter concerning the osmotically active substances applied, their concentrations and the duration of exposure of the cells to the osmotic stress are suitable for further testing. From the performed growth tests it could be included that the optimal test conditions for biochemical tests were a period of cell exposure to stressors for 6 days for the lower concentration and 3 days for the higher concentration. After these periods and concentrations of stress exposure most of the physiological processes should be initiated. In a final qualitative test it could be demonstrated that also for plantlets at least similar results were obtained. Among the in vitro plantlets the cultivar ´Unicopa´ showed a higher resistance towards osmotic and salt exposure (Figure 13). From the results previously obtained for cryopreservation at DSMZ and IPK a more pronounced difference of salt and osmotic tolerance has been expected at least between the cultivars ´Desiree´ and ´Ijsselster´. Nevertheless, it has to be taken into account that the results obtained for plant regeneration after cryopreservation is not only due to survival of the freezing and thawing process but also influenced by aspects like meristem activation

4.2. Measurement of biochemical stress markers

Proline is thought to play an important role as an osmoregulatory solute in plants subjected to drought and salt stress (Delauney and Verma, 1993) and in stabilizing cellular structures as well as scavenging free radicals (Hare and Cress 1997; Tripathi and Gauer 2004). Martinez and coworkers (1994) showed that Andean potato species with higher salt tolerance showed also a higher proline accumulation. They also pointed out that a correlation exists between the salt and the frost resistance among the investigated *Solanum* species. The results showed for the suspension cultures of all three potato cultivars an increase after treatment with the different osmotics compared to non treated control cultures. As in the growth experiments the increase of proline content was strongest in the cultivar ´Unicopa´. Proline accumulation in the ´Unicopa´cell culture was strongest after sucrose treatment, in comparison to the cell cultures of the cultivars 'Desiree' and 'Ijsselster' (Figure 14). A similar proline accumulation has been reported in several experimental systems (Gilmour *et al.,* 2000; Nanjo *et al.,*1999 and Sumithra *et al.,* 2006).The significantly higher proline accumulation of ´Unicopa´ cell suspension cultures than in ´Desiree´ and ´Ijsselster´ suggests that ´Unicopa´ possesses a higher potential to tolerate salt and osmotic stress.

Almost all biotic and abiotic stresses lead to oxidative stress by an increase of reactive oxygen species (ROS). For the elimination of ROS the cell has a wide range of compounds like vitamin C, vitamin E, phenolic compounds and glutathione. Glutathione plays a central role in oxidative stress response and is often considered as a marker for oxidative stress although its part in plant metabolism is a multifaceted one (Grill *et al.,* 2001). Glutathione was also measured in this study. After stress exposure the suspension culture of the cultivar ´Uncopa´ showed a decrease in the percentage of oxidized (GSSG) from total glutathione resulting from a constant level of oxidized and an increased level of total glutathione. Also the ´Desiree´cell culture showed a decreased percentage of oxidized glutathione but here also the level of total glutathione was reduced. In both cultures the strongest response was caused by salt treatment. In contrast the ´Ijsselster´ cell culture showed an increase in the content of oxidized glutathione as well as in the percentage of oxidized from total glutathione (Figure 15).At the same time a slightly decreased (salt) or constant level of total glutathione (sorbitol and sucrose treatment) was seen. Also cysteine content as a precursor molecule was measured. While in the cell culture of ´Unicopa´ an increase of total glutathione was accompanied by an

increase in cystein content, the cell cultures of ´Desirre´ and ´Ijsselster showed decreased levels of total glutathione as well as reduced cystein content after salt treatment and only moderate changes under sorbitol and sucrose treatment for both compounds. The measurements indicate that the cell line of ´Unicopa´ has the most balanced response to oxidative stress while the ´Ijsselser´ cell culture seems to face the highest level of oxidative stress (Figure 16). Nevertheless it has to be pointed out that only a time course analysis could clarify the occurrence and response of oxidative stress in the different cell cultures in detail. Also Sumithra *et al.,* 2006 demonstrated that oxidative stress plays a major role in salt stress in *Vigna* cultivars. Furthermore, the reduced forms of the two glutathione precursors, cysteine and γ-glutamylcysteine, and of hydroxymethylglutathione (hmGSH) and glutathione (GSH) were present in greater quantities after PEG treatment in two tolerant genotyes than in the sensitive ones. Osmotic stress also resulted in a higher ratio of the reduced to the oxidized form of the thiol compounds and in higher activity of γ-glutamylcysteine synthetase and glutathione reductase in tolerant genotypes compared to sensitive ones (Kocsy *et al.,*2004). Moreover, in 2004 Ball *et al.,* reported that the expression of 32 stress-responsive genes was influenced by a changed glutathione metabolism. They suggested that glutathione metabolism may play a key role in determining the degree of expression of defense genes controlled by several signaling pathways both before and during stress.

The accumulation of the soluble sugars sucrose, glucose and fructose in many plants is strongly correlated with the acquisition of drought tolerance (Crowe *et al.,* 1990; Vertucci and Farrant 1995; Hoekstra and Buitink 2001). In this study it was also demonstrated that for the cell cultures of all cultivars an increase in soluble sugar content occurred. Under control and sorbitol treatments, the ´Desiree´ suspension culture possesses the highest value of total sugars. The highest increase relative to the control level was found in the ´Unicopa´ suspension after sorbitol treatment (from 5 µmol/g fresh weigth to 12 µmol/g fresh weight). Remarkable is that the suspension culture of the cultivar ´Ijsselster´ showed a completely different ratio between the three sugars than the other cultivars. In the case of ´Unicopa´ and ´Desiree´, sucrose, glucose and fructose occur in almost the same concentrations. In the ´Ijsselster´ cell culture sucrose is present in a higher (4 fold) concentration than glucose and fructose (Figure 17). After osmotic treatment under the specific conditions of this study

mainly the sucrose concentration increases. When sucrose itself was used for osmotic treatment all cultures show a much higher intracellular concentration of all sugars than under sorbitol or salt treatment (data not shown). This is probably not due to intracellular sugar formation but to an uptake of sucrose from the medium and intracellular cleavage. Increased soluble sugar concentrations were also reported by Kerepessi and Galiba (2000). As in our study they reported that both ionic and non-ionic stresses lead to increased concentrations of the reducing sugar sucrose and also that of fructans in wheat seedlings under salt and osmotic stress. Furthermore, Watanabi et al (2000) showed that in vitro plantlets of *Populus euphratica* display tolerance for osmotic and saline stress and concluded that the accumulation of proline and total soluble sugars in leaves is related to osmotic and saline stress tolerance. Nevertheless it has to be pointed out that although sugar accumulation appears to be one of the factors linked to stress tolerance (Al Hakimi *et al.,* 1995; Kameli and Lösel, 1993; Munns and Weir, 1981), other authors have found a decreased or constant level of soluble sugar content (Hanson and Hitz, 1982; Morgan, 1992) during stress conditions.

Under the specific test conditions applied the quantitative and qualitative changes in free amino acid content of the different potato suspension cultures were also analyzed (Figure 18). In stressed suspension cells of ´Unicopa´ the content of free amino acids was increased 0.25 – 2 fold relative to the control. Similar results were reported for other salinity- and drought stressed plants and tissue cultures (Galiba *et al.,* 1989; Fougere *et al.,* 1991; Good and Zaplachinski, 1994; Cano *et al.,* 1996; Gzik, 1996). The ´Unicopa´ cell culture showed an increase of 80% of the assessed free amino acids relative to control, whereas there were fluctuations in the salt and osmotic stress responses in ´Desiree´ and ´Ijsselster´cells. Most of increased amino acids seemed to be linked to glycolytic intermediates or the tricarboxylic acid (TCA) cycle. Glutamate was the only amino acid which showed decreased content under salt and osmotic stress in all tested cell cultures. This result suggests that salt and osmotic stress causes great disturbances for nitrogen assimilation pathways in the potato cell cultures. Among the amino acids analyzed, valin (Val), leucin (Lue), isoleucin (Ile) and alanine (Ala) showed the highest increase during stress exposure in the suspensions of all three potato cultivars, especially under salt stress. This result is in agreement with findings of Rizhsky *et al.,* 2004. They reported a similar increase in the content of free amino acids such as isoleucine, leucine, valine, β-alanine but also tyrosine and isoleucine as response to heat and

drought stress. Kaplan *et al.,* (2004) measured increased proline, cysteine and serine concentrations following cold stress (Kaplan *et al.,* 2004). Alanine is the precursor of alanine-betaine, a quaternary ammonium compound that has similar osmoprotective function as glycine-betaine and accumulates in highly salt tolerant species belonging to the family *Plumbaginaceae*. (Hanson *et al.* ,1994). Under the conditions applied all potato suspension cultures did not show or only a very moderate increase of glycin or alanin content.

The previously described investigations could not give a detailed analysis of the stress response in the potato cell cultures of the different cultivars. It has been pointed out already that for such an analysis a time course of the increase of the different substances and mechanisms would be necessary. Nevertheless, it could be demonstrated that the response of all cell cultures is a concerted action where increased proline content, sugar accumulation, increased amino acid levels and oxidative stress response are included. An important result for the further work in this study is that previous results demonstrated that under the conditions used for measurement (method of cell cultivation, kind of osmotics, concentration of osmotics, duration of treatment) all the mentioned mechanisms are working in an early induced state, while active cell growth and thereby protein synthesis is still active. The same conditions were therefore applied also for the production of stressed cells for proteomic analysis.

4.3. Analysis of changes of the proteome pattern under stress conditions

Many studies on plant responses to abiotic stresses such as salt and osmotic stress have demonstrated the importance of proteomics for the identification of novel stress responsive proteins for subsequent genetic improvement of stress tolerant plants (Dubey&Grover,2001). In this study, we also studied changes in the proteome pattern induced by exposure to salt and osmotic stress. From the previous studies it can be concluded that under the conditions applied induced or up-regulated proteins should be detectable in the cell cultures. These conditions were not so much different from those described by other authors to induce hyperosmotic stress reactions. Liu *et al.,* 2000 and Bongani *et al.,* (2005) demonstrated that 200mM NaCl (iso osmotic to 400mM sorbitol) triggers salt and osmotic stress in *Arabidopsis*. In this study the aim was to perform a detailed analysis of induced proteins as a consequence of salt and osmotic stress. Other authors have already shown that sucrose treatment leads to a

high number of protein pattern changes (Carpentier *et al.,* 2005). The assumption made in the present study was that proteins induced *de novo* provided the most powerful tools for showing an effect after overexpression. Salt and osmotic stress responsive proteins identified in this study were subdivided into three functional categories:

a) Pathogenesis related proteins (PR): by mass spectrometry and data base search two of the strongly induced proteins were identified as STH2 (later termed PR10a) and STH21, belonging to the PR10 protein family. PR proteins have been identified first in biotic stress response and have recently been classified into17 families based on their biological function or similarities in primary structures (Van Loon and Vanstein 1999; Van Loon *et al.,*1994, 2006).

b) Detoxifying enzymes: another prominent spot identified was Benzoquinone reductase. This enzyme diverts quinones from participating in oxidative cycling that can generate reactive oxygen species (ROS) and from depleting intracellular glutathione. Quinones are common secondary metabolites with important roles in energy production, host defense and electron transport (Thomson, 1987). The cytotoxicity of quinones is a consequence result of reactive oxygen intermediates formed during redox cycling between oxidized quinones and reduced phenols (O'Brien, 1991) .

c) Amino acid biosynthesis: Glutamine synthetase enzyme (GS) was also strongly induced upon osmotic and salt stress. Glutamine synthetase is a key enzyme involved in the assimilation of inorganic nitrogen (O'Neal and Joy, 1973; Lea and Miflin, 1974). The enzyme catalyzes the condensation of ammonium with glutamate to yield glutamine. It also plays a major role in regulating proline production consistent with the function of proline (Norbert *et al.,*1995).This result is in agreement with the proline assessments in the investigated cell cultures treated with high salt and osmoticum concentrations.

d) Another protein spot was identified as miraculin-like protein (LeMir). This protein was reported to be associated with biotic stresses, since it was induced by a root-knot nematode. LeMir may have a role in defense against nematodes or other pathogens/pests (Brenner *et al.,* 1998). Also Lang *et al.,* 2005 reported that the LeMir gene may have a potential role in *Citrus unshiu* plants under abiotic stress conditions.

After all, among the protein spots that show strong *de novo* induction upon salt and osmotic treatment, the PR10a (formerly STH2) protein was selected for overexpression. The main reason for this decision was that although a lot of information is available on PR proteins no demonstration of their influence on salt and osmotic tolerance in potato was available and also no clear mode of action defined. Another interesting candidate would have been the miraculin-like protein.

4.4. Overexpression of the PR10a (STH2) protein

The necessary sequence information of the *sth2* gene was obtained from the NCBI database and allowed the design of primers for its amplification from genomic DNA of potato plantlets. The same primers were also used for amplification of a cDNA clone of the *sth2* gene. The genomic *sth2* gene and also the cDNA clone were isolated from potato (Solanum tuberosum cv. ´Desiree´). Since the cultivar ´Desiree´ is one the most widely used potato genotype for molecular studies, it was also selected for the molecular work in this study. For the transformation of cell cultures and plants the *sth2* gene was cloned into new dicistronic transformation vectors developed at DSMZ before (Ali 2007). In this vector system an IRES element from the Tobamo virus was used as a link between the *sth2* gene and the luciferase reporter gene. Both genes were under the control of the mannopine synthase promoter (MAS).This construct was introduced into two different pGreen vectors containing different selectable marker genes, the bar gene conferring phospninothricin and the nptII gene which confers kanamycin resistance. A similar construct was also obtained by inserting the cDNA sequence of the *sth2* gene (Figure 25).

Apart from normal selection for transformed cells by the resistance genes, this dicistronic vector system leads to the coexpression of both genes and thereby allows the monitoring of the expression of the target gene (here *sth2*) by measurement of the reporter gene. In these vectors the first cistron is translated by normal cap dependent ribosome binding. The IRES sequence in front of the second cistron leads to a cap independent ribosome binding and translation. The result is the formation of two physically independent proteins. Physical independence of the two genes should guarantee a normal physiological function of the target

gene, unaltered from possible structural changes which may occur from a fusion of target and reporter gene. The normal function of a transferred recombinant At-nhx1 gene by these transformation vectors had been demonstrated before (Ali 2007). Prior to transformation, the functionality of the constructs (pGreenII 0029 and pGreen 0229) was proven by transient expression using the leaf infiltration assay with *Nicotiana benthamiana* plants (Cazzonelli *et al.,* 2006).

At first tobacco (*Nicotiana tabacum* SR1) as a model system was used for transformation to safe time and effort. Transformed plants and subsequently cell cultures from these plants were easily obtained by standard methods and gene insertion proven by PCR. The proof of integration of the recombinant gene in tobacco was easier than in potato since here no endogenous sequence of the same gene was present. The first proof of an influence of *sth2* gene expression on salt and/or osmotic tolerance could be obtained from germination tests with seeds from T0 plants. Even under salt stress (150 mM) transgenic seeds germinated while wild type seeds failed (Figure 32). This result is consistence with those obtained from *Brassica napus*, where overexpression of another protein belonging to the PR10 protein family (PR10 1) enhanced germination and growth in the presence of 75mM NaCl (Sirvastava *et al.,* 2004).

A further proof of increased salt and osmotic tolerance could be obtained from growth experiments with transgenic calli, which were established easily from the transgenic T0 tobacco plants. The use of cell cultures allowed to acquire results fast but also to standardize experiments easily. The essential advantage of using cell cultures in this specific case was the availability of tobacco cell cultures expressing an At-nhx1 gene conferring increased salt tolerance. These cell cultures had previously been established at DSMZ (Ali 2007). The performed growth test demonstrated clearly that the *sth2* gene expression confers even a higher degree of salt tolerance than the At-nhx1 gene. The remarkable difference was that the *sth2* gene provided salt tolerance over a wider range of inhanced salt levels. The salt tolerance provided by At-nhx1 gene expression ranges only until concentrations of 100 mM. At levels of 200 mM salt At-nhx1 expressing cells died while *sth2* expressing cells still showed further growth (Figure 34). The limit of salt tolerance provided by the mechanism of the At-nhx1 gene in a cell culture system must be given by the saturation of the vacuole with NaCl. The level that the *sth2* gene provides salt tolerance at an even higher concentration or over a longer growth period indicates that it works by a different mechanism than At-nhx1.

At higher salt concentrations cells show also higher levels of luciferase activity (Figure 33). This effect could be explained in two ways, either Cells growing under salt stress are under selection for increased salt tolerance, which leads to the accumulation of the high expressing cells during the growth period.or the MAS promoter is induced by stress (salt and/or osmotic stress). Saito *et al.,* 1991 reported that the gene expression by the dual TR (MAS) promoter is regulated in several plant species in a tissue-specific manner and induced by physiological stresses.

Finally homologous expression of the *sth2* gene in potato was carried out. At first cell cultures were directly transformed. An efficient transformation protocol for suspension cultures developed at DSMZ was successfully used. For transformation the cDNA based construct was used. Integration of the gene proven by PCR leading to two bands one for the recombinant and a bigger one for the endogenous gene representing the full length sequence. Growth tests performed with the transformed cell cultures showed undoubtedly a higher osmotic and salt tolerance of the transgenic cell cultures (Figure 39 and 40). The transgenic cells were able to grow at concentrations of sorbitol or salt where the wild type cells clearly died. This growth occurred over a period of several weeks on solid medium until depletion of the medium. It has to be pointed out that it was possible to select highly expressive cell cultures by the qualitative luciferase assay carried out under sterile conditions. After testing the selection of callus regions showing high expression of luciferase, further cultivation of these callus regions is possible (Figure 36). It could be clearly demonstrated that osmotic treatment also leads to an accumulation of cells with high luciferase expression. Since the demonstrated effect of increased lucifersae activity at high stress levels occurs similarly after selection of transgenic cells by ppt application, probably a selection by exposure to salt and osmotic stress is the reason for the luciferase activity increase. Nevertheless, an additional contribution of an induced transcription of the mannopine synthase promoter cannot be excluded out.

4.5. Biochemical analysis

Interesting results were obtained from comparative biochemical assays done with the transgenic and wild type potato suspension cultures. Although a detailed biochemical analysis of the consequences of gene overexpression on the biochemical network of stress response was not possible due to the time constraints, some tests were carried out. Interestingly the transgenic cells did not show a much stronger response on salt and osmotic stress concerning

an increase of soluble sugar content. But the transgenic cells contained already constitutively much higher sucrose levels and lower levels of sucrose and fructose (Figure 46).
In contrast the glutathione content (% GSSG from total glutathione) is initially the same in wild type and transgenic cell cultures but under stress conditions the transgenic cells show a much lower percentage of oxidized glutathione (GSSG) than the wild type cells (Figure 47). A clear interpretation of the highly complex system of glutathione formation and function is not possible from our very preliminary experiments. Nevertheless, the result may indicate that the recovery of the reduced (antioxidant) form of glutathione is more effective in the transgenic cells. Alternatively the formation of reactive oxygen species under stress conditions could be lower in the transgenic cells and less reduced glutathione oxidized.

An also preliminary comparative study of the proteome pattern under normal growth conditions verified the presence of the PR10a (STH2) protein in the transgenic cells in a constitutive manner. But it also detected a number of other spots which clearly show increased formation in the transgenic cells even without a detailed statistical analysis of the gels (Figure 38).This indicates that secondary effects may also be involved in the enhanced stress tolerance.

4.6. Cryopreservation of transgenic and wild type cells

The transgenic cell cultures were also used for cryopreservation experiments by a controlled rate freezing approach. In controlled rate freezing, the slow ice formation in the surrounding medium leads to solute accumulation in the residual liquid phase and a mild and controlled dehydration of cells and finally to virtrification of the protoplast. Vitrification in this case means the formation of a glass in the cytoplasm of dehydrated cells (Leopold *et al.,* 1994; Buitink *et al.,* 1998). During the controlled rate cooling, cells are therefore also exposed to osmotic stress and may benefit from increased osmotic tolerance. The result was clearly that the transgenic cells showed a higher survival and better regrowth after cryopreservation (Figure 52). Nevertheless, the mode of action remains unclear and final conclusions need a more detailed study. Sugars are a major contributing factor to vitrification. Steponkus (1984) suggested that the accumulation of compatible solutes in the cytoplasm contributes to freezing

survival by reducing the rate and extent of cellular dehydration, by sequestering toxic ions, and/or by protecting macromolecules against dehydration-induced denaturation. But the overexpressed protein may also act more directly. WAP18, a PR-10 protein from mulberry (*Morus bombycis* Koidz.), exhibits in vitro cryoprotective activity against a freeze-labile model enzyme (L-lactate dehydrogenase-LDH), suggesting that some PR10 proteins might function as antifreeze proteins (Ukaji *et al.,* 2004). Moreover, an electrolyte-leakage analysis has revealed a significant correlation between the PR10 protein level and frost hardiness during seasonal cold acclimation (Ekramoddoullah *et al,*. 1995).

The presented results were mainly obtained from work with dedifferentiated heterotrophic cell cultures. An important aspect of salt tolerance is the influence of salt on ion homoeostasis and photosynthesis. It therefore has to be pointed out that our model system cannot mimic all aspects of salt tolerance. Therefore final control experiments should demonstrate that the basic effect of increased salt tolerance occurs also with differentiated plants. Transgenic potato plants were obtained by standard transformation methods using leaf discs and internode cuttings. It was an obvious result that wild type plantlets were affected to a greater extent by the NaCl (300mM) than the transgenic ones (Figure 50). Furthermore, the size of the root system is one of the most important factors involved in the mechanism of drought tolerance in potato cultivars under in vitro conditions (Gluska 1999). Under treatment with 200mM NaCl the transgenic potato cuttings could grew and gave long roots whereas negligible rooting was detected for the wild type plantlets (Figure 50). This result indicates that the overexpression of the PR10a (STH2) protein enhances salt stress tolerance also in intact plants.

Finally Southern blot analysis was carried for the transgenic cell cultures as well as for transgenic plants. The result confirmed not only the integration of the T-DNA but also suggests that two inserted copies of *sth2* gene conferred salinity tolerance to the transgenic potato cells and whole plants (Figure 51). Behnam (2006) reported that salinity tolerance depends on the number of inserted copies of the DREB1A gene in transgenic potato lines and that increasing the DREB1A insert copy number increased the salinity tolerance.

During recent years many researcher focused on the elucidation of the role of pathogenesis related (PR) proteins in biotic and abiotic stress tolerance in plants. PR proteins have recently been classified into17 families based on their biological function or similarities in primary

structures (Van Loon and Vanstein 1999; Van Loon *et al.,*1994, 2006). The PR10 proteins are unique in that they are located in the cytosol and unlike other PR proteins and their function is not yet established. In the present study proteome analysis revealed an induced formation of two members of this family STH2 and STH21 by salt and osmotic stress. The PR10 class proteins are normally induced by pathogen attack in a wide range of plant species including potato (Matton and Brisson 1989), parsley (Somssich *et al.,* 1986), Pea (Barratand Clark1991), sorghum (Lo *et al,.* 1999) and *Medicago truncatula* (Frank *et al.,* 2007) . PR 10 proteins from some sources have been shown to possess ribonuclease activity (Moiseyev*et al.,* 1994), which may be the reason for their antimicrobial and antiviral activity (Park *et al.,* 2004). Another possible function for PR 10 proteins may be in ligand-mediated signaling in response to plant stress. Evidence for this was concluded from the ability of many PR10 proteins to bind ligands including cytokinins, fatty acids, flavonoids (Fujimoto *et al.,* 1998, Mogensen *et al,.* 2002) and brassinosteroids (BRs) (Markovic-Housely *et al.,* 2003). Furthermore, Hashimoto *et al.,*(2004) discovered a novel PR 10 protein that induces a response to abiotic stress in rice plant roots via the jasmonate signaling pathway. Also recombinant expression of PR10 proteins has been carried out.Srivastava el al. (2004) demonstrated that the constitutive expression of a pea PR10 gene in *Brassica napus* enhances their germination and early seedling growth. Kav *et al.,* (2004) employed a proteomics based strategy to characterize changes in the PR10 content in the roots of pea (*Pisum sativum* L.) plants in response to salinity. Similar to our study for potato, their results revealed a significant increase in the level of several members of the PR10 protein family. A comparison between salt tolerant and salt sensitive peanut (*Arachis hypogaea*) callus cell lines (Jain *el al*. 2006) demonstrated that several proteins, identified as PR10 proteins, showed elevated levels in salt tolerant lines. Although there is a strong correlation between the expression of various PR 10 proteins and plant cell responses mainly to biotic stress, their intracellular functions remain unknown.

Summarizing the results of this study, it could be shown in comparative studies with cell cultures of different cultivars that salt and osmotic tolerance is exerted by a number of different biochemical mechanisms in potato. For the first time it was shown that the overexpression of the PR10a (STH2) protein in potato has a remarkable influence on salt and osmotic stress tolerance. It was demonstrated that overexpression of PR10a (STH2) exerts its action by influencing different metabolic pathways and stress mechanisms. How this

influence is exerted in detail cannot be concluded from the present study. Nevertheless the use of dicistronic vectors for overexpression, allowing a simple expression monitoring in potato cell cultures and plants creates an excellent model system for a further detailed analysis of the mode of action and by the way for cryopreservation research.

5. CONCLUSION

The study aimed to develop a strategy to investigate fundamental cellular mechanisms of salt and drought stress mechanisms in potato. Based on previous studies, cell cultures and *in vitro* plantlets were used as model systems (Dobranszki *et al.,* 2003). In comparative studies with cell cultures of three different potato cultivars it was demonstrated that the reaction of potato against osmotic and salt stress is a concerted action of different biochemical mechanisms like proline accumulation, accumulation of soluble sugars, accumulation of amino acids and changes in the levels of the reduced and oxidized forms as well as the total amount of glutathione.

Suitable conditions have been worked out for the comparison of the proteome pattern of potato cell cultures under normal growth and salt and osmotic stress conditions. Several protein spots could be identified by mass spectrometry which were induced under salt and osmotic stress conditions and not at all present in control cells.

Heterologous and homologous overexpression of the gene corresponding to one of the detected induced protein spots, the PR10a protein (formerly STH2), could be achieved in tobacco and potato cell cultures and differentiated plants. It was demonstrated for the first time that overexpression of the PR10a protein in potato leads to a strong increase in osmotic and salt tolerance and by the way increased cryotolerance.

It was further demonstrated for the first time that the overexpression of the PR10a protein in potato exerts its influence on salt and osmotic tolerance by altering different biochemical mechanisms like changed ratio and level of soluble sugars and influencing the protective metabolism against oxidative stress.

Finally, overexpression was achieved using expression vectors allowing a simple expression monitoring by coexpression of two genes leading to physically independent reporter and target gene products thereby creating novel model systems for future research.

6. OUTLOOK

Plant stress tolerance in most cases is achieved by networks of many different physiological and biochemical mechanisms. The present study exemplifies a strategy to elucidate the contribution a single protein may make. It could be demonstrated that proteomics may be used to find key proteins influencing such networks. By their respective over-expression, the different mechanisms, which are affected by such a protein, can be identified.

The use of the novel dicistronic transformation vectors allow a simple monitoring of gene expression by measuring a coexpressed reporter gene. This new method will open new avenues to correlate gene expression which caused a certain physiological effect or influenced by the overexpressed gene.

The novel approach can be specifically important if the overexpressed protein exerts its action by specific aspects of its molecular structure which might be changed by expression of a fusion protein.

Finally the present study demonstrated that the PR10a is a probably a good candidate for achieving salt tolerant either by genetic transformation or as a marker for breeding programs.

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8. Appendix

8.1 Appendix I: Potato and Tobacco in vitro culture medium

MS medium for Potato and tobacco plants *in vitro* propagation.

MS salt +vitamenes (Duchefa)	4.4 g/l
Sucrose	20 g/l
pH	5.6–5.8

8.2. AppendixII: 4X medium for suspension culture and callus induction

B5 basal micro- and macro salts + vitamins	3.16 g/l
Sucrose	20 g/l
Nz-Amin (Caseinhydrolysat)	2 g/l
2,4 D	2 mg/l
NAA	0.5 mg/l
IAA	0.5 mg/l
Kinetin	0.2 mg/l
pH	5.6
Plant Agar (for solid medium).	0.8 %

For transgenic callus or suspension, the same medium was supplemented with

Tic	150 mg/l (after autoclaving)
PPT	5 mg/l(after autoclaving)

8.3 Appendix III: Luria-Bertani (LB) Medium

LB medium (High salt) was used for growth of *E. coli* at 37°C.

Tryptone	10 g (1 %)
Yeast Extract	5 g (0.5 %)
Sodium chloride	10 mg (1 %)

H2O up to 1000 ml

Solified LB medium was prepared by adding 16 g agar/liter. Then the medium was sterilized by autoclaving. Filter-sterilized specific antibiotic was added to agar plates or into liquid medium just before use.

8.4 Appendix IV: YEB medium

Used for growth of *Agrobacterium* at 25-28°C.

Bovine extract	5 gm/l DIFCO
Yeast Extract	1gm/l Roth
Peptone	5 gm/l Roth
Sucrose	5 gm/l
MgSO4	0.493 gm/l

pH was adjusted to 7.0 with NaOH.

The final volume was adjusted to 1 liter.

8.5 Appendix V: SOC Medium

Soc medium was used for transformation of *E. coli and Agobacterium* competent cells to maximize the transformation efficiency.

Tryptone/Peptone from Casein	4.00 g (2%)
Yeast Extract	1.00 g (0.5 %)
Sodium chloride	116.90 mg (10 mM)
Glucose	37.28 g (20 mM)
1 M MgCl2 (sterile filtrated)	2.00 ml (10 mM)
1 M MgSO4 (sterile filtrated)	2.00 ml (10 mM)
H2O up to	200.00 ml

PH was adjusted to 7.5 with NaOH

8.6 Appendix VI: LB for Agrobacterium growth

LB medium (low salt) was used for growth of *agrobacterium* at 28°C.

Tryptone	10 g/l
NaCl	5 g/l
Yeast extracts	5 g/l

H2O up to 1000 ml

Solified LB medium was prepared by adding 16 g agar/liter. Then the medium was sterilized by autoclaving. Filter-sterilized specific antibiotic was added to agar plates or into liquid medium just before use.

8.7. Appendix VII: MMA mediun for leaf infiltration

MS salt	4.6 g /l
Sucrose	20 g
NAA	100 mM.
MES	1.95 g/l
PH, KOH	6.3
*Acetosyrinigone	100 μM

*Acetosyringone (filter sterilized) was added after autoclaving and before use.

8.8 Appendix VIII: MS liquid (cocultivation) Medium

MS salt +Vitamenes (Duchefa)	4.4 g/l
MES	0.25 g/l
pH	5.6 – 5.8

8.9 Appendix IX: Tobacco transformation medium

MS-1 Medium

MS medium	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
NAA	0.5 mg/l
BAP	1.0 mg/l
pH	5.6–5.8 with KOH
Plant Agar.	8.5 g/l

Tic, as required (post autoclave).

8.10 Appendix X: Tobacco transformation medium

MS-2 Medium

MS-salt +vit (Duchefa)	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
BAP	0.2 mg/l
pH	5.6 – 5.8 with 1N KOH
Plant Agar .	8.5 g/l
Tic	as required (post autoclave)
PPT	as required (post autoclave)

8.11.Appendix:XI: Tobacco transformation medium

MS 0 Medium

MS-salt +vit (Duchefa)	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
pH	5.6 – 5.8 with 1N KOH
Plant Agar.	8.5 g/l
Tic	as required (post autoclave)
PPT	as required (post autoclave)

8.13 Appendix XIII: Potato transformation medium

MS for potato callus induction medium

MS macro and micro salt's (Murashige and Skoog, 1962)	3.16 g/l
Sucrose	30 g/l
NAA	5mg/l
BA	0.1mg/l
*Ticarcillin	250 mg/l (Post autoclaving)
*Glucose	1.6% (Post autoclaving)
*Kanamycin	50mg/l
Plant agar	8.5g/l

PH (5.7)

*(filter sterilized) was added after autoclaving and before use.

8.12 Appendix XII: Potato transformation medium

MS for potato plant regeneration medium

MS macro and micro salt's (Murashige and Skoog, 1962)	3.16 g/l
Sucrose	30 g/l
NAA	20µg/l
GA_3	20µg/l
Zeatinribose	2mg/l
*Ticarcillin	250 mg/l (Post autoclaving)
*Glucose	1.6% (Post autoclaving)
*Kanamycin	50mg/l
Plant agar	8.5g/l

PH(5.7)

*(filter sterilized) was added after autoclaving and before use.

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CURRICULUM VITAE

Name : Antar Nasr Salem El-Banna

Date of Birth : 15 October 1969

Sex : Male

Nationality : Egyptian

Marital Statue : Married

Address : Dept. of genetics, Fac. of Agric. Dept.of Genetics ;Fac.of Agriculture; 33516 Kafr El-Sheikh University;EGYPT

Fax: 002-047-3232032

E.mail : antarsalem@yahoo.com

Current position: Assistant Lecturer, Department of Genetics, faculty of Agriculture Kafr El-Sheikh, Tanta University .Egypt.

Qualifications:

- **B.Sc**. June 1992, Tanta University .Egypt Agriculture science (genetics). With final grade, excellent with honour grade.

- **M. Sc**.: May 1998, Tanta Univ. Egypt (Biotechnology)
  M.Sc. Thesis title: The use of gametoclonal and somaclonal variations for releasing salt tolerant rice lines.

- **PhD** : October 2008 , Hanover university(Molecular genetics).
  PhD thesis title: Improvement of osmotic and salt tolerance in potato (Solanum tuberosum L.) by homologous protein overexpression.

Positions:

- Demonstrator, Genetics Dept.1993-1998.
- Assistant lecturer, Genetics Dept.(1998-till date).

Professional Experience:

Assistant in teaching the following courses in genetics Department, Faculty of Agriculture, Tanta University:

1-Genetics 101 (Fundamentals).

2-Genetics 105 (Plant Micotechniques).

3- Genetics 106 (Cytogenetics).

4- Genetics 102 (Cytology).

Professional affiliations:

- Membership in Egyptian journal of GENETICS AND CYTOLOGY.
- Member of Exterior government mission.

List of publications:

2- Abdel–Hamid A.Ali; Galal H.E.; and Naser M.A.and **El-Banna A.S**. (1998). In Vitro selection and somaclonal variation as tools for releasing salt tolerant rice lines. Procedings of the 26th Annual meeting of Genetics Alex. 29-30 sept.1998.

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Posters:

A El Banna, E Heine-Dobbernack1, M Hajirezaei, H Kiesecker and HM Schumacher Investigation of physiological mechanisms of osmotolerance using potato cell cultures SLTP Conference,12-14 September 2007.

A El Banna, E Heine-Dobbernack1, M Hajirezaei, H Kiesecker and HM Schumacher Investigation of physiological mechanisms of osmotolerance using potato cell cultures ICCC 11 Conference, 7-14 October 2007.

STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Signature Date