Polyamines and Calcium Signalling in Drought Tolerance of *Arabidopsis* and Barley

Dissertation

zur Erlangung des
Doktorgrades der Agrarwissenschaften (Dr. agr.)

der

Naturwissenschaftlichen Fakultät III

Agrar- und Ernährungswissenschaften,
Geowissenschaften und Informatik

der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von

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geb. am 10.11.1986 in Rawalpindi, Pakistan

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Halle, den 23. October 2017

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Abbreviations

$[Ca^{2+}]_{cyt}$	Free cytosolic calcium concentration
½ MS	½ concentrated Murashige und Skoog Medium
2,4-D	2,4-dichloro phenoxy acetic acid
ABA	Abscisic Acid
dd. H20	Double distilled water
Amp	Ampicillin
ANOVA	Analysis of variance
bp	Base pair
cDNA	Complementary DNA
cv	*Cultivar*
CO_2	Carbon dioxide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNAse I	Deoxyribonulcease I
dNTPs	Deoxyribonucleotide
E. coli	Escherichia coli
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylenglycol-bis(2-aminoethyl)-tetraacetic acid
et. al.	*et altera*
etc.	et cetera
GAPDH	Glycerinaldehyde-3-phosphate-Dehydrogenase
Hv	*Hordeum vulgare*
kb	Kilo base pair
max.	Maximal
min.	Minimal
min	Minutes
N-terminal	Amino-terminal

OD	Optical density
pH	Potential of hydrogen
PAO	Polyamine oxidase
PAM	Pulse amplitude modulation
PCR	Polymerase chain reaction
Put	Putrescine
RH	Relative humidity
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RNAi	RNA *interference*
ROS	*Reactive oxygen species*
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase-PCR
SD	Standard Deviation
sec	Seconds
SPAD	Soil and Plant Analyzer Development
Spd	Spermidine
spds	Spermidine synthase (Gen)
Spm	Spermine
spms	*Spermine synthase* (Gen)
T0	transgene Generation 0
T1	transgene Generation 1
t*Spms*	Thermospermine
tspms	Thermosperminsynthase (Gen)
Tris	Trishydroxymethylaminomethane
WT	Wild-type

Summary

To provide enough food for an expanding world population, a massive increase in crop production is required to meet the food demands of future generations, while preserving the ecological and energy-related resources of our planet. Agricultural production continues to be limited by a variety of biotic (e.g., pathogens, insects, and weeds) and abiotic (e.g., drought, salinity, cold, frost, and waterlogging) factors that can significantly reduce the quantity and quality of crop yield and are responsible for stunted plant growth. Among the abiotic stresses, drought is one of the major stress factors limiting crop production worldwide. Global temperature has been predicted to rise which will in turn bring a significant change in annual global rainfall and hence, drought incidences will increase. Therefore, drought resistance is a major goal for crop research. There is evidence that polyamines play a pivotal role in abiotic stress tolerance of plants in general and drought tolerance in particular. Common polyamines in plants are putrescine, spermidine, and spermine. Recent studies indicate that the latter is of particular importance for stress tolerance. It was hypothesized that spermine may alter cytosolic free calcium ($[Ca^{2+}]_{cyt}$) and thereby act as an indirect regulator for guard cell K^+ channels. This would in turn affect stress tolerance mechanisms. $[Ca^{2+}]_{cyt}$ is a universal second messenger, also integrated in guard cell signalling networks. The aim of the present research project is to investigate the role of spermine in drought tolerance of barley and *Arabidopsis*. We analyzed the effect of polyamines on stomatal regulation, which is a determinant of drought resistance. A principal aspect of the project is also to scrutinize the involvement of polyamines in Ca^{2+} signalling.

As key regulators of transpiration, stomatal guard cells are principal determinants of crop performance under limited water availability. Thus, the role of polyamines in stomatal regulation is studied. Stomatal bioassays were carried out to determine the involvement of spermine in drought responses. In stomatal bioassays with *Arabidopsis*, the application of polyamines, especially spermine, was found to induce stomatal closure

in a concentration-dependent manner. Chelation of external Ca^{2+} partially affected the spermine-induced stomatal closure. Treatment of entire *Arabidopsis* seedlings with spermine generated a biphasic $[Ca^{2+}]_{cyt}$ elevation. Spermine triggered changes in guard cell $[Ca^{2+}]_{cyt}$ levels by Ca^{2+} influx as well as release of Ca^{2+} from internal stores. A membrane-permeable fluorescent pH indicator revealed that intracellular pH may play a crucial role in the spermine-triggered generation of Ca^{2+} signals. Spermine caused an increase in pH_{cyt}, which may sequentially lead to the activation of Ca^{2+} channels and the increase of $[Ca^{2+}]_{cyt}$ causes an activation of various ion channels which eventually ends up with the closure of stomata.

To investigate the role of spermine in barley, two genes encoding *spermine synthase* proteins were identified. The full-length cDNA sequences were obtained. Transgenic barley lines, i.e. overexpressor and knockdown lines for the *Spermine synthase* genes, were generated. Genetic transformation of barley via *Agrobacterium tumefaciens* was carried out. The respective lines were analyzed genotypically, and lines with highest overexpression or knockdown levels were selected. In overexpressors, an increase in *SPMS* expression and concentration of spermine was noticed at different developmental stages as compared to the wild-type. Phenotypic analyses under non-stress conditions revealed a reduced stomatal conductance in *SPMS* overexpressors and a reduced water loss from excised leaves. In addition, the relative water content and chlorophyll concentration of the leaves were increased. Stomatal bioassay on epidermal strips confirmed the partial closure of stomata in *SPMS*-overexpressing transgenic lines. Drought stress experiments with these transgenic lines showed significantly increased relative water content under extreme stress, going along with increased chlorophyll concentration and PSII efficiency. An improved water conservation was indicated by increased stomatal conductance and excised leaf water loss under those conditions. The altered plant performance by *SPMS* overexpression was confirmed in hydroponics experiments with drought stress simulated by the addition of PEG6000 to the nutrient solution.

Furthermore, transgenic plants were grown to maturity which also indicated their better yield potential under environmental stress. Taken together, this study demonstrated in improving drought tolerance in barley

1 CHAPTER 1

1.1 General Introduction

1.1.1 Drought stress and drought tolerance of plants

Plants are exposed to different abiotic stresses due to certain environmental factors like heat, drought, salinity and light which are responsible for stunted plant growth, development and overall yield reductions (Ahmad *et al.*, 2010). These abiotic stresses are a major factor for the yield losses among crops globally accounting up to 70% and hence are considered as serious problems to agriculture which need to be tackled. Global temperature has been predicted to rise which will, in turn, bring a significant change in annual global rainfall and hence, the drought incidences will increase (OECD, 2012). According to the European Union, the area affected by drought stress has already doubled from 1991-2006 with an estimated yield loss calculated as up to 25%. So, it has been acknowledged as a threat by EU, and use of more drought resistant crops have been called for because environmental predictions estimate drastically reduced irrigated lands and reduced productivity in coming years.

Water is one of the most important factors necessary for proper growth, balanced development and high yield of all crops. Water deficiency affects plant growth and grain yield (Hussain *et al.*, 2004, Wajid *et al.*, 2004). Tillering, booting, and heading is negatively affected by water stress at the early growth stage. Water is the most abundant compound present on the earth´s surface with almost 70% area covered by water (Kuppers *et al.*, 2014), out of which only 2.5% is fresh consumable water in the form of glaciers, snow and aquifers (Gleick and Palaniappan, 2010, Sivakumar, 2011, Farihi *et al.*, 2013). Water shortages are one of the most threatening global problems as around 3 billion people do not have access to either safe drinking water or proper sanitation. The situation is expected to get worse as world’s population is projected to increase by 30% by 2050 with

1/5th of the global population likely to experience a shortage of freshwater in coming decades (Girones *et al.*, 2010, Godfray *et al.*, 2010, Schiermeier, 2014). Freshwater is consumed in large quantities in agriculture with estimates stating more than two-thirds of the total freshwater used (Gan *et al.*, 2013), and it is observed to become critical in various areas around the world due to over usage of irrigation water (Forouzani and Karami, 2010). In coming years, crops with maximum yield potential requiring least water for irrigation will be needed.

Stressful conditions bring different changes in plant mechanisms responsible for coping with such conditions. Drought can be battled with two strategies i.e. either escaping drought or resistance against drought. In physiological terms, *drought resistance* can be further classified into *drought avoidance* and *drought tolerance* (Price *et al.*, 2002) as shown in Figure 1. A plant adapts a strategy where it completes its life cycle before conditions become extreme to escape the drought conditions by shortening the flowering and maturity. Another strategy where plants develop a drought resistance against the stress environment is by avoiding the stress by attaining higher water potentials through longer roots, lower transpiration rate and reduction in leaf area under drought conditions. Resistance against drought can also be achieved by activating tolerance response of the plants under lower water potential by accumulating solutes and protective proteins to maintain turgor and help the plant to survive (Liu *et al.*, 2007, Ahmad *et al.*, 2012). Molecules known as ‘polyamines (PAs),' have also been known to be an integral part of plant stress responses (Bouchereau *et al.*, 1999, Walters, 2003, Alcazar *et al.*, 2006b). Polyamines (PAs) are low molecular weight ubiquitous nitrogenous compounds found in all living organisms (Kaur-Sawhney *et al.*, 2003). It has been observed that higher plants experience an increase in polyamines levels when abiotic stress is applied. Polyamines synthesis pathway has been reported to interact with different signaling molecules which are required for responses to abiotic stresses such as NO, hydrogen peroxide and ethylene (Alcazar *et al.*, 2010). Various types of abiotic stresses regulate genes involved in polyamines metabolism which

indicates the role of polyamines in abiotic stress signaling (Marco *et al.*, 2011).

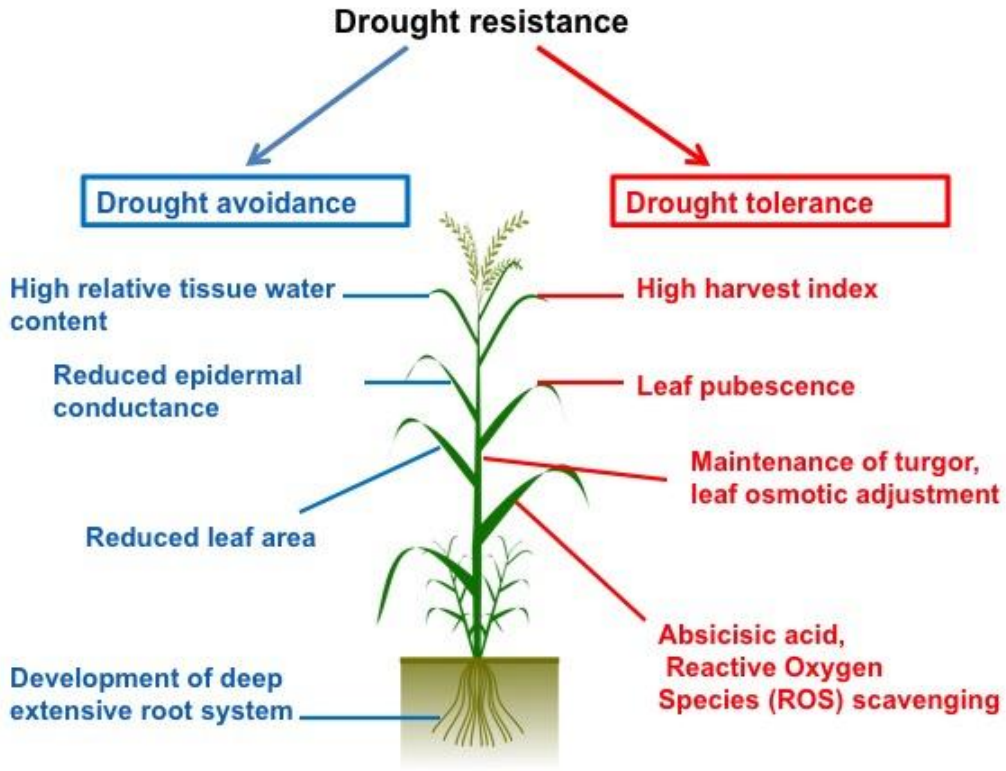


Fig. 1: Key traits associated with drought resistance in plants (Price *et al.*, 2002)

1.1.2 Polyamines and role in stress resistance

Polyamines, are vital for metabolic processes such as growth and development and flowering as well as responses to stress environment (Galston *et al.*, 1996, Kumar *et al.*, 1997, Tiburcio *et al.*, 1997).

Polyamines are known as aliphatic amines, namely putrescine, spermidine, and spermine , which can be found in quantifiable amounts. Polyamines occur in various soluble and insoluble forms due to being polycationic in nature (Childs *et al.*, 2003). Soluble forms of polyaminess consist of free polyamines and conjugated polyamine forms which are bound to phenolic acids, such as hydroxycinnamic acid. Insoluble forms of polyamines are linked to proteins, nucleic acids and phospholipids (Martin-Tanguy, 2001). Availability of polyamines in the cell varies with the development stage of plants and with plant species.In general terms, the

levels of putrescine in plants are higher than those of the other polyamines (Cohen, 1998).

The biosynthetic pathway and key enzymes of the polyamine metabolism are well documented (Tiburcio *et al.*, 2014). Briefly, putrescine is synthesized by the decarboxylation of ornithine, catalyzed by ornithine decarboxylase, or indirectly by the decarboxylation of arginine by arginine decarboxylase (ADC), via agmatine. Higher polyamines (spermidine and spermine or thermospermine) are produced by the sequential addition of aminopropyl moieties to the putrescine skeleton through enzymatic reactions catalyzed by the spermidine and spermine/thermospermine synthases (*SPDS* and *SPMS*/T*SPMS*) (Fig. 2). The donor of the aminopropyl groups is decarboxylated S-adenosyl-methionine, which is synthesized from S-adenosyl-methionine by S-adenosyl-methionine decarboxylase (SAMDC). Polyamines are catabolized by diamine oxidases (DAO) and polyamine oxidases (PAOs).

It has also been reported that polyamine levels increase when plants are exposed to environmental stress. Plants show higher tolerance levels when producing a greater level of polyamines by overexpressing genes encoding for enzymes involved in polyamine biosynthesis (Kasukabe *et al.*, 2004, Alcazar *et al.*, 2011, Fariduddin *et al.*, 2013, Minocha *et al.*, 2014).

The aliphatic polycationic compounds collectively known as polyamines have been described to play a significant role in multiple mechanisms in a plant cell i.e. gene expression and replication, growth, senescence, and response to biotic and abiotic stresses (Galston *et al.*, 1996, Zapata *et al.*, 2008). Polyamines are important growth regulators, but we still do not know their precise physiological function and mechanism of action.

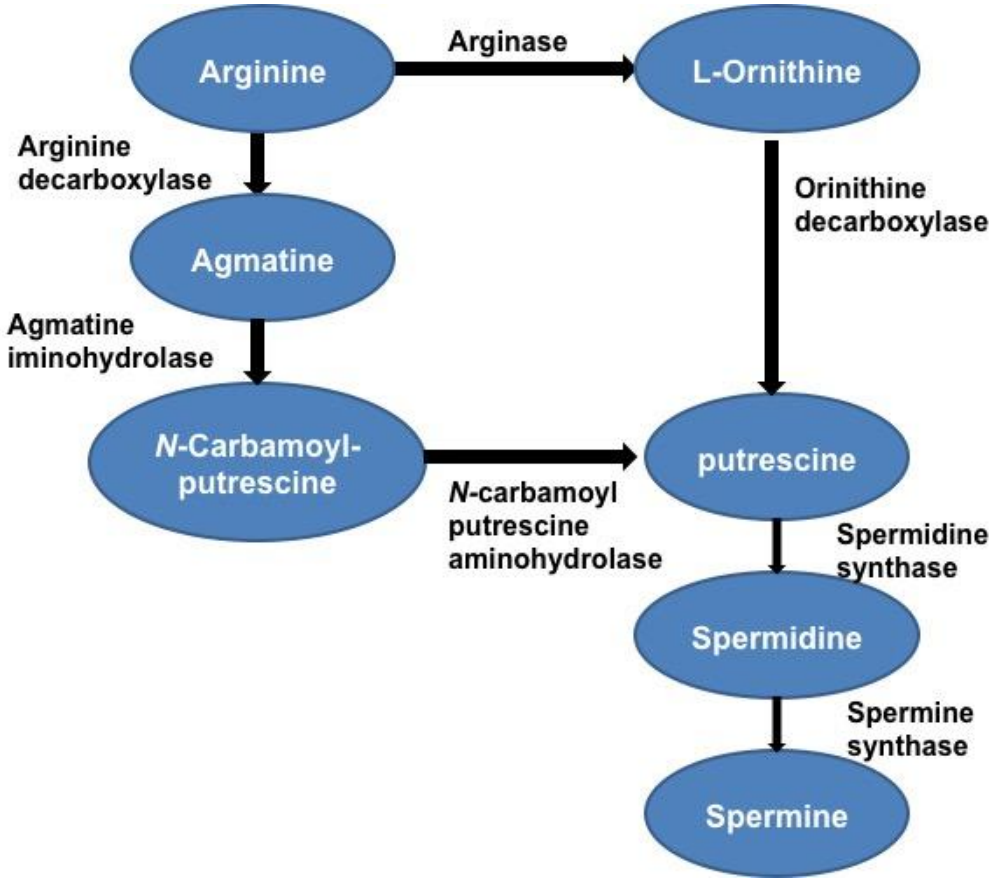


Fig. 2: Biosynthetic pathway of polyamines in plants

1.1.3 *Arabidopsis thaliana*: A model for polyamine research

A. thaliana has been selected as model plant species based on different characteristics, i.e. small size (phenotypically and genotypically), short generation time, good transformation efficiency, insertional mutant availability, expressed sequence tag (EST) libraries, microarrays, and a sequenced genome. *A. thaliana* is a model plant used for research purposes all over the world especially polyamine-related research due to some interesting facts. The *A. thaliana* genome lacks ornithine decarboxylase (ODC) pathway genes (Hanfrey *et al.*, 2001). Single gene copy of agmatine aminohydrolase (AIH) and N-carbamoyl putrescine amidohydrolase (CPA), were found (Janowitz *et al.*, 2003, Piotrowski *et al.*, 2003). Spermidine synthases (*SPDS*) are encoded by two genes, namely *SPDS*1 and *SPDS*2, whereas only one gene is found to encode *spermine synthase* (*SPMS*) (Alcazar *et al.*, 2006b).

ACL5 and *SPMS* genes were believed to encode Spermine Synthases for a long time, and *acl5* mutants were having reduced stem size (Hanzawa *et al.*, 2000). Recent studies have shown that conversion of spermidine to thermospermine was catalyzed by ACL5 recombinant protein instead of spermine indicating *ACL5* codes for Thermospermine Synthase and not for *spermine synthase* (Knott *et al.*, 2007) which is also indicated by *acl5* mutants showing no reduction in spermine content. Furthermore, thermospermine synthase activity was also detected in *A. thaliana* plants (Kakehi *et al.*, 2008). It was then reported that spermine is not essential for cell survival as double mutation of *ACL5* and *SPMS* does not affect the viability of the plant, whereas decreasing putrescine or spermidine levels make plants less viable (Imai et al. 2004; Urano et al. 2005). However, double mutant was shown to be sensitive to stress responses indicating the role of spermine in stress tolerance of the plant (Yamaguchi *et al.*, 2006a).

1.1.4 Drought stress in barley

Barley (*Hordeum vulgare* L.) is one of the oldest crops, which dates back to the old world agriculture and was one of the first domesticated cereals. Barley is an annual grain crop which is also used as a cover crop to help improve soil fertility. It is one of the fast growing annual plants which require cool season for its development and hence can be used for forage purposes as well (Ghanbari *et al.*, 2012). It is also the fourth largest crop in the world following wheat, maize, and rice with its annual production being around 136 million tons (Akar *et al.*, 2004). It has been used over time as human food, in animal feed, and mainly for brewing malts. Lowering of blood cholesterol with β-glucans (Behall *et al.*, 2004) has been known by utilizing barley in human food. Due to its short life cycle and morphological, physiological and genetic characteristics, it has been a model experimental system for research studies.

Barley belongs to the genus Hordeum in the tribe Triticeae of the grass family, Poaceae (also known as Gramineae). This tribe is a temperate plant group known to contain about 350 wild species along with several

major bowls of cereals and forages. The genus *Hordeum* includes both annual and perennial species which makes it atypical among the Triticeae (Bothmer *et al.*, 1992). Hordeum has a basic chromosome number of x=7. *Hordeum vulgare* L. ssp. *vulgare*, and its wild parent are diploid in nature, i.e. 2n=2x=14 chromosomes. There are other Hordeum species which are diploid, tetraploid (2n=4x=28) and even hexaploid (2n=6x=42) (Komatsuda *et al.*, 1999).

Drought stress is the most severe yield-limiting factor for crop production and hazardous to global food security; increased frequency of drought periods due to climatic changes require the design of better-adapted genotypes and drought-tolerant plant cultivars. Despite many efforts, the progress of conventional breeding to increase drought tolerance has been limited, likely due to the complexity of these traits. A thorough physiological and molecular understanding of the processes is thus required to identify critical processes and players, which then can be targeted individually by marker-assisted breeding or transgene technologies. Drought has been a major abiotic stress for barley due to its growth regions. It is usually grown in limiting-water environments which make barley the plant with the best water use efficiency compared to other cereal crops and thus it can survive limited irrigation (Fisheries, 2007).

1.1.5 Aim of the thesis

The objective of the research presented in this thesis was to get more detailed insight into the role of polyamines, especially spermine, in the drought tolerance of plants, mainly focusing on a monocotyledonous crop species (barley) and a dicotyledonous species (*Arabidopsis*) as a model plant, as introduced in **Chapter 1**. Polyamines, in particular, spermine, have been shown to improve tolerance and recovery from drought (Peremarti *et al.*, 2009). However, the mechanism of action of spermine is yet unknown. So, for that purpose, as part of the project, I will investigate the crosstalk between polyamines and Ca^{2+} signals in barley. To achieve that goal I studied the role of spermine in drought stress tolerance and found that Arabidopsis is perfect for this research as only one gene encodes for *SPMS*.

Chapter 2 describes the cross-talk of polyamine and Ca^{2+} signals in drought responses of Arabidopsis. Cytosolic free calcium ($[Ca^{2+}]_{cyt}$) is a central regulator of plant responses to stress, including drought. Plant lines transformed with luminescent and fluorescent Ca^{2+} reporters were available. Plants were analyzed for alterations in Ca^{2+} signals. Aequorin-expressing mutants for genes that may be involved in the Ca^{2+} response (e.g., TPC1) were also analyzed at the whole plant level. Stomatal assays were performed to understand the role of polyamines and their way of action in plants. Application of polyamines, especially spermine, induced the closure of stomata. We concluded that Ca^{2+} release from the vacuole plays a role in the spermine response, in addition to the Ca^{2+} influx. Intracellular pH may also play a part in the generation of spermine-triggered Ca^{2+} signals which ultimately leads to stomatal closure.

Chapter 3 deals with reverse genetic analysis approach to study the role of spermine in barley. *SPMS* homologs were identified and barley overexpressor and knockdown lines for those genes, i.e. *SPMS1* and *2* (*Spermine synthase1 and 2*), were generated in a previous project (Nancy Nowak, unpublished). The lines were screened by PCR, real-time PCR, Southern blotting, and HPLC analysis. Somatal assays were performed to

determine the stomatal response. Genotypic and phenotypic differences were found out during different develpmental stages in transgenic lines.

Chapter 4 describes the drought stress responses of transgenic lines. Drought experiments were done in soil with transgenic overexpressor lines to study physiological parameters. Hydroponics experiment was done with overexpressor and knowckdown transgenic lines to study responses to osmotic stress. Post-harvest parameters of transgenic plants were also analyzed. Water conservation under drought stress in transgenic overexpressor lines was determined, along with an increased PSII efficiency and stomatal conductance

Chapter 5 discusses the main findings of this thesis and the future perspectives of how the knowledge generated in this thesis can contribute to the improvement of drought tolerance.

2 CHAPTER 2

Cross-talk of polyamines and calcium signaling in drought stress response of *Arabidopsis*

2.1 Introduction

2.1.1 Generation of cytosolic calcium signals under stress conditions

Drought stress has an osmotic component due to decreased soil water availability. The plant responds to this challenge by regulating stomatal apertures, synthesizing compatible osmolytes, and increasing the capacity of ion uptake. The initiation of drought tolerance mechanisms is controlled by an intricate signaling network involving the plant hormone abscisic acid (ABA) and various second messengers. Upon perception of the stress, one of the first responses is a transient elevation of cytosolic free calcium (Ca^{2+}_{cyt}) (Knight *et al.*, 1997, Apse and Blumwald, 2007).

Calcium constitutes up to 5% of the total dry weight of the plant; however, it is present in only small quantities as cytosolic free ion, which concentrations usually being below 1 µM (White and Broadley, 2003). Large portions of the soluble Ca^{2+} are stored in different organelles of the cell, such as vacuoles. Plant cells contain large vacuoles, which can make up >90% of the cell volume and act as a store and buffer for cations (e.g., K^+, Mg^{2+}, Ca^{2+}). Many studies have shown that $[Ca^{2+}]_{cyt}$ elevations frequently originate from the vacuole and other internal stores (Peiter, 2011). Calcium is also present in the cell wall and in many anionic macromolecules. Large calcium stores, like vacuoles, have Ca^{2+} channels which are vital for Ca^{2+} homeostasis and Ca^{2+}-mediated signal transduction, making Ca^{2+} function as a second messenger in intracellular signals transduction (Bush, 1995, Hetherington and Brownlee, 2004).

A gene for a vacuolar Ca^{2+}-permeable channel, *TPC1,* has been identified (Peiter *et al.*, 2005). This channel has been known from classic electrophysiological studies as the Slow Vacuolar (SV) channel (Hedrich and Neher, 1987), but, due to its obscure molecular identity, role and regulation of this channel were a matter of speculation. In particular, the ability of the SV channel to release Ca^{2+} into the cytosol has been discussed controversially. The identification of TPC1 as a part of Ca^{2+}-dependent signaling networks (Peiter *et al.*, 2005), as well as more recent electrophysiological studies (Pottosin and Schonknecht, 2007), have now firmly established a role of TPC1 in Ca^{2+} signaling. However, it is unknown at present whether TPC1 plays a role in Na^{+}- or drought-specific Ca^{2+} responses and whether the role of TPC1 differs between dicots and monocots. Hence, our understanding of factors responsible for the generation of Ca^{2+} signals is sparse.

Polyamines also act as molecular chaperones by binding to negative surfaces, hence protecting proteins, membranes and nucleic acids which makes them unique polyvalent cationic metabolites (Rhee *et al.*, 2007, Igarashi and Kashiwagi, 2010). Polyamines also acts as free radical scavenger and generator of reactive oxygen species (ROS) as polyamines break down to release H_2O_2 (Ha *et al.*, 1998) which is responsible for the increase in cytosolic Ca^{2+} leading to the activation of subsequent signals responsible for adaptive responses to stress (Yoda *et al.*, 2006, Rodriguez *et al.*, 2009, Tisi *et al.*, 2011).

Polyamines are known to block cation transport by acting as substrate analogs due to their polycationic nature. In plants, polyamines block and inhibit voltage-dependent fast and slow vacuolar channels (Bruggemann *et al.*, 1999, Dobrovinskaya *et al.*, 1999). Polyamines can block these channels from either membrane side. Polyamines can not only block but also permeate some channels like ryanodine and acetylcholine receptor channels (Uehara *et al.*, 1996, Haghighi and Cooper, 1998).

2.1.2 Aequorin

Aequorin is a Ca^{2+}-sensitive photoprotein naturally produced by the jellyfish *Aequorea victoria* (Tsuji *et al.*, 1986, Cormier *et al.*, 1989). It is known as the first Ca^{2+}-sensitive photoprotein which is composed of apoaequorin, bound oxygen, and a prosthetic group, a luciferin molecule, coelenterazine. Apoaequorin is found in several forms which emit light when bound to Ca^{2+}. This ability enables apoaequorin to be used for the measurement of Ca^{2+} in the cell. The formation and reaction of aequorin are shown in Figure 3. Coelenterazine is a membrane-permeable molecule which is also hydrophobic and non-cytotoxic. It has a dihydropyrazinimidazolone ring structure. Oxygen binds to apoprotein and coelenterazine to stabilize the complex (Musicki *et al.*, 1986). Apoprotein contains three EF-hand Ca^{2+}-binding sites which bind to Ca^{2+}. Upon binding, oxygenase activity takes place which changes aequorin structure and converts coelenterazine into excited coelenteramide, and carbon dioxide is released as shown in Figure 3. Blue light is emitted when excited coelenteramide is relaxed to its original ground state (Mithofer *et al.*, 2009). High levels of aequorin synthesis are obtained by transforming the *Apoaequorin* gene with a strong promoter in the target organism. The expressed apoprotein generates aequorin in the cells by incubating cells with coelenterazine and contributes to measuring significant amounts of Ca^{2+}.

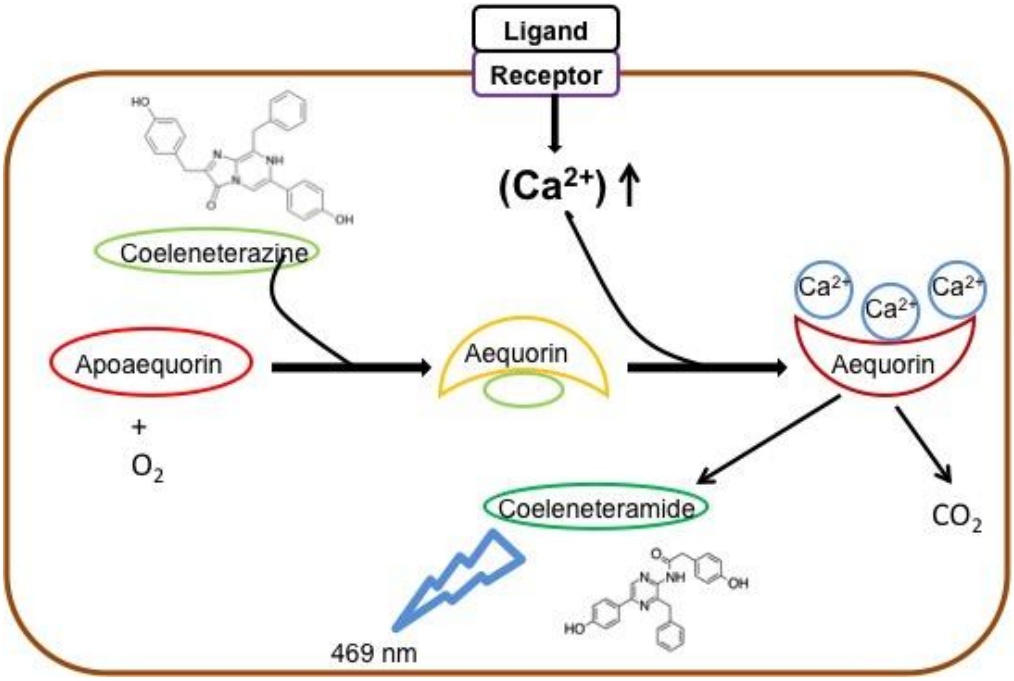


Fig. 3: Mechanism of light emission by AEQ upon Ca^{2+}-binding

2.1.3 Regulation of stomatal closure under drought stress

Higher plant leaves contain cells which are located within the epidermal cell layer known as guard cells. These cells control highly complex signaling and ion transport pathways which are activated by different parameters i.e. light, water, CO_2 and hence, determine the opening and closing of the stomata (Muller-Rober *et al.*, 1998). These guard cells support the regulation of different gas exchanges which helps in overall plant performance.

Stomata are responsible for the exchange of gasses in leaves; stomatal opening and closing has been mainly reported due to changes in K^+ levels in the guard cells. It is widely known that K^+ concentrations are increased during stomatal opening, and similarly, during stomatal closure, K^+ levels are decreased, which is linked to efflux from vacuole and cytosol (MacRobbie, 1988). Opening and closing of stomata are usually induced by changes in the level of CO_2, humidity, light conditions (Mansfield *et al.*, 1990), and ABA (Mittelheuser and Van Steveninck, 1969).

Ca^{2+} increase in the cytosol, which has been reported to happen earlier than K^+ efflux via plasma membrane, is said to be a precursor to stomatal

closure. It has been proposed for Ca^{2+} to play a role in closing when an increase in Ca^{2+} levels is observed (Gilroy *et al.*, 1990, McAinsh *et al.*, 1990, Gilroy *et al.*, 1991).

Abscisic acid (ABA) is one of the "classical" plant hormones, i.e. discovered at least 50 years ago, that regulates many aspects of plant growth and development. ABA treatment has been reported to be responsible for these increased $[Ca^{2+}]_{cyt}$ levels when increased spike patterns were observed. Non-selective Ca^{2+} permeable channels are activated in response to ABA which allows $[Ca^{2+}]_{cyt}$ levels to increase in the cell (Schroeder and Hagiwara, 1990).

ABA increases pH in maize and parsley cells (Gehring *et al.*, 1990) which points to a role of pH in stomatal closure. Acidification of guard cell cytosol was observed before stomatal opening whereas alkalinization of guard cell cytosol was measured before stomatal closure in response to ABA. This coupled with an increase in $[Ca^{2+}]_{cyt}$ exhibits role of pH in guard cell movement.

2.1.4 pH as a stress signal

Intracellular pH and its homeostasis are critical for the survival, metabolism, stability and ion channel activity of all cells. Different compartmental membrane trafficking events strongly show the need for pH balance (Casey *et al.*, 2010).

In the vacuole, an acidic pH is necessary for many proteolytic enzyme activities and is also required for the transport of sucrose and other solutes (Dettmer *et al.*, 2006, Muntz, 2007). Moreover, homeostasis of pH is essential for ATP synthesis in chloroplasts and mitochondria, posttranslational processing of different proteins in the secretory pathway, and maintaining the morphology and function of Golgi (Paroutis *et al.*, 2004, Schumacher and Krebs, 2010, Viotti *et al.*, 2010).

Alterations in intracellular pH can disrupt many intracellular events, which makes it very important to find ways to determine the cytosolic pH in order

to understand its effect on the function of cellular organelles and intracellular processes (Roos and Boron, 1981) like cell growth, proliferation, and differentiation.

Different pH-sensitive fluorescent dyes are used to assess the pH in living organisms. Apoplastic pH is determined by using dextran-conjugated forms of fluorescein isothiocyanate (FITC) (Hoffmann and Kosegarten, 1995) or Oregon Green (Hoffmann and Kosegarten, 1995) (Geilfus and Muhling, 2011). Cytosolic and vacuolar pH have been determined by using 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxy fluorescein (BCECF) and 5-(6)-carboxy seminaphthorhodafluor (SNARF) (Gehring *et al.*, 1997, Krebs *et al.*, 2010).

The BCECF dye can easily enter the cells with the help of acetoxymethyl (AM) ester coupling. This helps the dye which is hydrophilic in nature, to enter the cells through the plasma membrane. In the cytosol the acetoxymethyl ester is broken down with the aid of different esterases. This free form of the dye which is then present in the cytosol is retained in the cell. The pKa of BCECF is 6.97, which makes it suitable for measuring physiological changes in pH_i. The ratio of emission at the two excitation wavelengths of 490 and 440 nm, which is utilized for the determination of pH. This ratiometric method is very accurate and makes up for variable dye loading, cell thickness, dye leakage, and detector sensitivity (Bright *et al.*, 1989).

2.2 Materials and Methods

2.2.1 The effect of polyamines on stomata in Arabidopsis

2.2.1.1 Establishment of stomatal bioassays

Seeds of *A. thaliana* ecotype Columbia-0 (Col-0) were sown in a mixture of soil and vermiculite in 2:1 ratio. BIOMUKK was added as an insecticide. Plants were kept in a growth chamber at 22°C, 12 h photoperiod (light intensity 100-150 µmol $m^{-2}s^{-1}$) and 80% relative humidity. Plants were watered from the bottom.

Fully expanded leaves of 3-4 week-old plants were were chosen for stomatal assays. Epidermal strips were manually isolated with a razor blade and forceps. To open stomata, the isolated epidermis was floated for two hours in incubation buffer which contained 10 mM MES-KOH, 50 mM KCl, and 50 µM $CaCl_2$. After two hours of incubation, 10 µM of polyamines (putrescine, spermidine, or spermine) were added. Incubation conditions were lept similar to the one used for plant growth. Measuring of the stomatal aperture was done under a Zeiss Inverse microscope (Axiovert 40 CFL, AxioCAM).

2.2.1.2 Stomatal assay with calcium chelator

To assess the involvement of Ca^{2+} in the stomatal closure, *tpc1-2* mutant Arabidopsis plants were selected. Spermine was selected as a treatment after having been identified to exhibit the maximum response of stomata. Ca^{2+} chelator EGTA was used to bind extracellular Ca^{2+}.

Seeds of Col-0 and *tpc1-2* mutant plants were sown in a mixture of soil and vermiculite (2:1) with supplementation of BIOMUKK. Growth conditions were the same as mentioned in 2.2.1.1.

The stomatal assay was done with 3-4 week-old isolated epidermal strips which were put in the incubation buffer as in (2.2.1.1) for two hours for the opening of stomata. As a treatment, spermine (10µM) and spermine

(10µM) + EGTA (10mM) was applied. Strips in Petri dishes were incubated for two hours. The response of stomata was checked under a microscope where 35-40 apertures of stomata were measured in 30 min.

In addition to EGTA, BAPTA, which is known to be less pH sensitive, was used as Ca^{2+} chelator. BAPTA was added in the concentration of 100 µM along with spermine (10µM). Isolated epidermal strips obtained from the previous experiment were used.

2.2.1.3 Statistical analysis

Statistical analysis was done using ANOVA. One sample paired t-test was used to determine the differences between different treatments. All statistical analyses were performed in Statistix Version 8.1 (Analytical Software).

2.2.2 Polyamine effects on calcium signals in *Arabidopsis*

2.2.2.1 $[Ca^{2+}]_{cyt}$ response of the Col-0 and *tpc1-2* mutant to polyamines

All analyses were performed with *A. thaliana* ecotype Col-0 constitutively expressing *APOAEQUORIN* under the control of the cauliflower mosaic virus 35S promoter (Knight *et al.* 1991). *S*eeds were surface-sterilized by washing two times with 70% ethanol followed by one time with 100% ethanol. Thereafter they were dried under the sterile hood. Seeds were sown in 24 well plates with half-strength MS (Duchefa, Harlem, the Netherlands) supplemented with 0.25% (w/v) sucrose (adjusted to pH 5.8 with KOH). The plates were closed with parafilm, wrapped with aluminum foil, and seeds were stratified for two days at 4°C. The plates were then transferred to a Percival growth cabinet and grown under full spectrum fluorescent lights on a 16 h/8 h day/night regime and at a constant temperature of 22°C, with a photon flux density of 100-150 µM m^{-2} s^{-1} for 5-7 days.

One day before the experiment, seedlings were carefully transferred into a Petri dish containing 3 ml ½ MS containing 10 µM coelenterazine by using a pointed forceps. The Petri dish was wrapped with parafilm and incubated overnight in darkness at room temperature to reconstitute the aequorin. Coelenterazine was prepared from stock.

On the following day, seedlings were transferred to luminometer tubes containing ½ 500 µl MS medium. Plants were recovered from transfer for two hours; afterwards they were used for measurement.

Before $[Ca^{2+}]_{cyt}$ measurements, tubes were transferred to the chamber of a Sirius-1 luminometer for 4-5 min for baseline recording. 1 mM spermine was added as treatment as it was sufficient to generate cytosolic Ca^{2+} signals (Nancy Nowak, unpublished). Aequorin luminescence was recorded for 30 min. Total aequorin was discharged at the end of the experiment using discharge solution containing 2 M $CaCl_2$ and 20% ethanol. Three repetitions were performed. $[Ca^{2+}]_{cyt}$ was calculated as described by (Rentel and Knight, 2004).

2.2.2.2 Effect of Ca^{2+} chelators on Ca^{2+} signal generation

Ca^{2+} chelators (10 mM BAPTA and EGTA) were added to the medium to bind the available Ca^{2+} after which luminometer measurements were used to determine the cytosolic Ca^{2+} signal generation in Col-0 and *tpc1-2*. Measurements proceeded with the protocol mentioned above using similarly grown seedlings. Three repetitions were performed. $[Ca^{2+}]_{cyt}$ was calculated as described by (Rentel and Knight, 2004).

2.2.2.3 pH effect on calcium signals

To check the response of Ca^{2+} signals at different pH, above mentioned protocol for luminometer experiment was adopted. Two spermine formulations were tested, i.e. spermine, which was basic in nature and spermine tetrahydrochloride, which contains four molecules of HCl, hence contributing to lower pH. Both spermines in solution give pH 7.0 and 6.0,

respectively. Ca^{2+} signals in seedlings treated with spermine at different pH were determined as described earlier.

2.2.3 Determination of intracellular pH

Intracellular pH of the cell was determined by using the fluorescent pH indicator dye BCECF-AM. Epidermal strips were isolated as described previously (section 2.2.1.1). Whole seedlings were prepared as described for luminometer experiments (section 2.2.2.1).

All materials were incubated in dye loading medium with 1/10X MS containing 5 mM MES (adjusted to pH 5.7) and 0.5% (w/v) sucrose (Islam *et al.*, 2010). BCECF-AM dye was used at 5 µM. Selected material was incubated at room temperature for 30 min. Afterwards, washing was done two times for 5 min to remove the external dye. Slides were prepared and was checked under a microscope to confirm fluorescence.

2.2.3.1 Preparation of protoplasts

Protoplasts were isolated from 3-4 week-old *A. thaliana* plants (ecotype Col-0). Healthy and well-expanded leaves were harvested and placed in a beaker with distilled water. Plasmolysis buffer was poured into petri dish, and leaves were put in it and cut into 0.5 to 1 mm strips by using a fresh razor blade. The leaf strips were transferred into freshly prepared enzyme solution, vacuum-infiltrated two times for 5 min, and incubated in darkness for 3 h at 23°C. Protoplasts were separated from the undigested tissue by filtration through a 70 µm nylon mesh (BD Falcon, Dresden, Germany).

Isolated protoplasts were kept in dye loading medium for 15 min after which dye was applied at 5 µM. Incubation was done at 4°C in the fridge for 30 min. Washing was omitted to avoid loss of protoplasts.

Protoplasts were employed for intracellular pH determination. A microplate reader was used for determining fluorescence intensities to calculate intracellular pH values. Two excitation wavelengths, i.e. 490 and 450 nm, with emission at 535 nm, were taken for this analysis.

2.2.3.2 Optimization of BCECF-AM dye

To optimize the pH determination and check the change in fluorescence in protoplasts, two chemicals were used which are reported to increase or decrease intracellular pH of the cell. The pH of the solution was adjusted to pH 9.2 by 2 mM NH_4Cl to evaluate an increase in fluorescence and to pH 5.2 by 1 mM acetic acid to confirm a decrease in fluorescence.

2.2.3.3 Plate reader experiment

Protoplasts were prepared as described above (2.2.3.1). One mM spermine was used to assess the intracellular pH response in the cells. Baseline values of the cells were obtained after which the treatment solution was injected. Measurements were taken for 10 minutes. pH values were calculated by ratio measurements as described above. The response of spermine on intracellular pH was also assessed by adjusting the pH of the spermine-containing medium to 6.0. Different plant material were used i.e. protoplasts and whole seedlings of *Arabidopsis*.

2.3 Results

2.3.1 Spermine effects on stomatal aperture

A stomatal bioassay experiment with epidermal strips of *Arabidopsis* leaves was conducted to analyze the effect of different polyamines on stomatal aperture. To examine the response, treatments of different polyamines at 10 µM concentration were done. Polyamines examined were putrescine, spermidine, and spermine. The application of 10µM spermine and 10µM spermidine led to stomatal closure while putrescine-treated stomata remained open (Fig. 2.1). Spermine induced the maximum stomatal closure, indicating a potential role of spermine in drought tolerance. Differences between control and treatments were significant for both spermidine ($p < 0.01$) as well as spermine ($p < 0.001$).

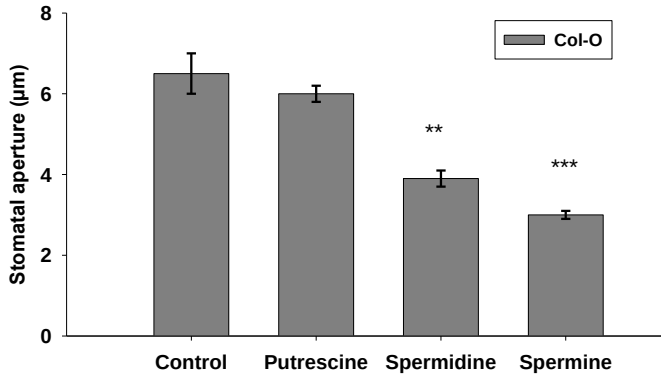


Fig. 2.1: Effect of different polyamines at the concentration of 10 µM on stomatal aperture. Significantly different from control at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data is presented as the mean of 20 replicates readings ± SE. The experiment was repeated two times with comparable results.

It was found that spermine treatment induced the closure of stomata. EGTA was used to chelate extracellular Ca^{2+} to examine whether Ca^{2+} plays a role in stomatal closure induced by spermine. Using the free Ca^{2+} calculator i.e. Ca-EGTA Calculator v1.3 software, it was found that there was nearly no free Ca^{2+} i.e. <0.00009 M in the solution when preparing the buffer with 10 mM EGTA and a final pH of 6.1. Stomatal assays were also performed with another calcium chelator, i.e. BAPTA. Using the

WEBMAXC software, it was determined that 100 µM concentration of BAPTA with final pH of 6.0 is needed to chelate nearly all of extracellular Ca^{2+} i.e. <.00009 M free Ca^{2+}. BAPTA is known to be less sensitive to pH than EGTA.

Epidermal strips of Col-0 plants were incubated with the calcium chelator EGTA, which led to the partial prevention of the spermine-induced stomatal closure (Fig. 2.2a). EGTA prevented spermine-induced stomatal closure only partially, which led us to believe that release of Ca^{2+} from internal stores might also be necessary for closure. For that purpose, seeds of the *Arabidopsis tpc1-2* mutant were obtained to further investigate the role of Ca^{2+} release from internal stores in stomatal closure. *tpc1-2* mutants showed slightly decreased stomatal apertures compared to Col-0 plants (Fig. 2.2b). Also, epidermal strips of *tpc1-2* mutants were incubated with EGTA, and it was observed that EGTA prevented stomatal closure to a larger extent as in Col-0 plants.

Differences between control and spermine were significant for both lines i.e. Col-0 ($p < 0.001$) and *tpc1-2* mutant line ($p < 0.001$) (Fig. 2.2). However, a significant difference between control and treatment with spermine + EGTA in *tpc1-2* mutant line was observed i.e. $P < 0.01$ which was lower to significance differences observed in Col-0 lines which was $P < 0.001$. It was concluded that internal Ca^{2+} stores might be involved in this induced closure.

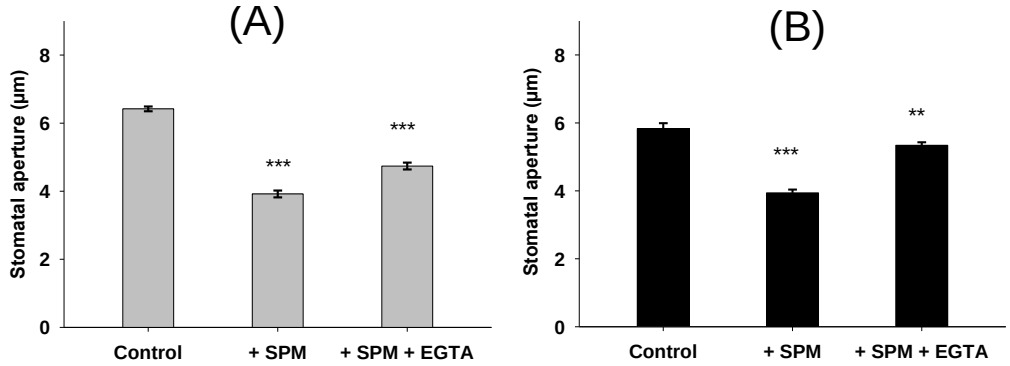


Fig. 2.2: Stomatal bioassays showing the effect of 10 µM spermine on stomatal aperture and the inhibition of closure after application of calcium chelators to Col-0 and *tpc1-2* mutant plants. (a) Stomatal aperture of Arabidopsis ecotype Col-0

after 2 hours incubation with spermine and spermine + EGTA (10 mM) (b) Stomatal aperture of *tpc1-2* mutant after incubation with spermine and spermine + EGTA (10 mM). Significantly different from control treatment at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data is presented as the mean of 20 replicates readings ± SE.

Experiment was repeated with Ca^{2+} chelator BAPTA which also exhibited a similar response with decreased stomatal aperture after spermine treatment. It was followed by partial prevention of stomatal closure upon pre-incubation with BAPTA chelator in Col-0 and *tpc1* mutant lines (Fig. 2.3).

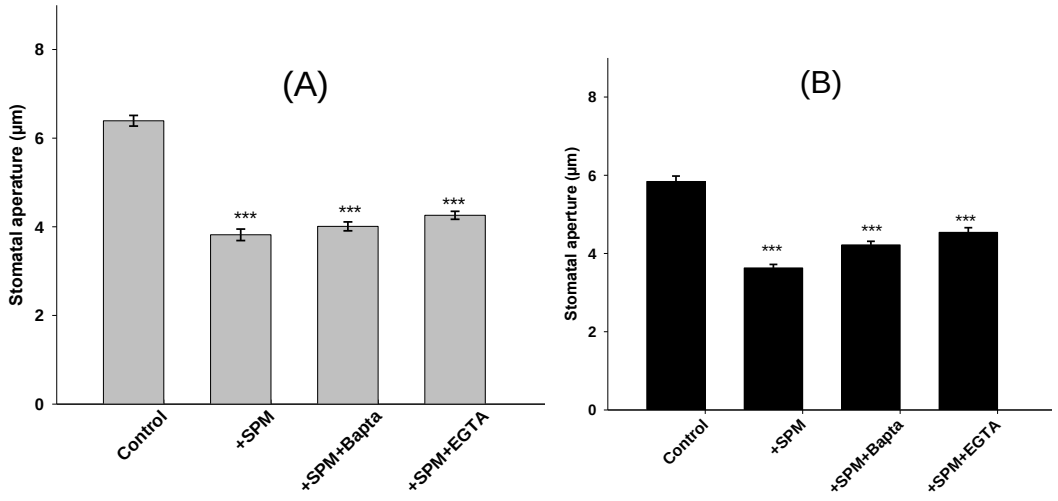


Fig. 2.3: Stomatal bioassays showing the effect of 10 µM spermine on stomatal aperture and the inhibition of closure after application of calcium chelators to Col-0 and *tpc1-2* mutant plants. (a) Stomatal aperture of Arabidopsis ecotype Col-0 after 2 hours incubation with spermine, with spermine + EGTA (10 mM) and Spermine + BAPTA (100 µM) (b) Stomatal aperture of *tpc1-2* mutant after incubation with spermine and spermine + EGTA (10 mM) and Spermine + BAPTA (100 µM). Significantly different from control treatment at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data is presented as the mean of 20 replicates readings ± SE. The experiment was repeated twice with comparable results.

2.3.2 Spermine effects on cytosolic free Ca^{2+}

Ca^{2+} ions play a role in stomatal closure. All experiments were performed with *A. thaliana* ecotype Columbia-0 constitutively expressing *APOAEQUORIN*. Luminometer experiments were done to analyze the effect of different spermine concentrations on the changes in cytosolic Ca^{2+} concentration in 7-days-old *Arabidopsis* seedlings i.e. spermine at different concentrations of 100 µM, 1 mM, and 10 mM. It was observed that 1 mM spermine was sufficient to generate cytosolic Ca^{2+} signals. Peak height was concentration-dependent and indicated that spermine application generates Ca^{2+} signals which may play a role in the closing of stomata (Nancy Nowak, unpublished). Similar to stomatal assays, this effect of spermine was examined on different Arabidopsis lines, i.e. Col-0 and *tpc1-2* mutant. Ca^{2+} signals were generated in both Col-0 and *tpc1-2* mutant plants. Nearly the same biphasic signal was obtained in both cases; however, the height of the initial peak in *tpc1-2* seedlings was lower than in Col-0 seedlings (Fig. 2.4).

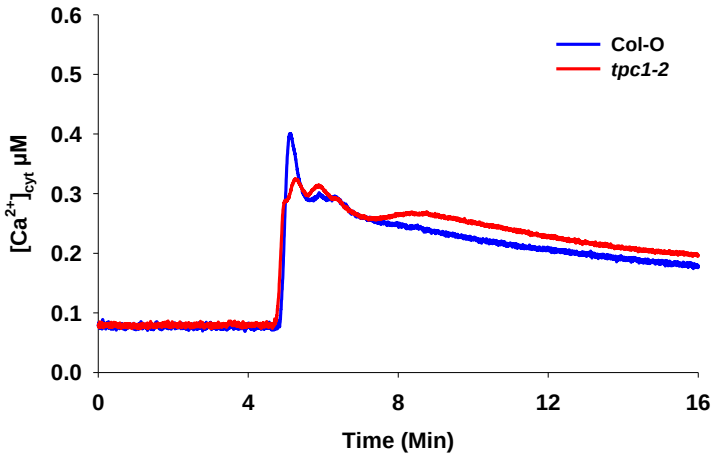


Fig. 2.4: Response of cytosolic free Ca^{2+} to application of 1 mM spermine. 7-day-old Arabidopsis ecotype Col-0 (blue line) and *tpc1-2* (red Line) seedlings were observed. Data show representative responses of three independent experiments.

Ca^{2+} signals generated by polyamine action may play an important role. To confirm the role of Ca^{2+} release from internal stores, Ca^{2+} chelators (10 mM EGTA or BAPTA) were used to bind Ca^{2+} in the buffer, and the generation of Ca^{2+} signals upon the application of spermine was examined in Col-0 and *tpc1-2.* Upon spermine treatment, cytosolic Ca^{2+} signal was observed with no biphasic signal. EGTA-treated seedlings exhibited a high steady-state $[Ca^{2+}]_{cyt}$. It might be due to high pH of treatment solution which had pH 6.9 and pre-incubation of 30 minutes might have caused increased background signal due to pH based Ca^{2+} signal (Fig. 2.5 a). Seedlings pre-treated with BAPTA at pH 6.0 showed a lower steady-state level after which the first peak was observed which continued to reduce until 15 minutes (Fig. 2.5 b). The results suggest that spermine triggers changes in $[Ca^{2+}]_{cyt}$ levels by Ca^{2+} influx as well as the release of Ca^{2+} from internal stores.

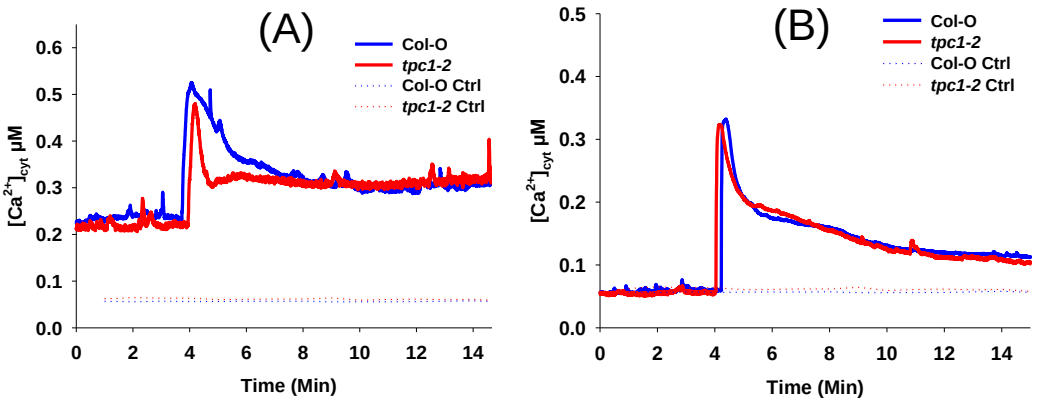


Fig. 2.5: Response of cytosolic free Ca^{2+} of wild-type (Col-0) seedlings (blue lines) and *tpc1-2* mutant (red lines) to 1 mM spermine. Water applied as a control. Steady-state signal was measured for four min before applying treatment (a) Addition of 1 mM spermine to 7-day-old seedlings pre-incubated with 10 mM EGTA for 30 min; (b) application of 1 mM spermine to 7-day-old seedlings pre-incubated with 10mM BAPTA for 30 mins.

2.3.3 pH dependency of calcium responses

The response of $[Ca^{2+}]_{cyt}$ to spermine application at different pH was determined. The previous experiment showed an increase in steady state level of $[Ca^{2+}]_{cyt}$ after preincubation with calcium chelators which had different pH i.e. 7.0. This led us to believe the role of pH on Ca^{2+} signal. For this purpose, spermine with different pH was tested, i.e. spermine at pH 7.0 and spermine tetrahydrochloride at pH 6.0. Ca^{2+} signals were generated during the treatment of 7-day-old Arabidopsis Col-0 seedlings with spermine at different pH. Higher pH induced an initial high peak followed by a sustained elevation of the signal (Fig 2.6 a), whereas lower pH did not provoke an initial peak but slightly increased $[Ca^{2+}]_{cyt}$ over time, after which it remained constant for at least xxx min (Fig 2.6 b). Ca^{2+} signal elevation observed at the end is comparable in both experiments.

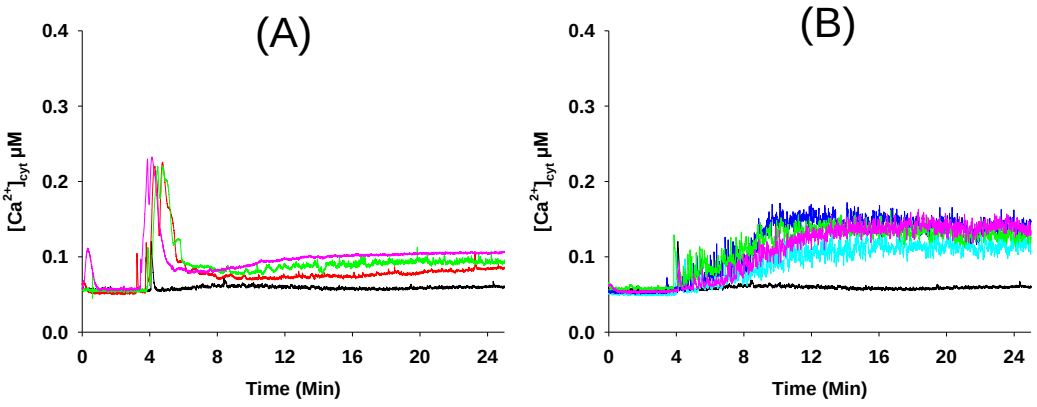


Fig. 2.6: Luminometer Measurements. (a) The addition of 1 mM spermine to 7-day-old Arabidopsis ecotype Col-0 seedlings with pH of the treatment adjusted to 7.0 (b) Application of 1 mM spermine to 7-day-old Col-0 seedlings with pH of the treatment adjusted to 6.0. Water applied as a control. Steady-state signal was measured for four min before applying treatment. Response measured for 20 min before discharging. Different colored lines indicate the number of replicates used with the same application of spermine

2.3.4 Determination of intracellular pH

Generation of Ca^{2+} signals might depend on increase or decrease in intracellular pH. Also, it was found that spermine-induced Ca^{2+} signals were elevated were pH-dependent. Keeping this in mind, it was planned to check the changes in intracellular pH while applying spermine treatment at pH 7.0. Spermine contains NH_2 groups which can react with cellular H^+ ions to increase the cytosolic pH. This parameter can be measured by a specific pH indicator dye, BCECF-AM, acting as a fluorescent indicator for intracellular pH. For this purpose, a dye loading medium consisting of 1/10 MS medium, 50 mM MES and 0.5% sucrose was used.

Different materials were selected for the determination of intracellular pH. Epidermal strips were used initially which were not successful. Epidermal strips do not have a cuticle on one side however, when incubated with dye medium strips were floating in a way which was preventing the non-cuticle side to come in contact with the medium and hence dye loading was not possible. Some successful cases has been shown in (Fig. 2.7 b & c), however, dye was entering the vacuole of the guard cells. SO, it was decided not to use this material. Subsequently, whole seedlings were observed under a microscope after 30 minutes of incubation with the dye. As they showed intensive fluorescence in guard cells , it was decided to use the 7- day old whole *Arabidopsis* seedlings to check the intracellular pH of whole plant (Fig. 2.7a). To check pH in individual cells, protoplasts were prepared and incubated with dye. It was observed that the dye entered the vacuoles which would make it impossible to determine cytosolic pH (Fig. 2.7 d). However, dye was confined to the cytosol when protoplasts were incubated at decreased temperature i.e. 4^oC for 15 min (Fig. 2.7e).

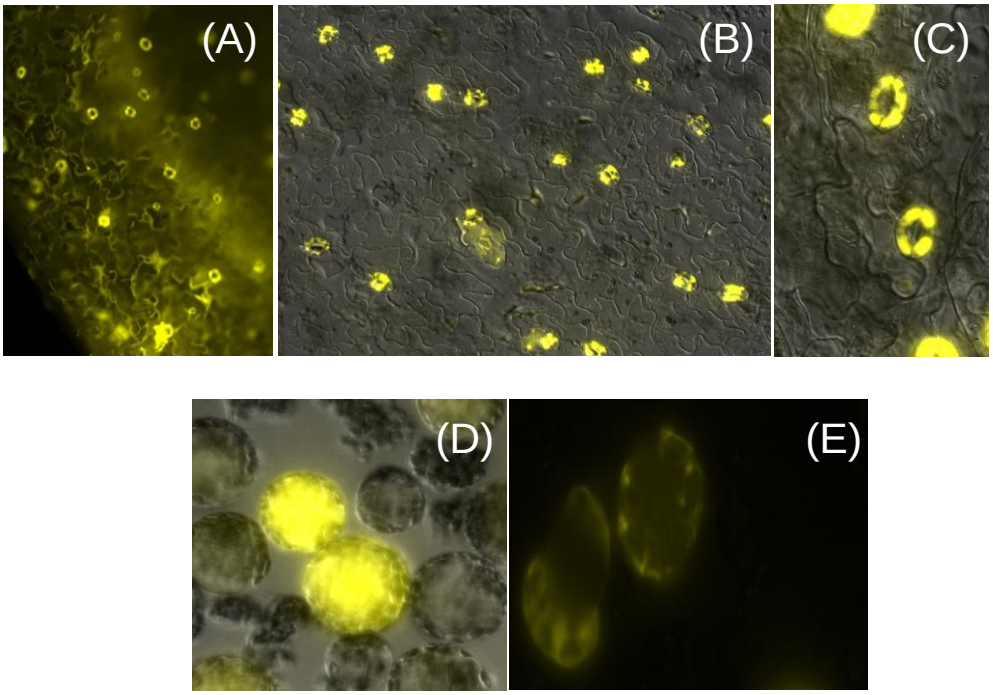


Fig. 2.7: BCECF-AM dye loading of different plant materials a) Whole 7-day-old Arabidopsis seedlings b) Epidermal strips obtained from 3-4 weeks old *Arabidopsis* plants c) Epidermal strip obtained from from 3-4 weeks old *Arabidopsis* plants fixed with adhesive on slides d) Mesopyll protoplasts e) Protoplast showing ideal dye loading with dye present only in cytosol and not in vacuoles, attained by keeping the cells at 4°C

2.3.4.1 Effects of spermine effect on intracellular pH

A microplate reader was chosen to measure the pH-dependent fluorescence intensity change. Optimization of the method was done by using two treatments, i.e. 2 mM NH_4Cl at pH 9.2, which increases the cytosolic pH (Fig. 2.8 a) and 1 mM acetic acid at pH 5.2 to decrease the cytosolic pH (Fig. 2.8 b). Measurements were taken at two excitation wavelengths, i.e. 490 and 450 nm, with emission at 535 nm .

After determining the conditions and software, 1 mM spermine (pH 7.0) was applied to protoplasts. This resulted in a sudden increase in fluorescence, showing that application of spermine increases intracellular pH of the cell (Fig. 2.8 c). This experiment was repeated by using the whole seedlings. Results showed a similar increase in fluorescence which kept on increasing constantly over measured time of 10 min.

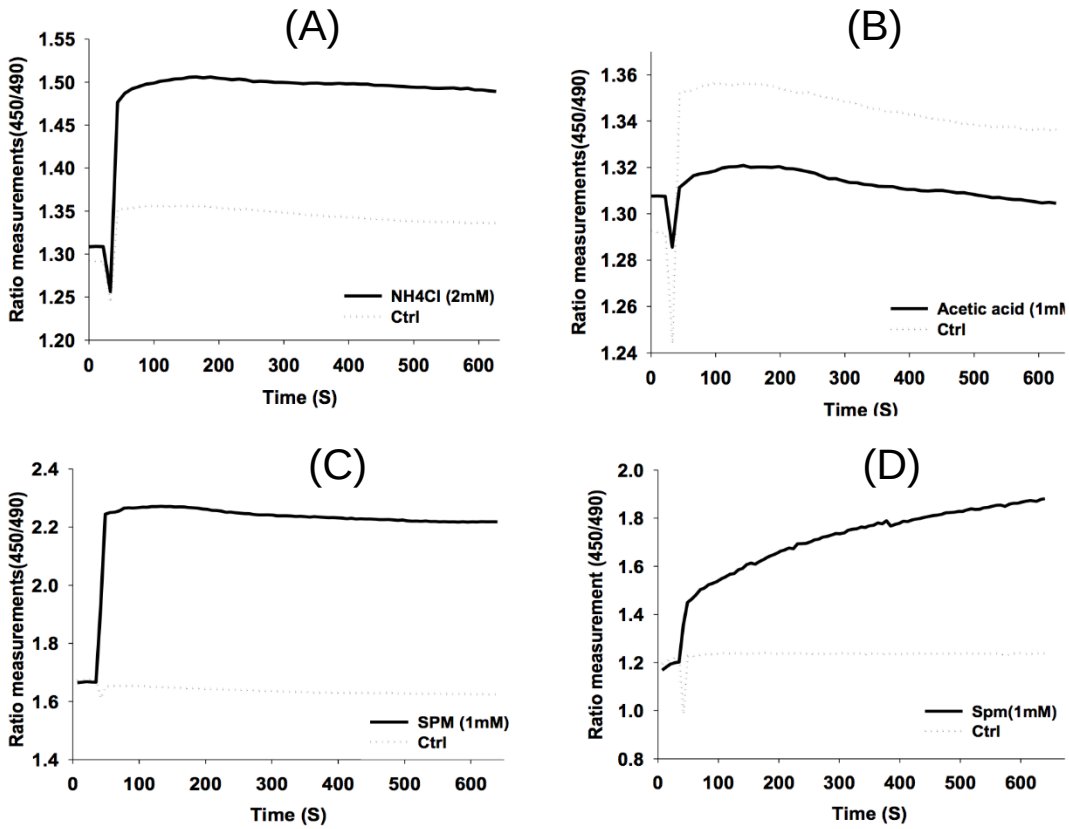


Fig. 2.8: Microplate reader experiment to determine intracellular pH by excitiation ratio measurement of BCECF-AM. (a) 2 mM NH_4Cl caused an increased fluorescence , (b) 1 mM acetic acid caused a decreased fluorescence (c) 1 mM spermine (pH 7.0) increased intracellular pH in protoplasts (d) 1 mM spermine (pH 7.0) applied to whole Arabidopsis seedlings increased intracellular pH.

2.4 Discussion

2.4.1 The polyamine spermine in plant stress responses

It has been observed that abiotic stress can induce increases in endogenous polyamine concentrations in various plant species, which is essential for adaptation to drought stress (Kusano *et al.*, 2008, Zhou and Yu, 2010, Hu *et al.*, 2012). Arabidopsis has been used as a model plant for polyamine research. In this project, the link between the polyamines, Ca^{2+} signals, and a drought stress response has been studied. It was found that addition of polyamines to epidermal strips partially closed the stomata and that this effect was strongest with spermine among the tested polyamines. It has been demonstrated before that the polyamine spermine, exogenously applied or endogenously enriched, has a role in the defense response of plants against both abiotic and biotic stresses, like salt, drought and heat stresses, and attack by viral pathogens (Yamaguchi *et al.*, 2006a, Yamaguchi *et al.*, 2007, Mitsuya *et al.*, 2009, Sagor *et al.*, 2012, Tiburcio *et al.*, 2014). Elevated endogenous polyamine content through exogenously applied spermidine or spermine led to enhancement in drought tolerance in *A. thaliana* (Kusano *et al.*, 2007b) and rice (*Oryza sativa*) (Farooq *et al.*, 2009). It was recently reported that exogenously applied polyamines i.e. putrescine , spermidine and spermine induce different membrane potentials and intracellular Ca^{2+} variations (Maffei *et al.*, 2004). (Liu *et al.*, 2000) showed that spermine, spermidine, Cad and putrescine strongly inhibited opening and induced closing of stomata, and that polyamines target inward potassium channels in guard cells, thus providing a link between abiotic stress, polyamine levels, and stomatal regulation.

2.4.2 Spermine involvement in cellular functions

Spermine has been established as the most active polyamine to induce stomatal closure when applied exogenously (Fig. 2.1), which corresponds to a role in the drought tolerance of plants. Generally, spermine may be

involved in the osmotic adjustment (Aziz *et al.*, 1999), stabilization of membranes (Tassoni *et al.*, 1998), scavenging of free radicals (Lester, 2000), and regulation of senescence (Lahiri *et al.*, 2004). It has been shown to participate in the prevention of electrolyte leakage and chlorophyll loss (Chattopadhayay *et al.*, 2002), as well as enhanced stem elongation growth (Rajasekaran and Blake, 1999). It is generally believed that higher polyamines (spermidine and spermine) can bind strongly to the negative charges in cellular components such as nucleic acids, proteins, and phospholipids, and thereby stabilize the membranes under stress conditions (Kasukabe *et al.*, 2004, Ma *et al.*, 2005).

The transport of polyamines across the plasma membrane of plant cells is energy-dependent, and Ca^{2+} is involved in the uptake mechanism (Walters, 2000).

2.4.3 Calcium signalling as pre-requisite for spermine-mediated drought responses

Transient increases in the concentration of Ca^{2+} ions in the cytosol has been known to be a prerequisite for drought responses, including stomatal closure. Ca^{2+} is known to be an important second messenger. Specific stimuli induce specified changes in the cytosolic Ca^{2+} concentration of the cell which in turn produce different downstream responses (McAinsh and Pittman, 2009, Kudla *et al.*, 2010).

In this work, stomatal assays were performed to measure the stomatal aperture on the epidermal strips of Col-0 and *tpc1-2* mutant plants. Mutant plants were used to determine whether Ca^{2+} ions from internal stores play a role in the stomatal regulation. These were preincubated with Ca^{2+} chelators to bind all the extraceullar Ca^{2+}. The results indicated only partial prevention of stomatal closure. These experiments showed that extracellular Ca^{2+}, as well as internal Ca^{2+} stores, play a role in stomatal closure. In tobacco, Ca^{2+} influx to the cytoplasm is a prerequisite for triggering a spermine signaling pathway where spermine application leads to ROS production stimulating Ca^{2+} flux (Takahashi *et al.*, 2003,

Takahashi *et al.*, 2004). This result was similar to our experiment where spermine application leads to increased Ca^{2+} influx as well as increased ROS production (Nancy Nowak, unpublished) Free spermine is accumulated in the apoplastic space and catabolized by extracellular polyamine oxidase (PAO), resulting in the production of H_2O_2 and the initiation of Ca^{2+} flux into the cytosol. The production of H_2O_2 and Ca^{2+} induces plant defense mechanisms under abiotic stress (Zhou and Guo, 2009, Gonzalez *et al.*, 2012). Putrescine (Put) exhibited similar effects of H_2O_2 production as well as cytosolic free Ca^{2+} level, which resulted in stomatal closure (An *et al.*, 2008).

Ca^{2+} was found to be important for stomatal closure. In *A. thaliana* guard cells, stimuli for stomatal closure, including abscisic acid (ABA), hydrogen peroxide, cold, elevation of external Ca^{2+}, and atmospheric CO_2, induce cytosolic Ca^{2+} oscillations (Allen *et al.*, 2001). The increase of cytosolic Ca^{2+} levels in guard cells can trigger stomatal closure, which demonstrates the importance of Ca^{2+} signaling for drought tolerance (Fu and Lu, 2007). Spermine-deficient Arabidopsis plants cannot grow well in Ca^{2+}-depleted medium (Yamaguchi *et al.*, 2006b), suggesting that the tetraamine is involved in Ca^{2+} dynamics and homeostasis. Polyamines are known to modulate Ca^{2+} dynamics via control of cation channel activity (Dobrovinskaya *et al.*, 1999 b, Pottosin and Shabala, 2014).

In this study, spermine elicited the generation of Ca^{2+} signals in the cytosol. A transient elevation was observed after the application of spermine, followed by a sustained elevated $[Ca^{2+}]_{cyt}$. A similar response was observed with *tpc1-2* mutants as well. However, magnitude of the Ca^{2+} signal was slightly lower compared to Col-0 seedlings indicating TPC1 proteins as the Ca^{2+} channels which supported the generation of Ca^{2+} signals from internal stores. Chelation of extracellular Ca^{2+} decreased the initial Ca^{2+} signal generated by spermine application followed by decrease in signal. A increase of $[Ca^{2+}]_{cyt}$ in guard cells is considered to be a center point leading to stomatal closure. When the increase in $[Ca^{2+}]_{cyt}$ is entirely prevented by microinjection of the Ca^{2+} chelator BAPTA into the guard cell cytosol, there is no loss of turgor which

indicated the failure in the generation of Ca^{2+} signals leading to closed stomata(Ng *et al.*, 2001).

Cytosolic Ca^{2+} concentration in guard cells is involved in opening, and closing of stomata, and spermine-deficient mutants with open stomata experienced impaired Ca^{2+} homeostasis (Allen and Sanders, 1996, Schroeder *et al.*, 2001, Yamaguchi *et al.*, 2006a). Polyamines especially spermine slowed down the opening and induced closure of stomata (Liu *et al.*, 2000). Spermine has been known to control putative Ca^{2+} channels such as the fast vacuolar (FV) channels and slow vacuolar (SV) channels, encoded by *TPC1* (Peiter et al., 2005), which ultimately leads to closure of stomata. In barley, it has been reported that polyamine inhibits or block the FV channels, and the effectiveness of polyamines to block FV channels was in the order spermine > spermidine > putrescine (Dobrovinskaya *et al.*, 1999). FV and SV channels are mainly responsible for the K^+ and Ca^{2+} ion release into the cytoplasm. The FV channel has a high selectivity for K^+ and do not release Ca^{2+} whereas SV channels are cation selective with monovalent cations and divalent cations (Dobrovinskaya *et al.*, 1999). While spermine block the monovalent cations through FV and SV channels, release of divalent cations such as Ca^{2+} is possible.

2.4.4 The role of pH in the generation of Ca^{2+} signals and the regulation of stomatal movements

Stomatal closure takes place through a net efflux of active solutes from the guard cells especially as a flux of K^+ from vacuole and cytoplasm through the plasma membrane which happens to cause a decline in guard cell turgor (Raschke, 1975, MacRobbie, 1988). Investigations into stomatal guard cells have led to the suggestion that pH may be a key regulator, modulating the activity of ion channels and thereby controlling osmotic solute fluxes for stomatal movement (Blatt, 1992, Blatt and Thiel, 1993). In this work, Ca^{2+} signal generation was found to be influenced by pH of the spermine solution. The pH specifically altered the initial signal, whereas the spermine-triggered sustained elevation of $[Ca^{2+}]_{cyt}$ was constant regardless of the pH of solution used. It was thought to be the effect of

intracellular change of pH which lead to the generation of the first phase of the Ca^{2+} signal. It was observed that Ca^{2+} signal response depends on pH, with higher pH inducing stronger signals. It is possible that the intracellular increase of pH is, in fact, responsible for spermine-triggered cytosolic Ca^{2+} signals which ultimately lead to stomatal closure. In this work, intracellular pH measurements showed that application of spermine increases the cytosolic pH, and increases in pH were dependent on the pH strength of spermine (not shown). Spermine contains NH_2 groups whose pKa values are 10.9 and 10.1 (Casero and Woster, 2009), so when spermine treatment enters the cell it reacts with cellular H^+ ions to increase the cytosolic pH. Spermine application gradually increases the pH which increases cytosolic Ca^{2+} signals. This result was consistent with the observation of (Gehring *et al.*, 1990, Irving *et al.*, 1992). Decrease in pH has been shown to be caused by weak acids (Brummer *et al.*, 1985) which happens to bring cytosolic acidification by increasing $[Ca^{2+}]_{cyt}$ in maize coleoptiles (Felle, 1988). High pH from the vacuolar side inhibited the $[Ca^{2+}]_{vac}$ which inhibited the SV channels identified as TPC1 (Peiter, 2011). Similarly, increase in cytosolic pH might inhibit the slow vacuolar channels from cytosolic side which are activated by $[Ca^{2+}]_{cyt}$ and in turn, reduces the vacuolar pH and activating the calcium influx into the cytosol from the vacuoles. Therefore, it appears that stomatal closure is preceded by an alkalinization of cytosol followed by an increase in $[Ca^{2+}]_{cyt}$, in the guard cells. Spermine induced stomatal closure is probably mediated by the alkalinization of cytoplasm and increase of intracellular Ca^2. Though relationship between pH and Ca^{2+} in spermine signaling leading to stomatal closure need to be further studied.

3 CHAPTER 3

Generation and characterization of transgenic barley lines misexpressing *Spermine Synthase*

3.1 Introduction

3.1.1 Barley transformation

Plant transformation is defined as the stable incorporation and expression of foreign genes (van den Eede *et al.*, 2004). Two methods have been used for the genetic transformation of plants, i.e. direct gene transfer or *Agrobacterium*-mediated gene transfer. Direct gene transfer is commonly employed in monocot species that are not amenable to *Agrobacterium* transformation. Microprojectile bombardment, electroporation and microinjection are different techniques used in direct gene transfer. *Agrobacterium tumefaciens* is a naturally occurring, soil-borne bacterium that causes crown gall disease in a wide range of dicot species. The bacterium contains a Ti (tumor-inducing) plasmid that interacts with the plant cells to provide a convenient mechanism for gene transfer into cells. During bacterial infection of plant tissue the T-DNA (transfer DNA), a mobile segment of the Ti plasmid, is transferred to the nucleus of the plant cell and integrated into a plant chromosome (Zupan *et al.*, 2000, Gelvin, 2003).

The first reported *agrobacterium*-mediated transformation for tobacco was described in 1984. The technique has been used for over 120 species since (Birch, 1997), including various monocot and dicot species (Birch, 1997, Gelvin, 2003). Two methods vastly reported used for stable barley transformation has been particle bombardment and *Agrobacterium*-mediated delivery over the years (Wan and Lemaux, 1994, Travella *et al.*, 2005, Hensel *et al.*, 2008). Barley transformation is genotype-dependent.

Various barley cultivars have been used for transformation, but it has been found the spring cultivar Golden Promise is the most responsive genotype with best transformation efficiencies (Finnie *et al.*, 2004). Similarly, Golden Promise is also reported to have a high regeneration rate (Dahleen and Manoharan, 2007). Other genotypes are reported to exhibit lower frequencies when using *Agrobacterium*-mediated techniques. For high transformation efficiencies, immature embryos have been identified as the optimum target tissue for barley transformation (Murray *et al.*, 2004). Different methods used for transformation over the years have indicated the *Agrobacterium* system as the most efficient, leading to higher transformation efficiencies and lower transgene copy number than biolistic-mediated transformation (Bartlett *et al.*, 2008). One of the most important aspects for a successful plant transformation is the host range of different *Agrobacterium* strains (Gelvin, 2003). For barley, *Agrobacterium* strain AGL1 is commonly used as the choice of *agrobacteria* strain is responsible for higher plant regeneration (Tingay *et al.*, 1997) . Barley transformation has been mostly reported to proceed with the *hpt* gene conferring resistance to the antibiotic hygromycin used as the selective agent. The CaMV35S promoter is often used to drive the hygromycin resistance gene which leaves stronger promoters such as the maize ubiquitin promoter (*ubi1*) available to drive a gene of interest .

3.1.2 Reverse genetic analysis approach

One of the most powerful tool to establish a link between the function of any specific gene and its role, is reverse genetics approach (Gilchrist and Haughn, 2010). Several methods have been developed that enable to change the nature of gene products for reverse genetics, such as an approach with reduced gene expression, and a gain-of-function approach (Bolle *et al.*, 2011).

Observations from the gain of function approach and the knock-down approach allow to deduce the role of the gene. The gain-of-function (over-expression) method leads to an increased expression of a wild-type gene (Prelich, 2012). This is achieved through the random activation of

endogenous genes by transcriptional enhancers or the expression of individual transgenes by transformation (Kondou *et al.*, 2010). The mutant phenotypes induced by loss-of-function and gain-of-function approaches are often complementary to each other (Bolle *et al.*, 2011).

Transgene-gene mediated gene silencing can be used to decrease (knock-down) but not entirely abolish the expression of the gene-of-interest (Bolle *et al.*, 2011). Silencing is generally achieved by post-transcriptional down-regulation of transcript accumulation via small RNAs that act in a sequence-specific manner by base pairing to complementary mRNA molecules. Based on gene silencing by small RNA, many strategies have been developed (Ossowski *et al.*, 2008). A widely used approach involves the activation of the RNA interference (RNAi) pathway, particularly by using microRNA (miRNA).

RNAi is a conserved pathway that post-transcriptionally silences RNA by recognition of target RNA. Double-stranded RNA (dsRNA) serves as the trigger for the RNAi pathway upon cleavage into duplexed small interfering RNAs (siRNAs) or hairpin microRNAs (miRNAs), which act in a sequence-specific manner to target and degrade ssRNAs (Baulcombe, 2004). Fire, Mello, and colleagues first described the RNAi pathway in 1998 using *Caennorhabditis elegans* (Fire *et al.*, 1998). RNAi has also been described as co-suppression of homologous genes in petunia plants and as RNAi in *Drosophila melanogaster*, mammalian and human cells (Romano and Macino, 1992, Hammond *et al.*, 2001, Liu *et al.*, 2004b). Another important factor associated with gene silencing is the number of transgenes per integration site (Kertbundit *et al.*, 1991, Prols and Meyer, 1992). Transgenes, often as part of the *Agrobacterium tumefaciens* T-DNA, integrate at different chromosomal locations leading to expression or inactivation of the T-DNA.

3.1.3 Polyamines in Plants

The concentration of free polyamines in the plants depends on their biosynthesis, catabolism, and conjugation (Alcazar *et al.*, 2010). Most commonly found polyamines synthesized in the plant cell are the diamine

putrescine, the triamine spermidine and the tetraamine spermine. Diamine oxidases (DAO) and polyamine oxidases (PAO) are two enzyme classes which catabolize polyamines. DAO catalyze putrescine oxidation whereas PAO catabolise or back-convert spermine into spermidine. DAOs are present in monocots and dicots (Cona *et al.*, 2006), whereas PAOs are present in higher amount in monocots (Alcazar *et al.*, 2010).

Polyamines may be present in conjugated form, mainly attached to hydroxycinnamic acids, forming amides of coumaric, ferulic and caffeic acid (Smith, 1985). In plants, polyamines also occur as free polyamines . The ratios between free and conjugated polyamines vary between plant species. Many methods have been developedto quantify polyamines, and HPLC has been reported as the most precise for this purpose (Muskiet *et al.*, 1995). Benzoylation (Kotzabasis *et al.*, 1993, Schenkel *et al.*, 1995) and dansylation (Marce *et al.*, 1995) is performed for pre-derivatization to determine the free polyamines present. Post-column derivatization with O-phthalaldehyde-thiol reagents has also been employed for polyamine analysis in several studies (Whitmore and Slotkin, 1985, Saito *et al.*, 1992). For quantification of conjugated polyamines, hydrolysis at high temperature is done to break the conjugates, producing the free form (Torrigiani *et al.*, 1987, Sharma and Rajam, 1995). As polyamine conjugates are hydrolyzed, most chemical information is compromised, but measurement of free polyamines is most easily performed using this method.

3.2 Materials and Methods

3.2.1 Generation of *SPMS*-misexpressing transgenic barley lines

3.2.1.1 Cloning of *SPMS* 1 and *SPMS* 2 overexpression and knockdown constructs

Two *SPMS* homologs, *SPMS* 1 and *SPMS* 2, were identified by Sebastian Pietack (MLU Halle-Wittenberg, Institute of Pharmacy; unpublished). To examine whether *Spermine Synthases* play a role during drought stress in barley, transgenic plants were generated to examine the effect of an increase or a knockdown of the expression level. For this purpose, two RNAi constructs and two constructs for the overexpression of both *spermine synthase* 1 and *spermine synthase* 2 had been created. To prepare the expression cassettes for over-expression, the full-length sequence of Hv*SPMS*1 and Hv*SPMS*2 was cloned into the vector pUbi-AB and subsequently cut out with SfiI. The respective expression cassettes were then successfully cloned into the binary vector pLH 6000 via the SfiI site (40) and the transformed into the *Agrobacterium*.

For the RNAi constructs, a 201 bp or 125 bp fragment of the Hv*SPMS*1/2-cDNA was first amplified by a PCR using primers for the restriction sites BAMHI/AscHI (RNAi-antisense) and Spe/Kpn (RNAi sense) respectively. The inserted interfaces are subcloned into the vector pStarling. This vector mediates resistance to ampicillin. The vector pStarling was then digested with HIND and cloned into the vector pUbi-AB. After that, restriction digestion was performed with SfiI, and the entire expression cassette was transformed into the binary vector pLH7000. In both cases, *Agrobacterium*-mediated gene transfer followed in barley (Nancy Nowak, Unpublished).

3.2.2 Transformation of barley with *SPMS1* and *SPMS2* constructs

3.2.2.1 Preparation of plant material

Barley cultivar Golden Promise was selected for the transformation. Mitscherlich-pots were filled with ED 73 soil substrate containing fertilizers. Plants were cultivated for three months in a walk-in growth chamber until grains are formed yet relatively green. Growth conditions used were 16 h/8 h photoperiod (light intensity 250-300 µmol m^{-2} s^{-1}) with 18°C/14°C temperature. Humidity was kept at 65-70%. Pots were watered every two days and fertilized with commercial WUXAL fertilizer every two weeks at 2ml/1L H_2O.

Immature seeds were separated from the spikes and sterilized with 70% ethanol followed by 20 minutes bleach (3% NaOCl + 1 drop Tween 20). Afterward, the seeds were washed 2-3 times with water.

3.2.2.2 Isolation of immature embryos

Immature embryos were isolated from immature seeds in a laminar flow hood under sterile conditions using a dissecting microscope. The seed was carefully cut in the center, and the coat was removed which allowed access to the immature embryo. The embryonic axis was removed without damaging the scutellum. The isolated embryo was transferred to a new petri dish for *Agrobacterium* inoculation.

3.2.2.3 Inoculation and Co-Cultivation

Agrobacterium was cultivated overnight in Yeast Extract Broth (YEB) containing beef extract (5g/L),Yeast extract (1g/ L), Casein Hydrolysate (5g/L), Saccharose (5g/L), $MgSO_4$ x $7H_2O$ (0,49g/L) and Agar(20g/L) with pH 7.2 at 28°C for 24 h in the dark while being continuously shaken at 120-150 rpm. Isolated embryos were placed in the center of the plate. *Agrobacterium* solution was poured on the embryos where 25 embryos were immersed in the 200 µl solution. The embryos were incubated for 40

minutes. Subsequently, ten inoculated embryos were transferred to a new plate with callus induction medium without antibiotic containing MS salt (4.4g/L), maltose (30g/L), myo-inositol (0.25g/L), L-Proline (0.69g/L), thiamine-HCl (1mg/L), $CuSO_4$ x $5H_2O$ (1.2mg/L) and Dicamba (2.5mg/L) maintained at pH 5.9. Co-cultivation was done for two days while calli were kept on callus induction medium in the dark at 24°C.

3.2.2.4 Regeneration of plants from transformed calli

Inoculated embryos were transferred to a new plate containing callus induction medium with Ticarcillin + Clavulanic acid (150mg/L) and cultivated for two weeks in the dark at 24°C to eliminate agrobacteria. The growth of calli was checked regularly. After every two weeks, the medium was changed. This was repeated three times. Six-week-old calli which survived on medium containing antibiotics and selective marker, i.e. hygromycin at 100µg/ml, were selected as transformed calli.

Transformed calli were transferred to new plates with growth regulators, i.e. 2,4- D (2.5mg/L) and BAP (1mg/L), along with antibiotics and selection marker. Calli were kept for two weeks at 24°C under low light which helped to produce small green shoots. These green calli with small shoots were shifted to test tubes with callus induction medium without growth regulators. The medium contained antibiotic and selection marker. Calli were kept in a growth chamber at 24 ^{0}C until rooted plants reached the top of the glass tube. Regenerated transformed plants were carefully transferred to soil.

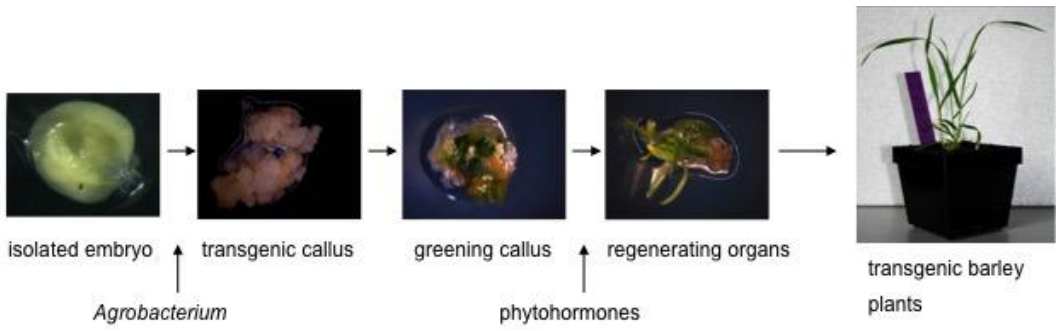


Fig. 4: Step-by-step overview of the Barley transformation protocol used (Pictures: Nancy Nowak, unpublished)

3.2.3 Molecular analysis of putatively transformed plants

3.2.3.1 DNA extraction

Molecular analysis of transformed plants was done to confirm the presence of marker gene and vector in the barley genome. To extract the DNA, a small piece of leaf was harvested, and 400 µL Extraction Buffer containing 200mM Tris-HCL (pH 7.5), 250mM NaCl, 25mM EDTA and 0.5% SDS was added to an Eppendorf tube over the leaf. Small steel beads were added, and a tissue lyzer was used to grind the material. The suspension was centrifuged for 5 min at maximum speed. The supernatant was transferred to a fresh tube, and isopropanol was added, vortexed briefly, and centrifuged for 10 min at maximum speed. A pellet became visible which was washed with 800 µl 70% ethanol after discarding the supernatant. The tube was centrifuged again for 10 min at maximum speed. Ethanol was removed, and the pellet was air-dried for 30 minutes after which it was dissolved in 50 µl water.

3.2.3.2 PCR

All PCR reactions were performed with a Mastercycler Gradient Thermal cycler (Eppendorf, Hamburg, Germany). Standard PCR was done to examine presence of the vector in transformants using Taq (*Thermus aquaticus*) polymerase (Fermentas native Taq Polymerase, Frankfurt/Main, Germany) in 25 µL reactions. The sequence of the primers for the detection of the pLH7000 vector is as follows:

Forward Primer: 5’ AAATGGGGGCTTAGATGAGA 3’

Reverse Primer: 5’ TGCGAAGGATAGTGGGATTG 3’

The composition of the reaction mixture is as follows:

10× Taq buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM forward primer	1.0 µl

10 µM reverse primer	1.0 µl
H_2O	15.0 µl
1U/µl Taq polymerase	1.0 µl
Total	21.0 µl

DNA template was added to the reaction mixture, i.e. 4 µl to give 25 µl volume of final reaction mixture. Reaction mixture without water instead of DNA sample served as negative control. Purified pLH 7000 plasmid (plasmid eluted and purified using HiYield Gel/PCR DNA Mini kit (YDF100/YDF300) served as positive control.

The PCR program used to amplify DNA was as follows:

95 °C for 7 min

95 °C for 30 s
60 °C for 30 s } 40 cycles
72 °C for 60 s

72 °C for 7 min

Approximately 35-40 cycles proved to be adequate for the PCR application.

The duration of the initial denaturation was selected due to the relatively large genome of barley. The annealing temperature (T_m) was the lowest melting temperature of the primers. The elongation time was taken according to the size of the amplified fragment (~ 1 kb/min) which was around 400-500bp.

3.2.3.3 Southern hybridization

I. **Preparation of probes**

Probes were prepared by standard PCR. PCR primers used were the same primers used previously in Section 3.2.3.2. Roche DIG (digoxigenin)-labeling kit containing 2 mM dATP; dCTP; dGTP and DIG-

labelled 0.19 mM dTTP and 0.1 mM DIG-11-dUTP was used instead of regular dNTP mix. Synthesis of the labeled probe was verified by agarose gel electrophoresis. DIG-labeled fragments appeared larger in size in comparison to non-labeled fragment.

II. Digestion of genomic DNA

DNA samples of 30 µg were digested overnight at 37 ^{0}C with 1 µl of EcoRI restriction enzyme (10-20U/µl) and 4 µl NEB 4 buffer in a total volume of 25 µL and incubated. A gel run was performed to confirm complete digestion.

III. Capillary transfer of digested DNA

Digested DNA was separated on a 1% (w/v) agarose TAE gel for 3-4 hours at 40 V, and digestion was visually confirmed using a Gel iX imager (Intas Science Imaging Instruments). The gel was equilibrated with 0.25 M HCl for 20 min followed by denaturation with 0.5 N NaOH for 20 min. The procedure was repeated two times on rotating tumbler after which the gel was neutralized with 0.5 M Tris-HCL and 3 M NaCl. DNA fragments were transferred overnight by capillary action to a positively charged nitrocellulose membrane (Hybond-N+, Amersham Pharmacia Biotech, Freiburg, Germany). To this end, the nitrocellulose membrane was immersed in 20x SSC (Appendix 3, B5) for an hour for equilibration. Subsequently, it was placed on filter papers of gel size (Whatman 3MM, Whatman, Dassel, Germany) which were placed on a stack of tissue papers after removal of air bubbles. The gel was placed carefully on the membrane and covered with three 20x SSC-soaked Whatman filters. One large Whatmann filter paper was cut and soaked with 20x SSC. Two glass dishes were placed on the sides of the construction and filled with 20x SSC. The long Whatman filter paper was passed from the left dish to the right one, so the middle part of the paper was on top of the construction, having three filter papers on top. It was pressed for supplying buffer for blotting. Finally, a small weight was placed on the blot construction to facilitate the transfer. After transfer, digested DNA was cross-linked to the membrane by UV exposure, i.e. 2*100 Units (Energy per unit area) and placed for one hour at 80 ^{0}C in an oven.

IV. **Hybridization**

The cross-linked membrane was placed in a hybridization tube. The pre-hybridization solution (Appendix 3, B6), which was pre-heated at 55 ^{0}C, was poured in. It was incubated at 50 ^{0}C for 2 h with 20 mL of prehybridization buffer in a hybridization oven (Biozym Scientific, Hess. Oldendorf, Germany).

For hybridization, the DIG-UTP-labeled probe was denaturated at 100 ^{0}C for 2 min in a heating block, cooled briefly on ice, and added to the prehybridization buffer in the tube. Hybridization was performed at 50°C for overnight. Salmon sperm DNA was added at 2 mg/20 ml in the buffer for blocking to avoid non-specific binding of the probe.

V. **Detection of bound probe**

The membrane was washed stepwise in the tube for 5 min with 2x SSC at room temperature followed by 15 min with 0.5x SSC pre-incubated at 68°C and 15 min with 0.25x SSC preincubated at 68°C in an oven at 68°C after which it was equilibrated for 5 min with Solution M (Appendix 3, B13), at room temperature. The membrane was allowed to run for 3-4 h with a 1x blocking solution (Appendix 3, B10),, and anti-DIG antibody-coupled alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) was added for 30 min. The membrane was washed three times for 10 min with solution M, and afterward immerse it in detection buffer (Appendix 3, B11), for 2 min. The membrane was put in plastic seal bag, 1% CSPD solution (Roche-CSPD-Ro, Sigma-Aldrich) was added, and the membrane was incubated in the dark for 5 min, immediately followed by 15min at 37 ^{0}C, after which the membrane was shifted to a new plastic seal bag. Emitted chemiluminescence was documented with an X-ray film for 18 h (Hyperfilm ECL, Amersham Pharmacia Biotech, Freiburg, Germany) and was developed with an Optimax TR device (MS Laborgeräte, Heidelberg, Germany).

3.2.3.4 Expression analysis

a) Extraction of RNA

For RNA extraction, leaves of transgenic barley plants which were 5-6 weeks old, were taken. They were grinded in liquid nitrogen into a fine powder using a mortar and pestle. Approximately 100 mg of the resultant powder was filled into a 1.5 ml Eppendorf tube. Trizol reagent (Appendix 3, B16) was used to resuspend tissue and incubated for 5 min at 30 ^{0}C. After centrifugation for 10 min at 12000 x g, 200 µl 1-bromo-3-chloropropane was added to clear supernatant in a new 2-ml tube, shaken and incubated at 15 to 30 ^{0}C for 3 min. The aqueous upper phase was transferred into a new 2-ml tube after centrifugation, and 500µl 1-bromo-3-chloropropane was added. The previous step was repeated, and the aqueous phase was transferred and mixed with isopropanol in equal volume and incubated for 10 min. A pellet appeared after centrifugation at 12000 x g and 4 ^{0}C for 10 min. The RNA pellet was washed with 1.5 ml of 70% ethanol and centrifuged at 7,500 × g for 5 min. The pellet was dried under vacuum in a desiccator and dissolved in RNase-free water. Quantification was done using a Nanodrop 2000 Spectrophotometer.

For gDNA digestion in extracted RNA, 10 µg RNA was used with 1X DNase buffer and 1 µl of DNaseI in a final volume of 100µl and incubated at 37 ^{0}C for 30 min. Enzyme heat inactivation was done at 75 ^{0}C for 10 min after addition of 0.5M EDTA. RNA was precipitated by adding 100 µl 8M LiCl and incubating on ice for 30 min. A pellet was obtained by centrifugation at max speed at 4 ^{0}C. It was washed with 70% ethanol, centrifuged again, and the step was repeated for a second wash. After removing supernatant, the pellet was dried under vacuum and dissolved in RNase-free water and quantified with a Nanodrop.

b) cDNA synthesis

RT-PCR was performed using the M-MuLV RNaseH$^-$ Reverse Transcriptase (NEB, 200U/µl). One microgram of RNA was used for cDNA synthesis. RNA was added along with 2 µl Oligo $(dN)_6$ primers (25 µM) and filled to 16 µl with H_2O. The tubes were incubated at 65°C for 5 min and then put on ice immediately. Five µl of 5x M-MLV buffer, 1 µl dNTPs (10 mM each) and 1 µl M-MLV RT enzyme (200U) were added, followed by quickly flicking and spinning the microtube. Afterward, the following

incubation steps were applied: 5 min at 25°C; 60 min at 42°C, followed by 5 min at 70°C. After adding 1 µl of RNAse A, the reaction was incubated at 37°C for 20 minutes. The reaction was filled up with 24 µl of H_2O for a total volume of 50 µl and stored at -20°C.

c) Real-Time PCR

qPCR reaction was done with 96-well plates (MicroAmp; Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The instruments used was a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Reaction conditions were as follows: one step at 95°C for 10 min, and 40 cycles of 95°C for 15 s denaturation and 60°C for 1 min annealing and extension. The reference gene used for the experiments was *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*). The reaction master mix consisted of the Real-time PCR Master mix (TaqMan Universal PCR Master Mix; Applied Biosystems), reverse and forward primers, and a template. Template for the real-time PCR was 1:20 dilution of the synthesized cDNA. and five µl of the diluted template were used in 12.5 µl reaction volume. Primers utilized for the expression analysis of *SPMS*1 and *SPMS*2 genes are as follows:

Primer Sequence *SPMS1* (Real-Time PCR):

Forward Primer: 5’ GCTGCTTTTATTCTGCCAACA 3.'

Reverse Primer: 5’ CTGTCCCGCATTACCTTCA 3.'

Primer Sequence *SPMS2* (Real-Time PCR):

Forward Primer: 5’ CAACTTCCTGGCACCCATAAA 3’

Reverse Primer: 5’ CTGGTTCTGCTGATGTTTCCTCT 3.'

The threshold cycle number for product detection (CT value) was used to calculate the relative expression levels.

3.2.3.5 Polyamine analysis

For isolation and quantification of polyamines, plant material was grinded with a pestle and mortar in liquid nitrogen, and 100 mg was mixed with 1.5 ml of a 5% solution of perchloric acid, and shaken for 2 h at 4°C in 2 ml tubes. The supernatant (S1) was transferred to a new 2 ml tube after 20 min centrifugation at 15,000 rpm. The remaining pellet P was resuspended in 1 ml of a 1N NaOH solution. Afterward, 250 µl of the supernatant S1 and 250 µl of the dissolved pellet P were each transferred to a separate hydrolysis flask (NEOLAB - spit ampoules, Heidelberg, Germany) and 250 µl of a 12N HCl solution were added to each flask. The hydrolysis flasks were then sealed airtight by melting the glass over a Bunsen burner. The hydrolysis was carried out in an oven (Memmert) at 110°C for at least 18 h. The remaining supernatants S1 were stored overnight at 4°C. After hydrolysis in the oven, hydrolysis flasks were opened, and hydrolysates were transferred to a 2 ml reaction tube and centrifuged for 2 min. Then 100 µl of the supernatant were transferred into a new reaction tube and dried at 60°C for 2 h in a speedvac (RVC 2-25, Martin Christ Freeze Dryers, Osterode am Harz). The dried residues were resuspended in 100 µl of a 5% perchloric acid. At the end of the purification, the following samples were available for further analysis:

• 100 µl S1 (free polyamines - original supernatant)

• 100 µl S2 (soluble polyamines - hydrolysed supernatant)

• 100 µl P (insoluble polyamines - hydrolysed pellets)

i. Sample preparation for HPLC analysis

Following extraction, derivatization of the polyamines took place by dansylation with 5- dimethylamino-1- naphthalin sulfochloride (dansyl chloride) (Smith, 1985) to detect fluorescent derivatives.

In a reaction vessel, 100 µl of the sample was mixed with 100 µl saturated sodium carbonate solution and 400 µl of a dansyl chloride-acetone solution (7.5 mg/ml). The reaction vessels were shaken for 1 h at 60°C in

a thermal shaker. Then 100 µl of a proline solution was added (100 mg/ml) to the samples, and the probes were left at room temperature in the dark for another 30 min to prevent side reactions with other functional groups. After reacting with proline, the extraction was done with 500 µl toluene. The toluene extracts were evaporated to dryness at 40°C for about 1 h in the speedvac (RVC 2-25, Martin Christ Freeze Dryers GmbH, Osterode am Harz). The dried residues were stored at -20°C.

ii. Quantitative Polyamine analyses by HPLC

The dried samples were then treated with 400 µl of acetonitrile medium and analyzed by HPLC (Merck Hitachi - LaChrom D7000). Two detectors were used, i.e. a diode array detector (DAD, L7455) and a fluorescence detector (FL, L7485). A Merck LiChroCART 250-4, LiChrospher 100RP-18 (5 µm) column was employed. The mobile phase used was acetonitrile: water (9:1); the injection volume of the sample was 50 µl. An autosampler (Hitachi L-7200) was utilized. All area readings and peak values were recorded and analysed by the HSM D7000 software installed on the PC connected to the HPLC setup.

3.2.3.6 Selection of homozygous lines

Seeds of initially transformed barley lines were harvested and denomiated T0. Homozygous transgenic plants were determined by sowing T0 seeds to get first generation (T1) plants. The Mendelian ratio was checked. Plants were taken to maturity, and T1 seeds were harvested. Afterwards, T1 seeds were sown to determine the homozygous lines based on analysis of the second-generation (T2) seedlings.

3.2.4 Phenotypic analysis of transgenic lines at early developmental stages

Overexpressor and knockdown transgenic lines were compared with wild type plants by sowing in the glass house for the determination of different parameters at early growth stages. Similar conditions were created as mentioned in (Section 3.2.2.1). Genotypic analysis was done and

compared with wild-type i.e. Golden Promise. DNA was extracted as mentioned above and PCR performed to confirm transgenes. RNA was extracted, cDNA was synthesized as explained before and real-time PCR was done for expression analysis. Polyamines were obtained with the above-mentioned protocol to determine the change in spermine concentration. Samples were taken at early developmental stages i.e. two weeks, four weeks and six weeks.

I. Chlorophyll content index

Chlorophyll content is one of the major factors affecting photosynthetic capacity. Transgenic Overexpressor and knockdown plants which were kept at similar available water conditions were checked for chlorophyll concentration index. Readings were taken from the middle part of the primary leaf: using a SPAD (Soil Plant Analysis Development) analyser (Minolta, http://www.konicaminolta.eu) which measures transmittance of red light (650 nm) and infrared (940 nm) radiation through an intact leaf. The relative SPAD values are related to the chlorophyll content in a linear manner (Zhu *et al.*, 2012). Three different areas of a single leaf were measured, and three biological replicates were used to determine relative chlorophyll concentrations.

II. Transpiration rate and stomatal conductance

Stomatal conductance estimates the rate of gas exchange (carbon dioxide uptake) and transpiration (water loss) through the leaf stomata as determined by the degree of stomatal aperture. Primary leaf of 6-8 weeks old plants were measured. Measurements were taken at midday. Stomatal conductance of three different leaves from each plant with three biological replicates was measured by a Porometer (AP-4, Delta T-Devices, Cambridge UK) . The porometer was calibrated before every experiment.

III. Canopy temperature

To measure leaf temperature, an infrared thermal camera (FLIR T640) was used which was calibrated. Plants were moved to the middle of the table, one day before the measurements. Infrared thermal images were taken such that plants were not moved from their position. Results

regarding the change in temperature were calculated by FLIR Tools software, Version 5.2.

IV. Relative water content (RWC)

Under similar conditions as mentioned in Section 3.2.2.1, relative water content (RWC) was determined. Falcon tubes (50ml) were weighed without leaf samples and with leaf samples to subtract the difference to obtain leaf sample weight (W), after which the sample is immediately immersed in water and hydrated to full turgidity for 3-4 h under normal room light and temperature. After hydration, the samples were taken out of the water, dried with tissue paper, and immediately weighed to obtain the fully-turgid weight (TW). Samples were then oven dried at 80 ^{0}C for 24 h and weighed again after being cooled down in a desiccator to determine the dry weight (DW). RWC was calculated by the formula below:

RWC (%) = [(W-DW) / (TW-DW)] x 100

W – Sample fresh weight, TW – Sample turgid weight, DW – Sample dry weight

V. Excised leaf water loss (ELWL)

Restriction of water loss from leaf surfaces during periods of severe water stress is an important drought survival mechanism. The flag leaf of 6-8 weeks old plants was sampled in each of the three replicates. The leaves were weighed immediately after sampling (FW) after putting into an incubator at 28 ^{0}C for 6 h, and after oven-drying for 24 h at 90 ^{0}C. The excised leaf water loss (ELWL) from the excised leaves was determined by the formula given:

ELWL (%) = Fresh weight - weight after 6 h / Dry weight x 100

3.2.5 Statistical analysis

Statistical analysis was done using ANOVA. Randomized factors design was selected based on variation with plant type. One sample paired t-test was used to determine the differences between plant genotypes, i.e. Wild-

type vs. Overexpressor (OX) or Knockdown (RNAi) lines. All statistical analyses were performed in Statistix Version 8.1 (Analytical Software).

3.3 Results

3.3.1 Screening of putatively transformed plants

Spermine plays a role in stomatal closure which can lead to drought tolerance. *Spermine synthase* homologs had been identified in barley, and constructs were prepared to overexpress as well as knock down *SPMS* genes in barley via *Agrobacterium*-mediated transformation. Molecular analysis of transgenic lines was done to check the presence of transformed genes in barley plants.

3.3.1.1 PCR analysis for T-DNA presence

Screening of the transgenic lines was performed by PCR to confirm presence of the T-DNA insertion containing the overexpressor construct. Out of 30 lines generated for *SPMS*1 overexpression, 25 were tested positive (Fig. 3.1 a) whereas 16 out of 25 lines were positive for *SPMS*2 (Fig 3.1 b).

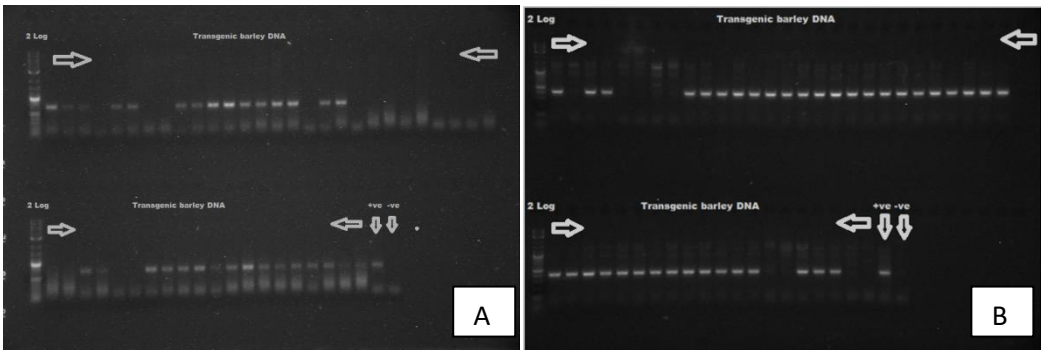


Fig. 3.1: PCR amplification with pLH7000 primers to screen transgenic overexpressor lines. a) *SPMS1* overexpressors, b) *SPMS2* overexpressors. a, b: Lane 1= 2 log DNA ladder, lane 2-50= DNA amplification of overexpressors, lane 51= positive control, Lane 52= negative control

3.3.1.2 Expression analysis

Positively confirmed lines were subjected to expression analysis by realtime PCR. Two *SPMS1* and eight *SPMS2* lines were found to

overexpress the target gene. Significant differences (P<0.05) were observed in only one *SPMS1* overexpressor line. However, SPMS2 expression was highly significantly increased inall eight *SPMS*2 overexpressor lines (P<0.001) (Fig. 3.2 a & b).

RNAi constructs were used to generate 11 transgenic plants, which were all positive for the T-DNA as confirmed by PCR. Expression analysis indicated five transgenic lines with significantly reduced *SPMS*1 expression (Fig. 3.2 c).

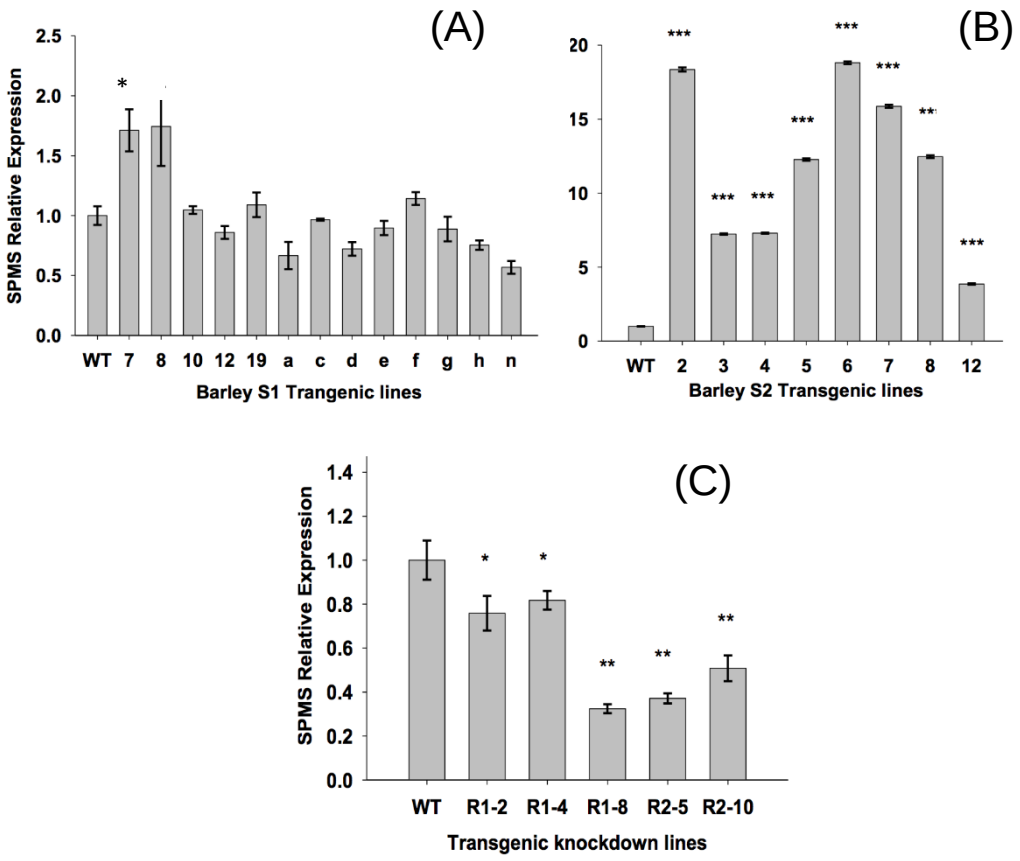


Fig. 3.2: Relative expression level of *SPMS1* and *SPMS2* in overexpressor and RNAi knockdown lines (a) S1 lines overexpressing *SPMS1* gene, (b) S2 lines overexpressing *SPMS2* gene, (c) knockdown lines generated via *RNAi* constructs R1 and R2 showing *SPMS1* expression. Significantly different at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level.

3.3.1.3 Southern hybridization

Southern hybridization was performed to identify transgenic lines with single gene inserts present. Ten lines which showed increased expression based on the real time PCR results were selected for Southern blotting. Results indicated that all lines carry more than one inserts (Fig. 3.3 a). Pooled DNA of T1 plants were used for the analysis. Some lines showed two inserts indicating the presence of two single insert or inserts linkage. To determine the linkage of inserts in transgenic lines DNA from each, individual line was selected instead of pooled DNA. Lines which were used were the one with two inserts. Blot results indicated same inserts pattern in all the plants suggesting inserts to be linked (Fig. 3.3 b).

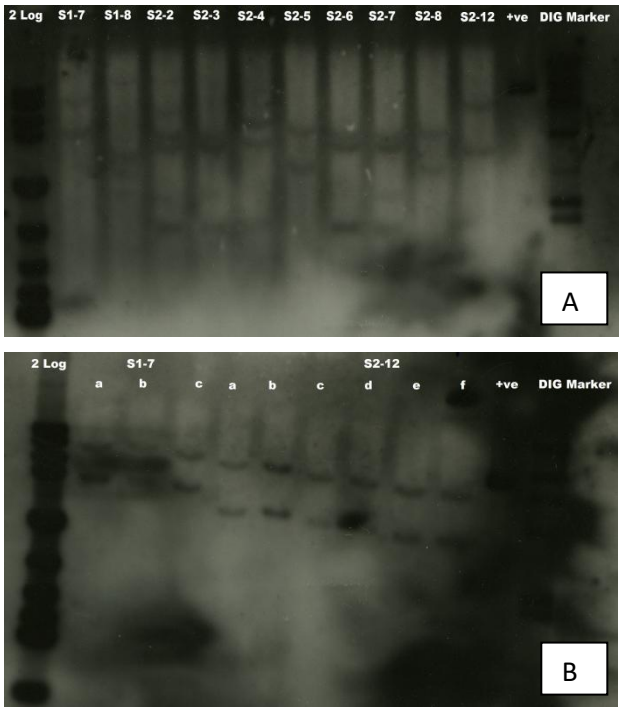


Fig. 3.3: Southern blot analysis of genomic DNA derived from 10 *SPMS* overexpressor lines (S1 and S2 lines) to determine the number of inserts. (a) Genomic DNA of pooled transgenic lines digested with EcoRI enzyme and detected with labeled probe. (b) DNA derived from individual positive plants of overexpressor lines S1-7 and S2-12.

3.3.1.4 Quantitative polyamine analysis by HPLC

Transgenic lines with overexpression and knockdown of *SPMS* genes had been identified. The effect of this altered expression on the production of spermine was determined quantitativly by HPLC. These analyses indicated an approximately two-fold increased spermine concentration in four of the overexpressing lines (Fig. 3.4 a). Plants carrying the *SPMS*1 overexpression construct did not show an increase in spermine concentration. Hence, only *SPMS*2 overexpressor plants were selected to be used for further experiments.

Transgenic knockdown lines showed reduced spermine levels (Fig. 3.4 b).

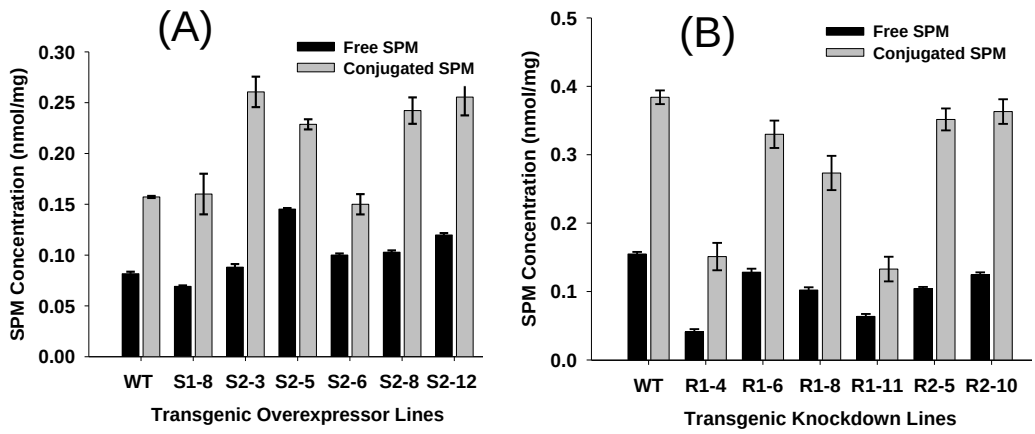


Fig. 3.4: Free and conjugated spermine concentrations in overexpressor (a) and *RNAi* Knockdown lines (b) which were 7-8 weeks old and analysed by HPLC. Black bars: Free spermine; gray bars: conjugated spermine, i.e. soluble and insoluble spermine.

3.3.2 Analysis of transgenic lines at early developmental stages

3.3.2.1 Genotypic analysis

Experiments were performed to analyze transgenic lines during early developmental stages,i.e. at 2, 4 and 6 weeks. Strong overexpressing (S2-3, S2-5 and S2-12) and knockdown lines (R1-4, R1-8 and R2-5) were selected from T1 generation plants. Expression analysis indicated

increased expression in early stages which continued to rise to 100 fold that in the wild type, whereas RNAi lines showed reduced expression by up to 50% which is upto 30% reduced after 6 weeks. The results indicated a highly significant increase in *SPMS2*expression in all overexpressor lines at different developmental stages (P< 0.001). RNAi transgenic lines showed a significant reduction in expression of *SPMS*1 gene which diminished with the age of the plants. However, despite an increase in the relative expression of the *SPMS1* gene in RNAi lines, a significant difference at P<0.01 level was still apparent after 4 and 6 weeks of plant growth (Fig 3.5 a, b & c).

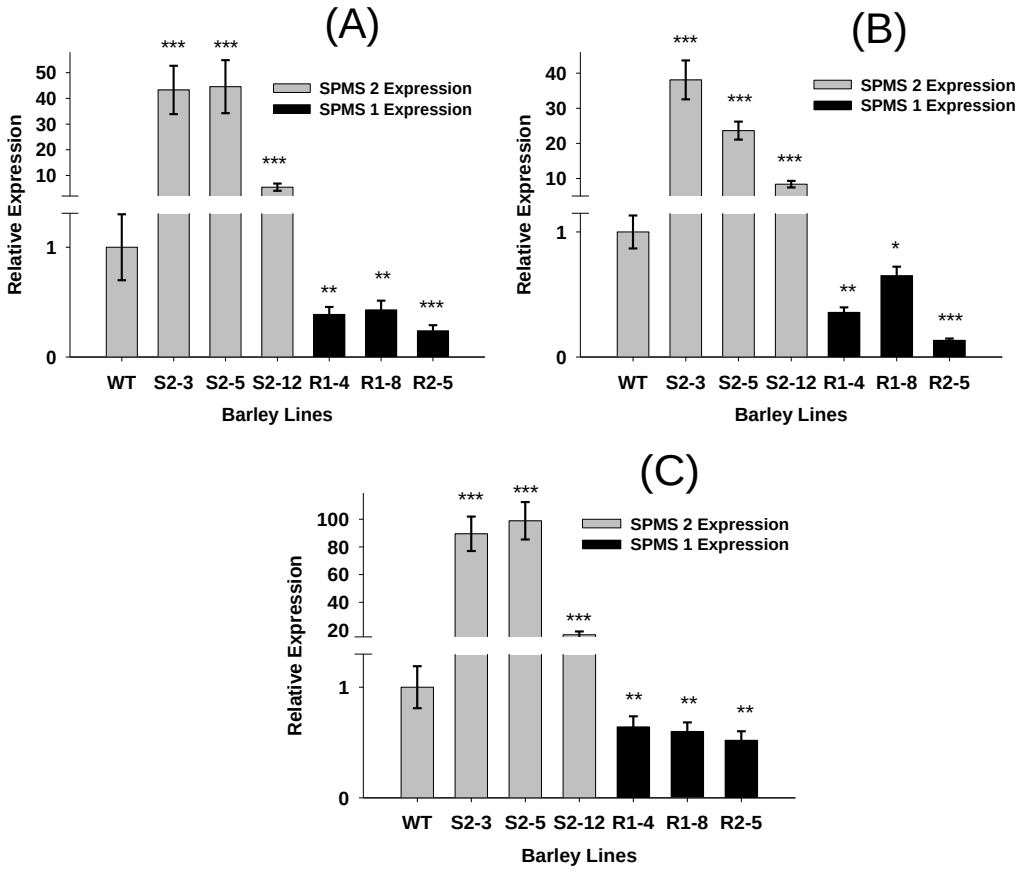


Fig. 3.5: Relative *SPMS* expression levels in overexpressor lines (S2-3, S2-5, S2-12) and knockdown lines (R1-4, R1-8, R2-5) compared to wild-type after (a) 2 weeks, (b) 4 weeks, (c) 6 weeks of growth. Significantly different to wild type at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data is presented as the mean of three biological replicates ± SE.

Overexpressor line (S2-5) showed an increase in spermine levels by up to 2 fold whereas 1.5 fold increase was observed in other two overexpressor

lines (S-3, S2-12) (Fig. 3.6 a). A significant increase in spermine concentration in overexpressor lines was observed after 2 weeks of growth. Though, an increase in endogenous spermine decreased the difference between transgenics and wild-type, and, after 4 weeks, a significant difference in spermine concentration could be seen in only two overexpressor lines (P<0.05). RNAi lines showed a tendencially reduction in spermine concentration that was not significant at early stages. Nevertheless, a significant decrease in one RNAi line (P<0.05) was found after 4 weeks due to an increase in endogenous concentration of SPM (Fig 3.6 a & b).

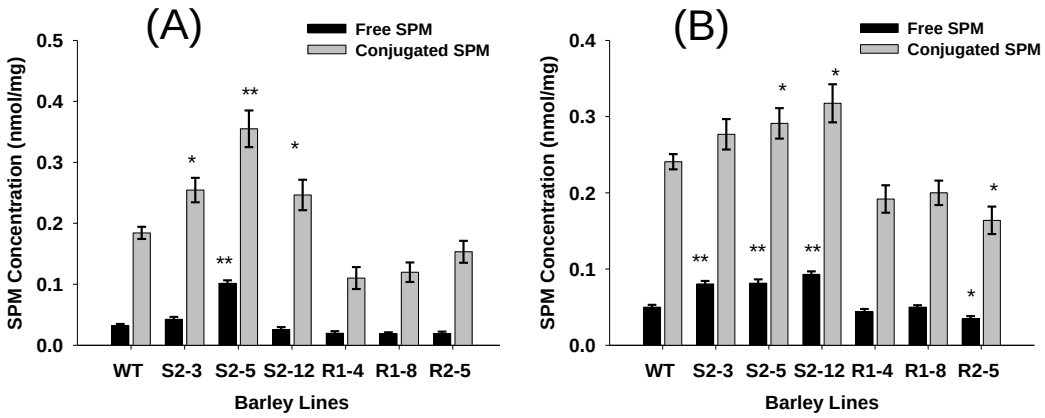


Fig. 3.6: Spermine level of overexpressor and *RNAi* knockdown lines after (a) 4 weeks and (b) 6 weeks of growth. Significantly different from wild type at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Black bars: Free spermine; grey bars: conjugated spermine, i.e. soluble + insoluble spermine. Data is presented as the mean of three biological replicates ± SE.

3.3.2.2 Phenotypic analysis

Overexpressor and knockdown plants were subjected to a preliminary phenotypic analysis. Chlorophyll concentration index recordings indicated a significantly increased chlorophyll content in two overexpressor lines (P<0.05), whereas no change was observed in knockdown lines (Fig 3.7 a).

Measurement of stomatal conductance revealed high transpiration rates in knockdown lines (P<0.05) (Fig 3.7 b). In contrast, in overexpressor plants

a reduced stomatal conductance as compared to the wild type was observed (P < 0.01). This indicated that high *SPMS* expression resulted in partial stomatal closure resulting in reduced conductance.

The two overexpressor lines displayed a reduction in excised leaf water loss as compared to the wild type (P<0.05), whereas the knockdown lines revealed no significant change (Fig 3.7 c).

Leaf temperature (data not shown) and relative water content (RWC; Fig. 3.7 d) of plants grown under optimal growth conditions were not significantly different in transgenics and wild-type plants.

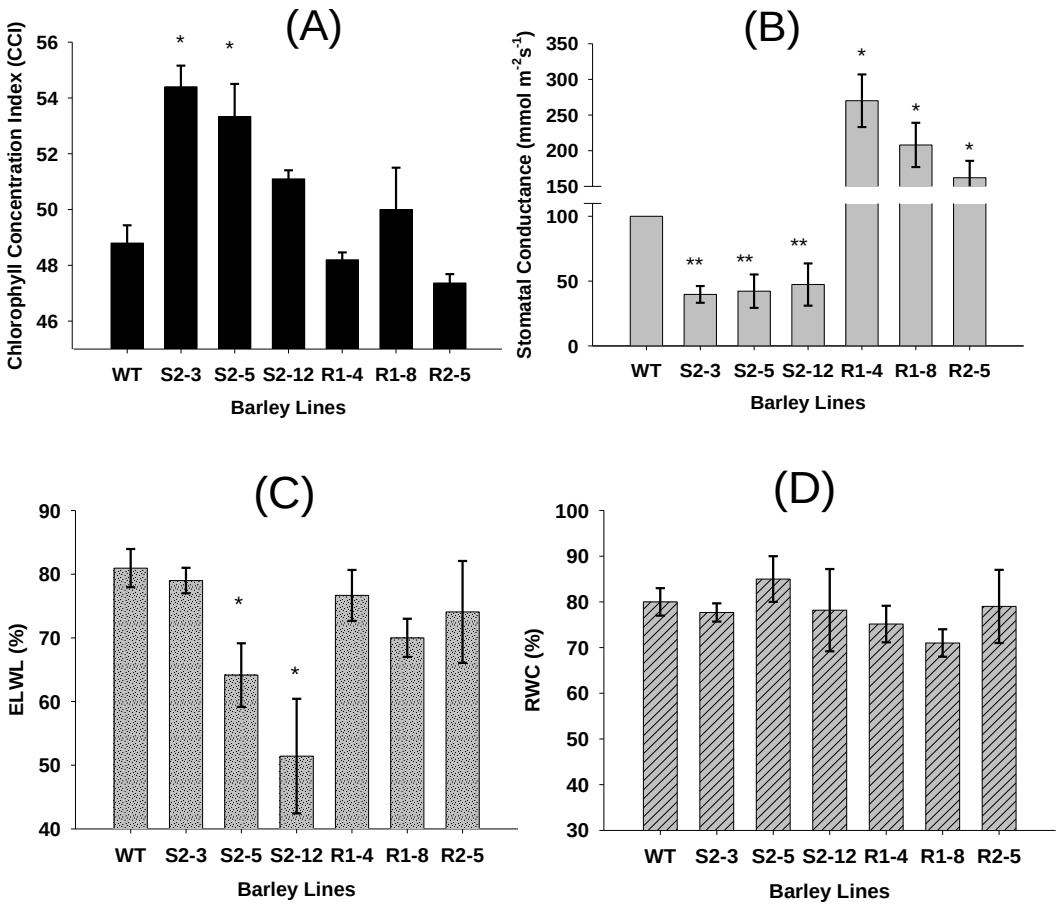


Fig. 3.7: Physiological analysis of overexpressor and *RNAi* knockdown lines and wild type after six weeks of growth. (a) Stomatal conductance (b) Chlorophyll concentration index (SPAD) values (c) Relative water content (d) Excised leaf water loss. Significantly different at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data is presented as the means of three biological replicates ± SE.

3.4 Discussion

It has been found that spermine plays a role in stomatal closure, which is a significant step in a drought tolerance response as observed in chapter 2 of this thesis. Spermine has also been attributed other important roles regarding plant defense under conditions of abiotic stress. Water-deficit stress has been observed to result in increased levels of spermine in drought-tolerant rice plants (Yang *et al.*, 2007). In support of these observations, Spermine-deficient *Arabidopsis* mutants have been reported to be more susceptible to drought (Yamaguchi *et al.*, 2007), while exogenous application of SPERMINE has been observed to enhance drought tolerance (Pang, 2007, Farooq *et al.*, 2009).

3.4.1 *Agrobacterium tumefaciens*-mediated barley transformation

Since modulation of spermine homeostasis appeared as a promising way to increase abiotic stress resistance, this study was conceived to investigate drought tolerance in barley, a monocot and major economic crop. *Spermine synthase* (*SPMS*) genes, which code for spermine-producing enzymes, were found in barley with two homologs, named *SPMS*1 and *SPMS*2. Constructs had been prepared for overexpression and knockdown of those genes. Using those constructs, transgenic barley lines had been generated in preparation of the present study. Some of the overexpressor and knockdown lines displayed an increased and decreased expression of *SPMS*, respectively.

In the past, barley has been successfully transformed through various methods, such as electroporation (Salmenkallio-Marttila *et al.*, 1995), microinjection (Holm *et al.*, 2000), particle bombardment (Hagio *et al.*, 1995, Manoharan and Dahleen, 2002) and *Agrobacterium tumefaciens* (Tingay *et al.*, 1997, Murray *et al.*, 2004). The latter method has been particularly successfully demonstrated in barley. However, its success is limited mostly to cultivars, such as Golden Promise, that are easily regenerable. The first report of *Agrobacterium*-mediated barley

transformation was provided by (Tingay *et al.*, 1997). They used the Golden Promise cultivar for transformation and immature embryos as explant for *Agrobacterium* infection. In that study, a binary vector was used that carried the *bar* gene as selectable marker, and bialaphos was used as screening agent. A range of tissues other than immature embryos have also been used as explants for the transformation of barley using *Agrobacterium*; this includes mature scutellum (Kovalchuk *et al.*, 2013), microspores (Kumlehn *et al.*, 2006) and young ovules (Holme *et al.*, 2008). Recently, a high-throughput *Agrobacterium*-mediated barley transformation was developed with 25% transformation efficiency based on the infection of immature barley embryos with Agrobacterium (Bartlett *et al.*, 2008).

To generate transgenic plants for this work, the transformation was performed by the *Agrobacterium*-mediated technique. (Trifonova *et al.*, 2001) examined some of the variables that can affect the efficiency of transformation of Golden Promise by *Agrobacterium*, such as growth regulators, osmotic treatment, wounding, and acetosyringone treatment. Acetosyringone has been known to increase the effectiveness of wheat transformation if used during co-cultivation. Preculturing immature embryos for 2 d before inoculation was better than for 1 d, and acetosyringone in the media increased transient expression in wheat by (Wu *et al.*, 2003), who also found that larger embryos and longer immersion and co-cultivation times increased transient expression. Better efficiency was obtained without the addition of acetosyringone. In the generation of lines for the present study, green calli were subjected to phytohormones for the regeneration of organs shoot and roots; antibiotics and selection marker were added to eliminate the negative transformants. Finally, transformants were moved to soil to obtain mature plants. Transformation efficiency was around 45%.

3.4.2 Screening for gene of interest in transgenic barley

Screening methods used to confirm the positive transgenic plants included a PCR analysis as first step. PCR of transgenic plant material allows the

detection of parts of the inserted T-DNA (Tao *et al.*, 2001). Successful PCR was done after design of primers, minimizing theformation of primer dimers. In addition, thermocycler settings were optimized before starting the analysis of transgenic plant material.

Transgenic barley lines misexpressing *SPMS* genes have been successfully identified by PCR. This was followed by an expression analysis of the target gene. Real-time PCR has been known as the most efficient method for the determination of gene expression. To determine the concentration of target product, i.e. spermine, HPLC was used, which is known to allow very accurate and precise determination via separation. In some transgenic overexpressor lines, *SPMS* transcripts were accumulated, and Spermine content increased. The levels of transcripts were increased approximately 4 to 20 folds in all *SPMS2* overexpressors compared to those in WT. *SPMS1* overexpressor lines did not show gene upregulation in all but two lines which exhibited an increase of upto 2 folds. *SPMS1* overexpressors did not show increased expression of more than 2 folds with most lines having similar values as WT. This can be due to the lethal effect of overexpression of *SPMS1* gene where plants did not survive with high transcript level and only plants surviving displayed smaller relative expression values compared to wild type. A 30-50% decrease in transcript level in *SPMS* knockdown transgenics was observed. Two RNAi constructs were transformed each having the potential to knock down both *Spermine synthase* genes. RNAi constructs were able to decrease *SPMS1* expression whereas *SPMS2* expression did not significantly change as compared to wild type. Spermine content was also found to be increased by up to 2 folds in overexpressors, whereas a 50% reduction in spermine concentration was measured in knockdown lines.

Transgene expression has been positively correlated with the number of copies of T-DNA present in transgenic plants (Gittins *et al.*, 2003, Afolabi *et al.*, 2004). However, single insertions are needed for the future breeding of the plants. So in the present study, Southern blot analysis was employed to determine T-DNA copy number in the transgenic plants.

Southern blotting proved to be an effective technique for this purpose. However, due to the large genome size of barley, many difficulties arose initially, starting from digestion to background on the films. Many problems were faced and eventually overcome during this technique like optimization of primers to prepare probes, blocking of membrane, hybridization temperature optimization, hybridization of probes and development of chemiluminescent films. The following parameters were changed from the standard protocol: (1) Probes were prepared and used to detect the T-DNA. (2) Digested DNA was fixed to the membrane by UV treatment as well as heating at high temperature to enhance chances of linking. (3) Hybridization was done for two days wherein salmon sperm DNA was added to exclude all non-specific binding and hence decrease the background effect on the film. (4) Stringent washing conditions were applied which led to satisfactory results on chemiluminescent films. The Southern blot analysis showed that overexpressor lines had either multiple inserts or single linked inserts present. Three transgenic lines were selected based on least number of linked inserts. In future experiments, the number of inserts may be reduced by chosing another explant tissue.(Grevelding *et al.*, 1993) demonstrated in *Arabidopsis* that the explant type had an effect on the number of T-DNA copies inserted. Root-derived transgenic plants contained fewer multiple insertions of the T-DNA, i.e. 36%, compared to leaf disc-derived plants, which had 89%.

3.4.3 Physiological confirmation of transformed plants during developmental assay

After the successful screening, we selected three barley *SPMS2* overexpressor lines and three *SPMS* knockdown lines for further analysis. A developmental assay was performed to study *SPMS* expression and phenotypic parameters of the transgenic barley plants. It was confirmed that transgenic plants were altered in *SPMS* expression. Overexpressor lines showed an increased expression, which became higher with plant age. Conversely, the RNAi constructs limited the *SPMS* expression. However, an increase in relative expression compared to wild-type was

visible as the plants developed, which was due to the decrease in endogenous absolute expression of the *SPMS1* gene while the plant grows. Similarly, comparable results were obtained for polyamine concentrations, with a consistent increase in spermine levels in overexpressor lines in early growth development stages compared to wild type plants.

Drought in plants initiates a complex set of responses which begins with the perception of stress, triggering various levels of physiological, metabolic and developmental responses (Mahajan and Tuteja, 2005). In the initial characterization of the transgenic lines, drought-relevant phenotypic parameters were considered. Transpiration rate, determined by stomatal conductance, of transgenic lines was determined. Overexpressor lines for *SPMS* genes showed a decreased transpiration rate whereas knockdown lines exhibited an increased transpiration compared to wild-type. Most of the total water absorbed by the roots is lost via transpiration (Sterling, 2004). The main driving force of transpiration is the water potential gradient between the inner space of stomata and the atmospheric air (air-water potential).

Chlorophyll content, which has been considered as an important parameter to measure the plant´s ability to survive drought, was determined non-destructively by a SPAD meter. Overexpressor lines had higher relative chlorophyll values, indicating a better photosynthetic potential of the transgenics. Chlorophyll levels are closely related to the photosynthesis rate because they allow plants to absorb energy from light (Porra *et al.*, 1993). With a higher amount of chlorophyll in the leaves, a higher photosynthesis rate can be maintained (Kura-Hotta *et al.*, 1987).

High rate of photosynthesis can be obtained by retention of chlorophyll which is achieved by low transpiration rate to produce required amount of assimilating (Flexas *et al.*, 2013).

Misexpression of *SPMS* genes did not lead to changes in relative water content of the leaves under optimum growth conditions. However, excised leaf water loss (ELWL) was a vital tool under normal conditions. It is the

ability of excised leaves to lose water after they are cut from the plant grown in controlled environment. Overexpressor lines showed less water-losing potential than wild-type and knockdown lines. ELWL may more closely reflect the balance between water supply to the leaf and transpiration rate. Genotypes indicating a low excised leaf water loss have a better capability to maintain water balance in their leaves under stress conditions, which plays a considerable role in stress tolerance (Behl, 1998).

Taken together, this study identified SPMS-misexpressing barley lines which were altered in spermine content and which showed physiological differences to wild type plants that may contribute to an increased drought tolerance.

4 CHAPTER 4

The role of spermine in drought tolerance of barley (*Hordeum vulgare*)

4.1 Introduction

4.1.1 Polyethylene glycol (PEG) 6000 as drought stress mimic

Stress experienced by plants due to unexpected environmental conditions is termed abiotic stress (Gupta and Kaur, 2005). One of the factors most responsible for decreasing crop yields is considered to be drought stress (González *et al.*, 2008). Drought affects plant growth, metabolism, and hence crop yield (Lu and Neumann, 1998, Szilgyi, 2003). This stress, induced by lack of water in the soil, affects various physiological processes of the plant, starting with reduced leaf development (Lu and Neumann, 1998). It affects the chlorophyll contents by reducing chlorophyll levels, which in turn inhibits photochemical processes and decreases the enzymatic activities of enzymes involved in the Calvin cycle (Monakhova and Cherniad'ev, 2002). Another drought effect observed in plants is the production of ROS which stimulates the up-regulation of oxidative stress protectors (Horling *et al.*, 2003).

A highly controlled methods to limit water availability to plants experimentally is by employing hydroponic systems (Munns *et al.*, 2010). Hydroponics, commonly termed as ‘water culture’ of plants, has been used for research purposes for a long time. It was developed following the understanding that plants need only a small number of inorganic elements to grow along with water, carbon dioxide, and light. It allows studying the plants in controlled conditions under different elemental deficiencies or toxicities, or different stress environments. Hydroponics systems are commonly used under controlled regimes of light and temperature in

glasshouses or growth cabinets. The osmotic stress applied in hydroponic culture is more controlled and homogeneous as compared to drought applied in soil-based systems.

One of the commonly used approaches to induce osmotic stress in hydroponic culture involves the application of high molecular weight osmotic compounds, e.g. polyethylene glycol (PEG) (Türkan *et al.*, 2005), which forms hydrogen bonds with water and makes it unable to enter the cell (Rubinstein, 1982). The addition of osmotic compounds in the hydroponics system may reduce the osmotic potential of the water and water available to the plants (Michel, 1983). PEG with higher molecular weight cannot be absorbed by the plant and hence is unavailable for plant uptake (Tarkow *et al.*, 1996). So, increasing the concentration of PEG can restrict water availability to the roots of the plants to create different stress intensities. Therefore, PEG has been used frequently to examine the effects of drought in physiological studies (Bradford, 1986).

Besides PEG, high concentration of macronutrients or mannitol have been used to impose water deficit conditions in nutrient solutions. However, mannitol has been known to be absorbed by the roots of the plants (Hohl and Schopfer, 1991), which affects drought responses and metabolism of the plant. PEG has been reported to be least likely to have this problem. It is not absorbed by intact plant roots, only through damaged roots which should be avoided (Miller, 1987). However, the application of osmotic stress by PEG introduces some problems. Particularly the aeration of the culture media is impeded due to the high viscosity of PEG. Hence very careful aeration is required (Verslues *et al.*, 1998).

4.1.2 Parameters for the monitoring of plant growth under drought stress

Plant phenotyping is the comprehensive assessment of complex plant traits. The plant phenotype includes different measurement parameters, such as root and shoot morphology (Walter *et al.*, 2009), biomass (Golzarian *et al.*, 2011), leaf characteristics (Arvidsson *et al.*, 2011), yield-

related traits (Duan *et al.*, 2011), photosynthetic efficiency, (Balachandran *et al.*, 1997, Bauriegel *et al.*, 2011), or biotic and abiotic stress responses (Balachandran *et al.*, 1997). Leaf water stress can be determined by different parameters such as stomatal behavior or leaf rolling and expansion (Dhanda *et al.*, 1998).

4.1.2.1 Stomatal conductance and density

Another parameter that affects the resistance of plants to drought stress is the stomatal density. The number of stomata per unit leaf surface is, within certain limits, a characteristic of a particular plant species or variety. It also has been shown that environmental conditions influence the stomatal density (Teare *et al.*, 1971).

Stomatal pores are major ports of the exit and entry of gasses into a leaf. The rate of gas diffusion is directly proportional to the cross-sectional area of the path and the difference in gas density along the path, and is inversely proportional to the length of the path. The "efficiency" of stomatal pores as pathways for gaseous diffusion depends primarily on pore size and distribution in the epidermis. The larger stomatal openings or a greater number of stomata per unit leaf area permit higher rates of photosynthesis by decreasing the diffusive resistance to CO_2 uptake.

The stomatal conductance regulates water loss from plants and carbon dioxide uptake (Cowan, 1978). This is a crucial process that usually depends on the rate of open stomata and differs in light and darkness or under different water stress conditions (Franks and Farquhar, 2007, Brodribb *et al.*, 2009). Stomatal conductance has been linked with water use efficiency (WUE) as well as photosynthetic capacity of the plant. Limitation of water and high evaporative demands of the plant leads to a closure of stomata to decrease the water loss (Ludlow, 1989, McDowell *et al.*, 2008). Therefore, this parameter has been commonly used to determine the plant response to drought stress.

4.1.2.2 Leaf Area

Leaf area is an important parameter that determines evapotranspiration, photosynthetic efficiency, water use efficiency, crop growth, and yield potential. Hence, this parameter is an important tool to asses primary plant production, and estimation of the leaf area can provide informations about the soil and water relation and transpiration in plants (Blanco and Folegatti, 2005). Larger leaf area has been known to produce larger spikes, higher grain weights and higher yield which was achieved by higher photosynthetic rates (Rasmusson, 1987). Larger leaf area also results in greater water loss through the leaves which decreased the plant growth. The reduced plant growth was the result of no turgor maintenance while lower turgor pressure can affect leaf area and shape (Kramer and Boyer, 1995).

4.1.2.3 Excised leaf water loss

The most common methods to determine leaf water stress are the determination of leaf water loss and leaf water potential. Reduction of water loss from leaf surfaces during periods of severe water stress is a significant drought survival mechanism (Rawson and Clarke, 1988). Evapotranspiration of drought-stressed plants and rate of water loss from excised leaves were compared successfully to determine drought resistance in wheat (Dhanda *et al.*, 1998).

4.1.2.4 Leaf temperature

Stomatal closure, known to be the first event in adaptation to drought results in increased leaf temperatures (Jones *et al.*, 2009). This increased temperature can have multiple effects on the plant, such as damage of cell membranes, which play a role in all cellular activities, and in turn weakens the plant's response to drought stress (Torres-Franklin *et al.*, 2007). Leaf temperature can be monitored by infrared thermal imaging and thus allows conclusions about the plant water relations (Merlot *et al.*, 2002). The leaf temperature is linked with many physiological processes such as stomatal

conductance, transpiration rate, plant water status, water use, leaf area index and crop yield (Lopes and Reynolds, 2010). Measurements of the leaf temperature are technically difficult, as it is dependent on many factors, like air temperature, humidity, light, and the wind (Jones *et al.*, 2009). Nevertheless, it has been used successfully for drought studies, for example in wheat and rice (Reynolds *et al.*, 1998).

4.1.2.5 Relative water content

Relative water content (RWC) is a simple yet reliable method to measure the water status of leaves. It requires no specialized equipment and has been shown to be a more accurate method to determine the plant response to different stress conditions than the determination of the leaf water potential (Sade *et al.*, 2009, Sade *et al.*, 2012).

Increasing the relative water content is one mechanism to improve drought stress tolerance. Decreasing the dehydration and thus increasing the RWC depends on many factors, like the thickness of the cuticle, water storage mechanisms, and stomatal closure. It is thus reported that high relative water content is the result of increasing the osmotic potential or decreasing the elasticity of cell walls (Ritchie *et al.*, 1990).

4.2 Materials and Methods

4.2.1 Drought experiment

4.2.1.1 Cultivation of *Hordeum vulgare* under drought conditions on soil

To determine differences in drought tolerance, growth of homozygous barley lines overexpressing the *Spermine Synthase 2* (*SPMS2*) gene was compared to the wild-type under increasing drought stress conditions. As control, the barley cultivar Golden Promise was used. Plants were cultivated on soil (ED 73) in 5-l Mitscherlich pots in an air-conditioned greenhouse under long day conditions with a photoperiod of 16 h and a temperature of 21 ^{0}C and 18 ^{0}C in the night. The relative humidity was 60-70% throughout the experiment.

Relative soil water content was calculated by weighing the pots. The weight of saturated soil was determined as 100 % relative soil water content, while the weight of desiccated soil (dryed for five days at 80 ^{0}C) was determined as 0% relative soil water content. The relative water content of the soil was adjusted to 65%, which was 550 ml water per 1.5 kg of soil.

Seeds were stratified for 48 h at 4 ^{0}C in wet filter paper. For germination seeds were transferred to room temperature and left in the dark for another day. 10-12 germinated seeds were transferred to Mitscherlich pots with 1.3 kg soil and covered with another 200 g of soil. To induce drought stress under environmentally relevant conditions, the soil was allowed to dry stepwise. After four days of cultivation only the pots of the control conditions were irrigated further. For randomization, the position of the individual pots was changed daily. Three lines were selected i.e. two overexpressor transgenic lines (S2-3, S2-5) and wild type. Ten replicates of each line were sown for the experiment under both normal and stress conditions. The experiment was repeated twice.

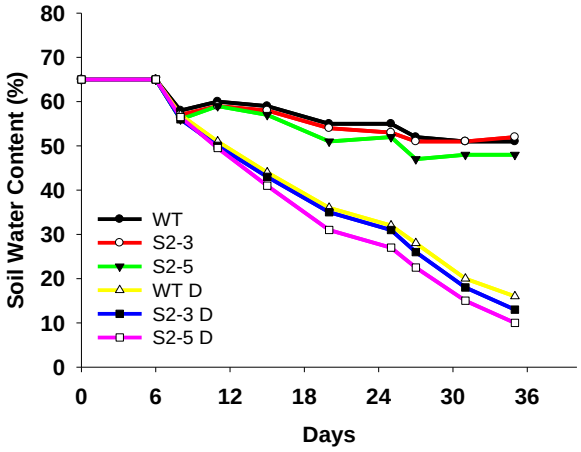


Fig. 5: Soil water content data during the drought stress experiment in overexpressor transgenic lines (S2-3, S2-5) and wild type. Black=WT, Red=S2-3, Green=S2-5, indicating control conditions. Yellow=WT D, Blue=S2-3 D, Pink=S2-5 D, indicating drought conditions

To determine differences in drought stress tolerance, different parameters were assessed at six different time points, i.e. 6, 12, 18, 24, 30, and 35 days after planting of the seeds. Drought was applied after six days.

4.2.1.2 Genotypic analysis and polyamine quantification

The primary leaf was used for analyses. Five biological replicates were harvested into liquid nitrogen for isolation of RNA and polyamines. RNA extraction was performed manually and cDNA was synthesized using M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase**)** enzyme for quantitative real-time PCR analysis (Section 3.2.3.4). Polyamines were extracted from the same leaf, and their concentration was determined by high performance liquid chromatography (HPLC) as described before (Section 3.2.3.5). Sampling for the extraction was done at 10:00 am.

4.2.1.3 Phenotypic analyses

All measurements were taken using the primary leaf at the indicated six time points with five biological replicates. A thermal imaging camera was used to determine the leaf temperature as described before (Section 3.2.4, Part III). To determine the stomatal conductance, Porometer (AP-4, Delta

T-Devices, Cambridge UK) measurements were performed as described before (Section 3.2.4, Part II). Measurements were done starting at 12:00 PM. Chlorophyll concentrations were determined non-destructively by a SPAD meter as described before (Section 3.2.4, Part I).

Length of the primary and secondary leaves was determined using a measuring tape or ruler. Relative water content (RWC) was determined as previously described (Section 3.2.4, Part IV).

4.2.1.4 Chlorophyll fluorescence

The photosynthetic efficiency of transgenic lines was determined by using a Photosynthesis Yield Analyzer (Mini-PAM, Walz) using the pulse amplitude modulation (PAM) method (Genty *et al.*, 1989). Leaves were dark- adapted for 10 min before measurements. The effective photochemical quantum yield of PS II Y(II) was calculated as mentioned in (Genty et al. 1989; Kramer et al. 2004).

4.2.1.5 Statistical analysis

Statistical analysis was performed to compare lines and treatments. RWC, photosynthesis, chlorophyll content, leaf temperature, and stomatal conductance data were analyzed separately for plants under control and stress conditions using repeated measures ANOVA with two factors: Plant type (WT vs. transgenics), and time (day of treatment). Variation between pots (nested within treatment) was included as a random factor. The analysis was represented by the linear model. For interaction effects, Tukey's pairwise comparison after (Tukey, 1949, Haynes, 2013) was used to determine differences between plant type at each time point, i.e. different treatment levels.

To determine differences of the plant type in response to control and drought conditions, one sample / paired t-test was used. Within a day of treatment, differences between plant type (wild-type vs. transgenic lines) are indicated in figures with asterisks,* = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. All statistical analyses were performed in Statistix Version 8.1.

4.2.2 Hydroponics experiment

Two independent homozygous *SPMS2*-overexpressing lines, S2-3 and S2-5 , and two knockdown lines, R1-4 and R2-5, were used. The barley cultivar Golden Promise served as control. Seeds were stratified and allowed to pre-germinate as previously described (Section 4.2.1.1) and cultivated on liquid culture media (1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.0 mM NH_4NO_3, 1.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 mM NaCl, 2.4 mM MES (2-[N-Morpholino]ethanesulfonic acid) with pH adjusted to 5.8 with 1M NaOH, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 0.2 mM FeNaEDTA).

Osmolality of the PEG 6000 solutions at different concentrations were measured by osmometer (Fischer Scientific). Osmotic stress was applied by adding 0% (control), 14% (moderate stress), or 18% (severe stress) polyethylene glycol 6000 (PEG 6000) to hydroponic culture media after seven days. Plants were cultivated for another week under the same conditions. The osmotic pressure of the solutions containing different concentrations of PEG 6000 was determined. The medium was changed twice a week.

4.2.2.1 Physiological parameters of plants grown on hydroponics

Stomatal conductance was determined with the help of a porometer as previously described (Section 3.2.4, Part II). Chlorophyll content was measured by SPAD meter as mentioned earlier (Section 3.2.4, Part I). Biomass was determined by weighing the plants after washing with distilled water and drying with blotting paper to remove the entire nutrient solution from the roots. Leaf area of the primary leaf as well as the whole plant, was determined by using a LI-3100 Area Meter (LI-COR, Lincoln, Nebraska, USA). Relative water content (RWC) was determined as previously described (Section 3.2.4, Part IV). Measurements were performed at day 7 and day 14.

4.2.2.2 Statistical analysis

Statistical analysis was done to compare lines and treatments. RWC, biomass accumulation, leaf area and stomatal conductance data was analyzed separately for overexpressor and knockdown lines using repeated measures ANOVA with two factors: Plant type (WT vs mutants), and treatment (control vs stressed). Variation within treatment was included as a random factor. This analysis is represented by the linear model. For interaction effects, Tukey's pairwise comparison after (Tukey, 1949, Haynes, 2013) was used to determine differences within plant type at different treatment levels, i.e. 0%, 14%, and 18% PEG. One sample / paired t-test was used to further examine the treatment effect between different plant type. All statistical analyses were performed in Statistix Version 8.1.

4.2.3 Long-term drought experiment

To determine post-harvest parameters under drought stress, plants of overexpressing mutant lines S2-3 and S2-5 were cultivated on soil as previously described (Section 4.2.1.1) and allowed to grow till maturity. As control, the cultivar Golden Promise was used. The relative water content of the soil was adjusted to 65% for the control plants. Drought stress was applied at an intensity of 35% soil water content by drying out, which started at the 10^{th} day after sowing, followed by controlled watering to maintain the moisture level till harvesting. Plants were cultivated under glass house conditions with a photoperiod of 16h and a temperature of 21 ^{0}C (day)/ 18 ^{0}C (night). The relative humidity remained at 60%-70% throughout the experiment. The experiment was performed with five biological replicates per line and treatment.

4.2.3.1 Post-harvest parameters

Plants were harvested after four months to determine post-harvest parameters. The tiller number was counted after two months and four months of growth. The height of each plant was measured by using a

measuring tape. Plants were carefully cut above soil level to determine the above ground biomass. The number and weight of spikes was determined for each plant. Seeds were obtained by threshing, and seed weight was determined. Harvest index of each line under control and drought conditions was determined by dividing the total weight of seeds by total weight of above-ground biomass.

4.2.3.2 Statistical Analysis

All post harvest parameters recorded were analyzed by using repeated measures ANOVA with two factors: Plant type (WT vs mutant lines), and drought treatment (control vs stressed). Randomized factors design was selected based on variation within treatment with linear model used for analysis. For interaction effects, Tukey's pairwise comparison was used to analyze differences between plant types at treatment level, i.e. control (65% Soil water content) and drought (35% Soil water content). One sample / paired t-test was used to further examine differences between treatment in each plant type. All statistical analyses were performed in Statistix Version 8.1.

4.2.4 Stomatal bioassays on transgenic lines

The number of stomata per cm^2, stomatal size, and stomatal aperture of *SPMS* overexpressors (S2-3 and S2-5) and RNAi lines (R1-4 and R2-5) were compared to those of the wild-type. Seeds were sown on soil and cultivated under glass house conditions as previously described (Section 4.2.1.1). Epidermal strips of two-week-old plants were isolated, transferred to opening buffer (10 mM MES-KOH, 50 mM KCl, and 50 µM $CaCl_2$), and incubated at above mentioned conditions for two hours. Measurement of stomatal aperture and stomatal area was performed by microscopic analysis in AxioVision Software. The number of stomata was determined by counting. Three biological replicates were performed, and 3-5 frames (375000 μm^2) per epidermal strip were counted. Stomatal apertures were measured under a Zeiss inverted microscope (Axiovert 40 CFL, AxioCAM) with a 40x lens.

4.2.5 Statistical analysis

Stomatal bioassays were analyzed using ANOVA. Randomized factors design was selected based on variation with plant type. For interaction effects, Tukey's pairwise comparison was used to analyze differences between plant types, i.e. WT and transgenic lines. One sample / paired t-test was also used to further confirm the differences between each plant type. All statistical analyses were performed in Statistix Version 8.1.

4.3 Results

4.3.1 Drought Experiment

4.3.1.1 Expression Analysis and polyamine quantification

Drought stress experiments with *SPMS2*-overexpressing lines (S2-3 and S2-5) were performed, and typical parameters to characterize the stress response of the plants were analysed.

An expression analysis was conducted at five different time points under control and drought stress conditions (Fig. 4.1). The absolute expression data indicated an increase of *spermine synthase 2* (*SPMS* 2) expression in the first 24 days in lines under both control condition and drought stress. Reduction in expression was observed after 30 days of growth compared to earlier time points under both conditions. Expression of *SPMS* 2 under drought was increased in overexpressor lines compared to control conditions. There was no significant increase in expression under drought stress compared to control condition in either of overexpressor lines throughout the experiment. However, a decrease in expression of *SPMS*2 of wild-type plants was observed after 30 days under drought stress which was also not significant when compared within genotype.

The calculation of relative expression values (not shown) of overexpressor liunes to wild type showed an increase of *SPMS*2 expression under drought. Overexpressor lines exhibited a higher *SPMS2* expression under control conditions, with an increase by up to 20–80 folds. Gene expression increased under increasing drought stress. It can be observed that wild-type plants were wilting under extreme drought conditions (see below), and led to decreased absolute expression values, hence relative expression values increased *SPMS2* expression in transgenic lines under drought.

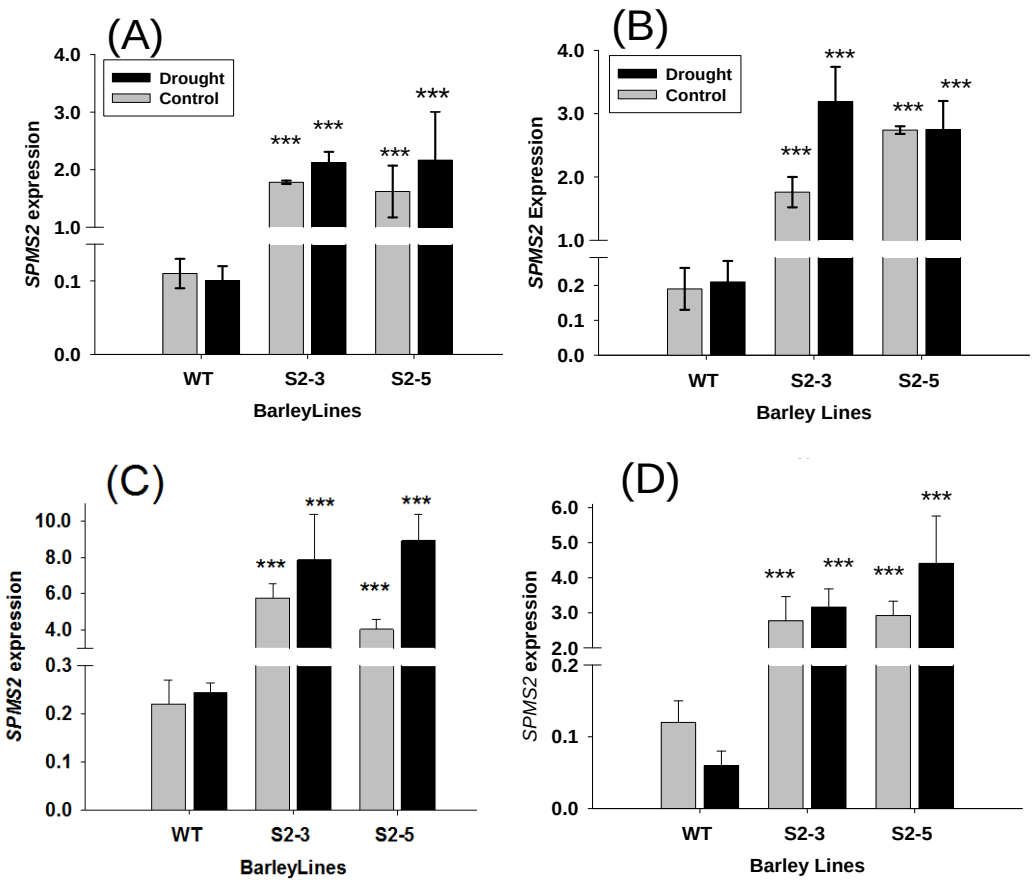


Fig. 4.1: Expression of *SPMS2* (normalized to *GAPDH*) of *SPMS2* overexpressor lines S2-3, S2-5 compared to the wild-type under control and drought conditions. The measurements were performed at (a) 12 days, (b) 18 days (c) 24 days (d) 30 days. Significantly different compared to wild type at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data represent means (± SE) of four biological replicates.

4.3.1.2 Phenotypic analyses

Spermine concentrations (free and conjugated) were found to be increased in the *SPMS2* overexpressor lines under both, unstressed and drought conditions. (Fig. 4.2) Under drought conditions, an increase in spermine concentration was observed in leaves of all plants of the wild-type and the overexpressor lines. There were no significant differences within genotype under control and drought conditions. However, significant increase in spermine was observed in overexpressor lines.

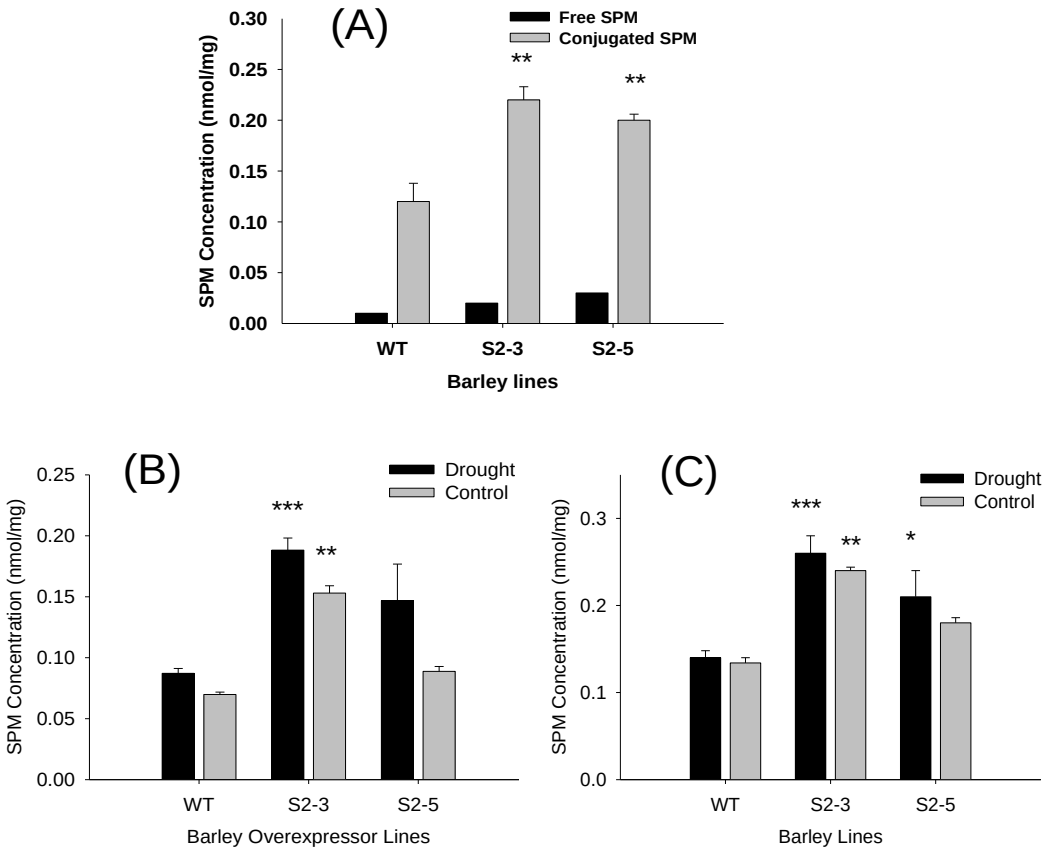


Fig. 4.2: a) Spermine level of overexpressor lines compared to wild-type cultivar Golden Promise under control conditions after 6 days under control condition B) Total spermine concentration of overexpressor lines compared to wild-type cultivar Golden Promise under control and drought conditions after 18 days c) after 30 days. Significantly different at * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 level. Data represent means (± SE) of four biological replicates.

Significant differences to wild type plants were observed in the transpiration rate of wild-type at different time points after the application of drought stress. In all lines, an increased transpiration under control conditions was observed after 12 days, which was significant compared to all other sampling time points. It was also noted that the transpiration rate between wild-type and the overexpressor lines were significantly reduced during the whole experiment under control conditions (Fig. 4.3 a). However, under increasing drought stress, the *SPMS2* overexpressing lines showed an increased stomatal conductance in comparison with the wild-type as soil water content remains only 20% (Fig. 5), probably due to less water availability in the leaves. Significantly reduced conductance in

the *SPMS2* overexpressor line S2-3 was observed at the beginning of the drought stress (d 12). After 24 days, the overexpressor line S2-5 was found to have considerably increased conductance values compared to wild-type. Under higher drought stress, both overexpressor lines showed a significantly increased transpiration rate than wild-type (Fig 4.3 b).

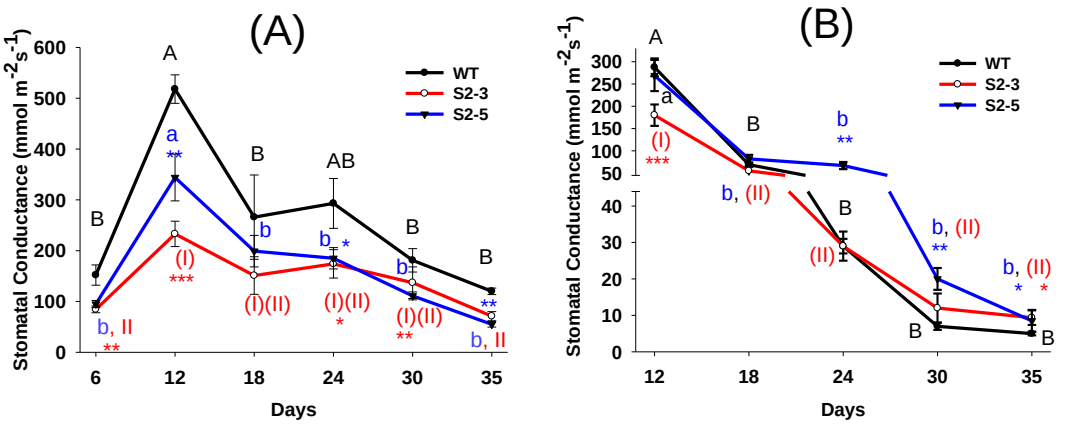


Fig. 4.3: Stomatal conductance determined by porometer of *SPMS2* overexpressor lines compared to the wild-type cultivar Golden Promise under a) well-watered conditions and b) drought stress. Significant differences between days are indicated with Roman alphabet letters (S2-3 OX transgenic line), lower case letters (S2-5 OX transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.01$. Within a day, significant differences between overexpressor lines and the wild type are indicated with asterisks,* = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the means of five plants ± SE.

Thermal imaging of plant leaves showed that the temperature of leaves of both *SPMS* overexpressor lines were tendentially increased under well-watered conditions, which corresponds to the decreased transpiration rate of those lines. However, these differences were not significant (Fig. 4.4 a). The application of drought stress resulted in a significantly lower leaf temperature of the overexpressor lines compared to wild-type after day 24 and day 35 at $P< 0.05$ (Fig. 4.4 b). Whereas the wild type showed a steady increase of leaf temperature over time under drought conditions, leaf temperature of the mutant remained constant (Fig. 4.4 b). This result may be explained by the reduction in stomatal conductance in wild-type, but not overexpressors, under severe drought (Fig. 4.3 b)

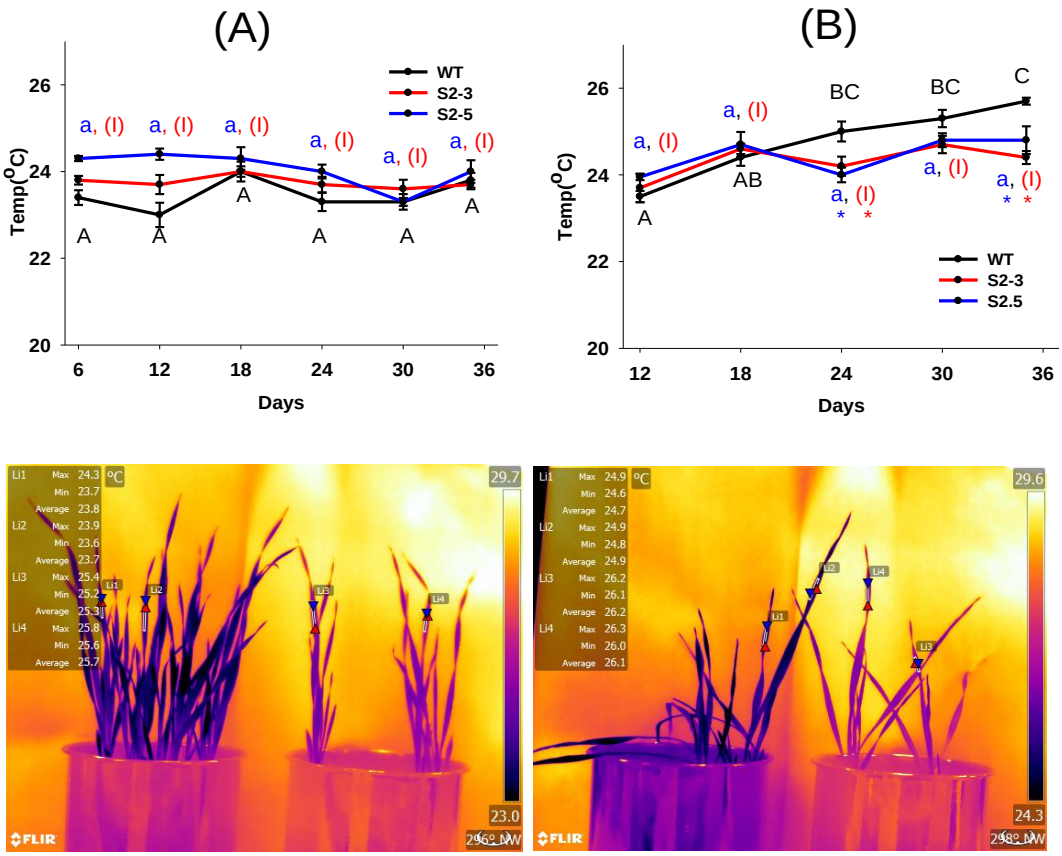


Fig. 4.4: Leaf temperature determined by thermal camera imaging of *SPMS2* overexpressor lines compared to wild-type a) under well-watered conditions and b) under drought conditions. c) thermal image of OX line (S2-3) after 35 days under control and drought d) thermal image of WT after 35 days under control and drought. S2-5 line thermal image not shown. Significant differences between days are indicated with Roman alphabet letters (S2-3 OX transgenic line), lower case letters (S2-5 OX transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.01$. Within a day, significant differences between overexpressor lines and the wild type are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of five plants ± SE.

The relative water content (RWC) of leaves measured under control conditions was comparable in all lines and did not change over time (Fig. 4.5 a). In contrast, leaf RWC showed a progressing reduction in the wild type under drought stress, whereas *SPMS2*-overexpressing lines showed the ability to retain water under those conditions, with no significant decrease in leaf water content (Fig. 4.5 b). This lead to highly significant

differences between the overexpressing lines and the wild-type under extreme drought.

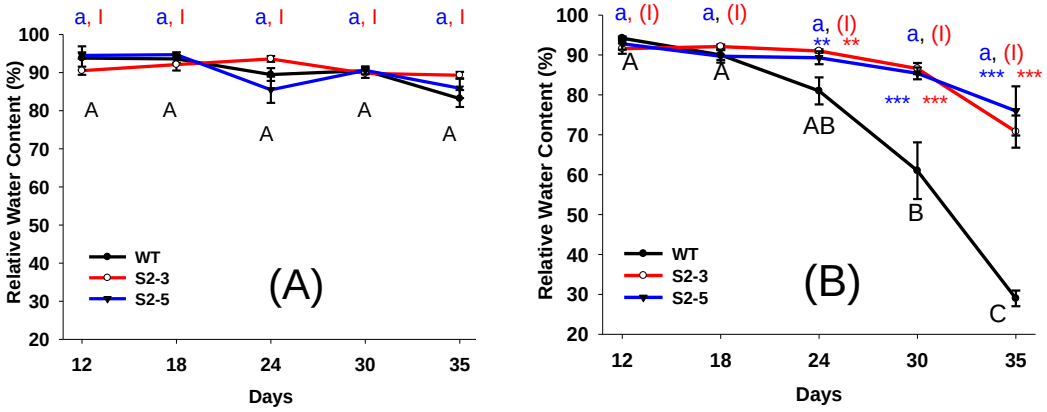


Fig. 4.5: Relative water content of *SPMS2* overexpressor lines compared to wild-type (a) under well-watered conditions and b) under drought conditions Significant differences between days are indicated with Roman alphabet letters (S2-3 OX transgenic line), lower case letters (S2-5 OX transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.01$. Within a day, significant differences between overexpressor lines and wild type are indicated with asterisks,* = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of five plants ± SE.

Chlorophyll concentrations under control conditions were similar on the six time points measured (Fig. 4.6 a). Lines were not signifiantly different under control condition. However, a significant decrease in CCI values under extreme drought conditions was observed in wild-type compared to the overexpressing line S2-3, which did not lose chlorophyll (Fig 4.6 b). In contrast to the overexpressor line S2-3 that showed no significant changes in CCI values under drought, the line S2-5 showed a decrease after 30 days but showed higher chlorophyll content than wild type though it was not significant.

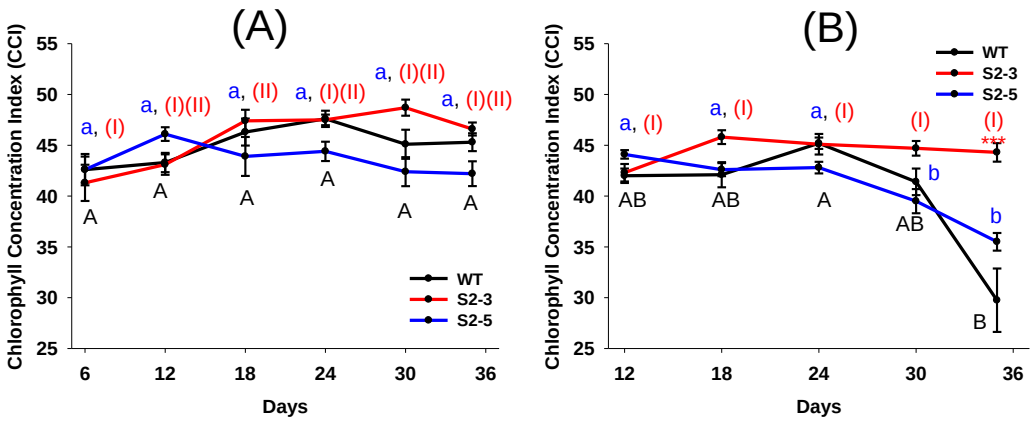


Fig. 4.6: Chlorophyll concentrations determined by SPAD meter of *SPMS*2 overexpressor lines and the wild-type cultivar Golden Promise under a) well-watered conditions and b) drought stress. Significant differences between days are indicated with Roman alphabet letters (S2-3 OX transgenic line), lower case letters (S2-5 OX transgenic line) and upper-case letters (Wild-type plants) when level for significance was P < 0.01. Within a day, significant differences between overexpressor lines and the wild type are indicated with asterisks, * = P < 0.05, ** = P < 0.01, and *** = P < 0.001. Data are the mean of five plants ± SE.

The efficiency of photosystem II (PSII) was determined by pulse amplitude modulation (PAM) imaging. Under control conditions, there were no significant differences in this parameter between the *SPMS2* overexpressor lines and the wild-type at different time points (Fig. 4.7 a). Under drought stress, PSII efficiency exhibited a significant decrease in wild-type after 35 days (P<0.01 level) (Fig. 4.7 b), which did not occur in both overexpressor lines. Hence, PSII efficiency was significantly lower in the wild type than in the mutants at days 30 and 35.

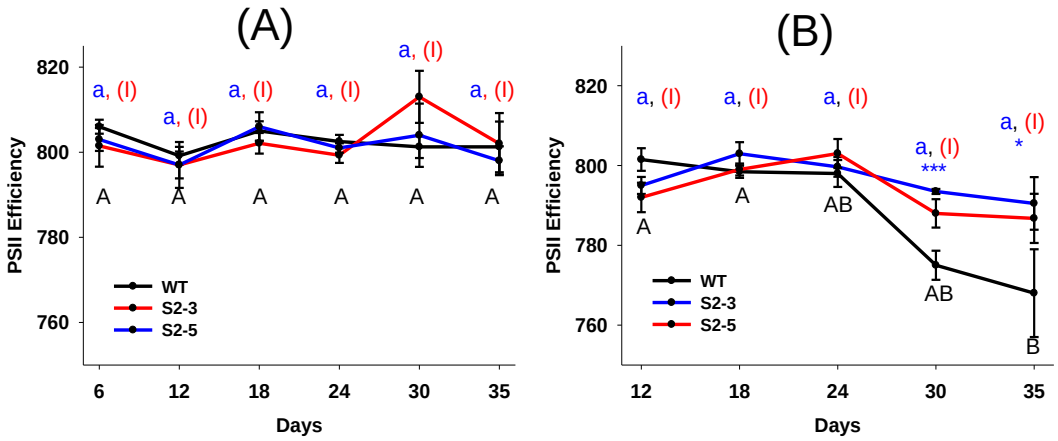


Fig. 4.7: PS II efficiency determined by PAM imaging of *SPMS2* overexpressor lines compared to wild-type (a) under well-watered conditions and b) under drought stress. Significant differences between days are indicated with Roman alphabet letters (S2-3 OX transgenic line), lower case letters (S2-5 OX transgenic line) and upper-case letters (Wild-type plants) when level for significance was P < 0.01. Within a day, significant differences between overexpressor lines and wild type are indicated with asterisks,* = P < 0.05, ** = P < 0.01, and *** = P < 0.001. Data are as the mean of five plants ± SE.

4.3.2 Hydroponics experiment

The previously described drought experiment showed some impressive results, indicating improved water relations and photosynthetic capacity under drought stress in *SPMS2* overexpressor lines. To further examine this improved performance, drought stress was mimicked in form of osmotic stress by PEG6000 in an hydroponic culture system. Osmolality was calculated for different PEG6000 solutions and it was found out that 14% PEG = 300 mOsm/kg will be considered as medium stress whereas 18% PEG = 600 mOsm/kg will be considered as high stress for our experiment. Plants were exposed to stress for 7 days after which they were analysed. Measurements were started after noon.

The stomatal conductance, determined by porometer measurements, was significantly reduced in both overexpressor lines compared to the wild-type under control conditions (Fig. 4.8 a). Osmotic stress caused a strong reduction of stomatal conductance in the wild type and a weak, but significant reduction in the overexpressor S2-3, but not in the second overexpressor line. Consequently, under osmotic stress wild type and overexpressor did not differ in their conductance.

One RNAi line indicated a slightly, but not significantly, increased transpiration rate as compared to the wild-type. Transpiration rate was found to be decreasing in all lines under increasing stress conditions in the same manner as found before (Fig. 4.8 b).

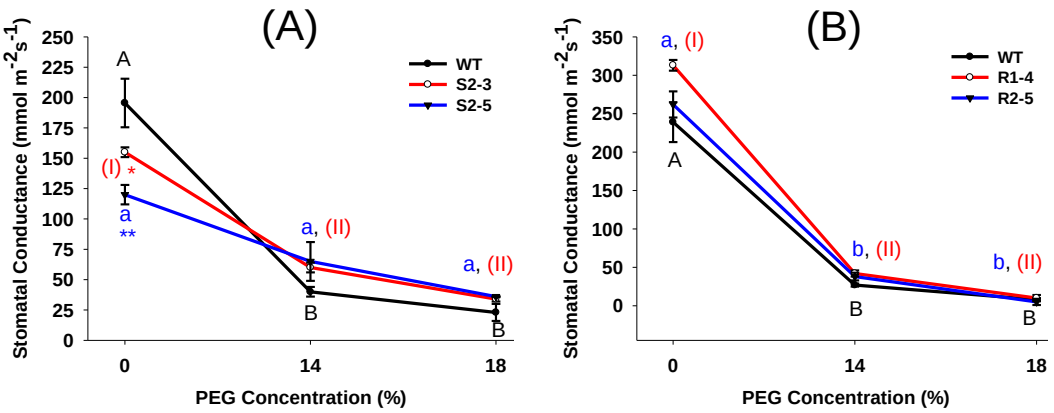


Fig. 4.8: Stomatal conductance of hydroponically cultivated overexpressor (a) and knockdown lines (b) compared to wild-type. Significant differences between treatments (control VS moderate stress VS extreme stress) are indicated with Roman alphabet letters (S2-3 OX or R1-4 RNAi transgenic line), lower case letters (S2-5 OX or R2-5 RNAi transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.05$. Within a treatment, significant differences between transgenic lines and wild type are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of three replicates ± SE.

The relative water content (RWC), determined after 7 days of stress, showed similar trends as previously obtained in the drought stress experiment on soil. Under extreme stress conditions, the *SPMS*2 overexpressor lines conserved more water in their leaves compared to the wild-type, which showed a stronger decrease in moisture content (Fig. 4.9 a). Stress was constant throughout the experiment. Consequently, RWC was significantly higher in the overexpressors under moderate and extreme osmotic stress. Both RNAi lines showed no significant difference in RWC as compared to the wild-type.

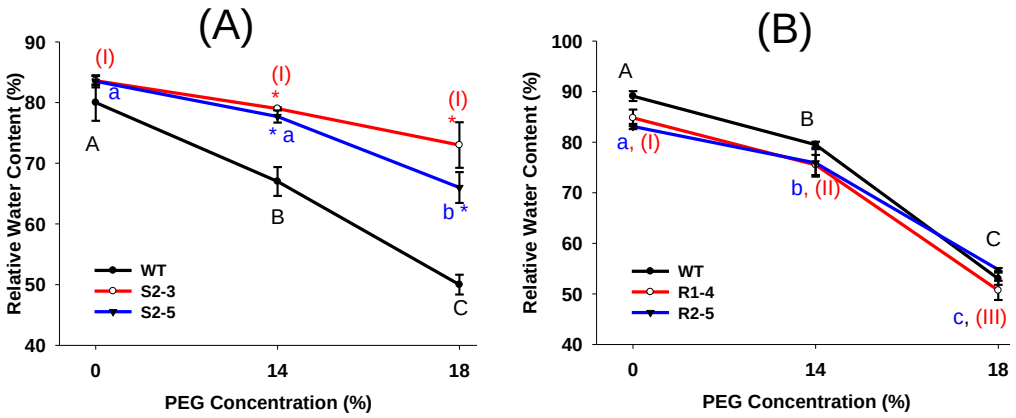


Fig. 4.9: Relative water content of overexpressor (a) and knockdown lines (b) compared to wild-type. Significant differences between treatments (control VS moderate stress VS extreme stress) are indicated with Roman alphabet letters (S2-3 OX or R1-4 RNAi transgenic line), lower case letters (S2-5 OX or R2-5 RNAi transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.01$. Within a treatment, significant differences between transgenic lines and wild type are indicated by asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of three replicates ± SE.

Biomass i.e. fresh weight of shoots and roots of wild type and overexpressors was similar under unstressed conditions (Fig. 4.10 a). A significant reduction in biomass was observed in wild-type plants, but not in the overexpressors, under severe stress, which was reflected in an higher biomass of overexpressor lines which was not significant compared to wild type plants (Fig. 4.10 a). Control plants, but not overexpressor lines were wilting in the severe stress environment (not shown). This result hinted at better adaptation of overexpressor lines to those conditions.

The RNAi lines showed no significant differences in biomass compared to the wild-type. Under increasing drought stress, the RNAi lines, like the wild-type, showed a significantly reduced biomass compared to control conditions (Fig 4.10 b) .

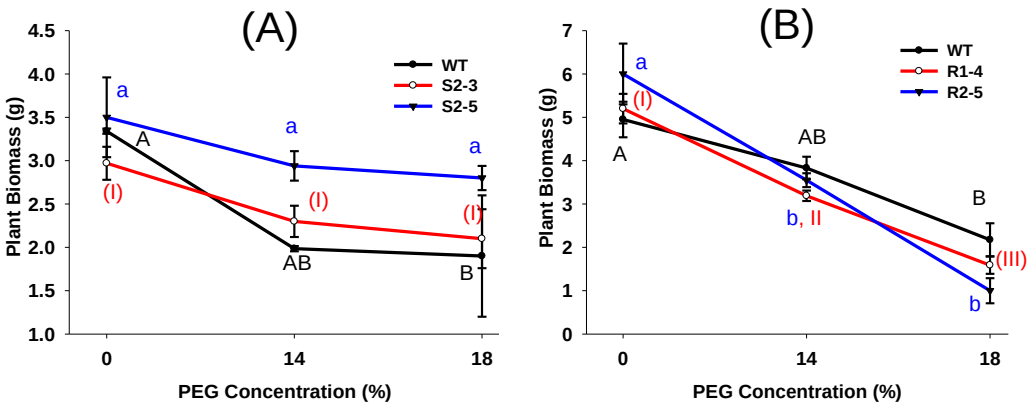


Fig. 4.10: Biomass of overexpressor (a) and knockdown (b) lines compared to wild-type. Significant differences between treatments (control VS moderate stress VS extreme stress) are indicated with Roman alphabet letters (S2-3 OX or R1-4 RNAi transgenic line), lower case letters (S2-5 OX or R2-5 RNAi transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.05$. Within a treatment, significant differences between transgenic lines and wild type are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of three replicates ± SE.

Fresh weight (FW) of plants was determined at the end of the experiment. Plants were separated into roots and shoots. The fresh weight of shoots and roots of the wild-type was higher than that of the overexpressor lines under control conditions (not shown). However, under drought stress, the overexpressor lines showed a lesser decreased fresh weight in roots and shoots compared to wild-type which is also visible in biomass (shoots + roots) measurements where increase in biomass of overexpressor lines can be observed compared to wild type. Furthur this effect was measured by calculating root-shoot ratio. Under high osmotic stress, a significant increase in root-shoot ratio of overexpressor line (S2-5) was observed compared to wild type, whereas a no significant differences in root-shoot ratio was observed in the RNAi lines compared to the wild-type (Fig. 4.11). Though, the differences were not significant in one OX line (S2-3) but the significantly increased ratio in overexpressor lines (S2-5) were evident between different treatments as well. This increase may be explained by a smaller effect of PEG on root biomass in the overexpressor lines, whereas the RNAi lines were not able to accumulate biomass in the same way as

the wild-type with increasing osmotic stress. This indicates an higher tolerance of the overexpressor lines to the harsh environment.

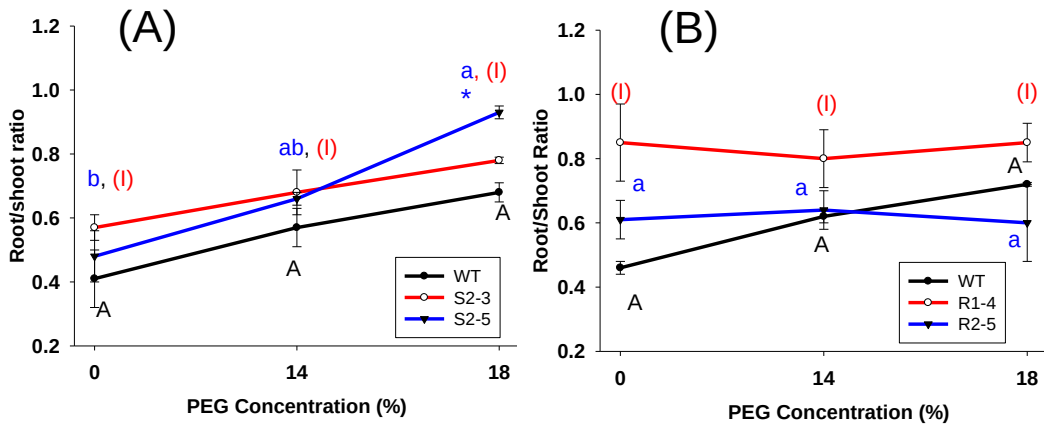


Fig. 4.11: Root-shoot ratio of overexpressor lines (a) and knockdown lines (b) as compared to wild-type. Significant differences between treatments are indicated with Roman alphabet letters (S2-3 OX or R1-4 RNAi transgenic line), lower case letters (S2-5 OX or R2-5 RNAi transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.05$. Within a treatment, significant differences between transgenic lines and wild type are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of three replicates ± SE.Data are the mean of three replicates ± SE.

Another parameter that is responsive to drought stress is the leaf area. Under control conditions, all transgenic lines showed similar leaf areas (Fig. 4.12 a). However, under severe stress conditions, reduction in leaf area was more pronounced in the wild-type (62.5% as compared to unstressed conditions) then in the overexpressor line S2-3 (46%) and S2-5 (40%). The overexpressor line S2-5 had a significantly larger leaf area than the other lines under high osmotic stress (Fig. 4.12 a).

RNAi knockdown lines showed a reduction of the plant leaf area under osmotic stress in the same way as the wild-type (Fig. 4.12 b).

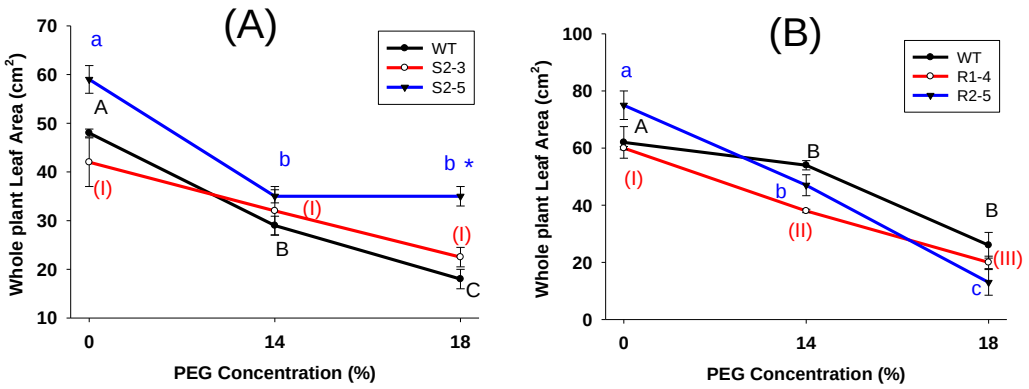


Fig. 4.12: Whole plant leaf area of overexpressor lines (a) and knockdown lines (b) compared to the wild type. Significant differences between treatments are indicated with Roman alphabet letters (S2-3 OX or R1-4 RNAi transgenic line), lower case letters (S2-5 OX or R2-5 RNAi transgenic line) and upper-case letters (Wild-type plants) when level for significance was $P < 0.05$. Within a treatment, significant differences between transgenic lines and wild type are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of three replicates ± SE.

4.3.3 Stomatal bioassays on transgenic lines

The drought and osmotic stress experiments (Section 4.3.1, 4.3.2) showed that water retained in the plants may contribute to a better performance of *SPMS*2 overexpressor plants. Since water loss by plants is determined by transpiration, stomata of those lines were investigated. The density of stomata as well as their seize and aperture were the parameters considered. Results indicated a significant decrease in the stomatal aperture in the overexpressor lines as compared to the wild type ($P<0.001$) under light conditions with fully open stomata while no difference was recorded in the RNAi knockdown lines (Fig. 4.13). In the same experiment, the number of stomata per area was calculated, and it was found it was comparable in all plants, being 30+/-2 stomata/mm^2 (mean +- SE of 10 plants), concluding that not the difference in the number of stomata, but reduced stomatal aperture was responsible for reduced stomatal conductance.

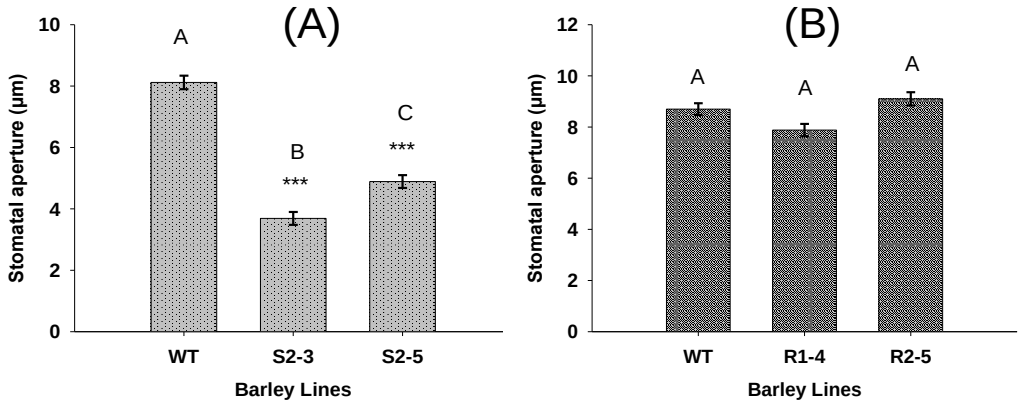


Fig. 4.13: Stomatal bioassay to compare stomatal aperture of (a) overexpressor lines and (b) knockdown lines compared to the wild type. Significant differences between the control line and transgenic lines are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data is presented as the mean of 40 stomata replicates readings ± SE.

The stomatal length, width, and area of overexpressor line S2-5, but not line S2-3, were reduced compared to wild-type plants (Fig 4.14 a). Knockdown lines were not significantly different from the wild type in those parameters (Fig. 4.14 b).

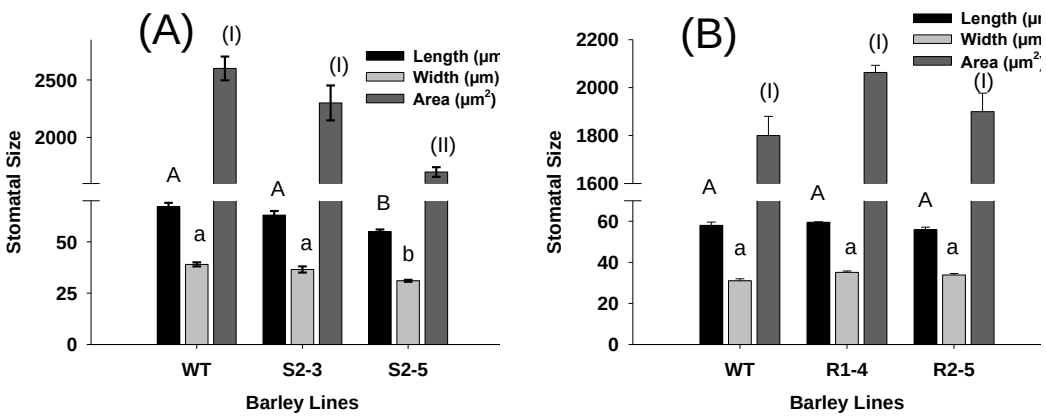


Fig. 4.14: Stomatal bioassay to determine length, width, and area of stomata (a) overexpressor lines and (b) knockdown lines as compared to wild type. Significant differences between the transgenic lines and the wild type are indicated with upper-case letters (length), lower case letters (width) and Roman alphabet letters (area) when level for significance was $P < 0.05$. Data are the mean of 20 replicates ± SE.

4.3.4 Long-term drought experiment

Short-term stress experiments did not allow to study the effect of *SPMS* misexpression on postharvest parameters. Therefore, an experimental setup was conceived to apply constant drought stress (35% soil water content) and cultivate plants until maturity. Different post-harvest parameters were studied in two overexpressor lines as compared to wild-type plants under well-watered and drought conditions.

Tiller number was not significantly different between the lines under control conditions (Fig. 4.15 a). However, under drought stress, both overexpressors produced significantly more tillers than the wild type. Accordingly, the reduction of tiller number by drought was highest in the wild type and no significant reduction was visible in the S2-5 overexpressor line (Fig. 4.15 a).

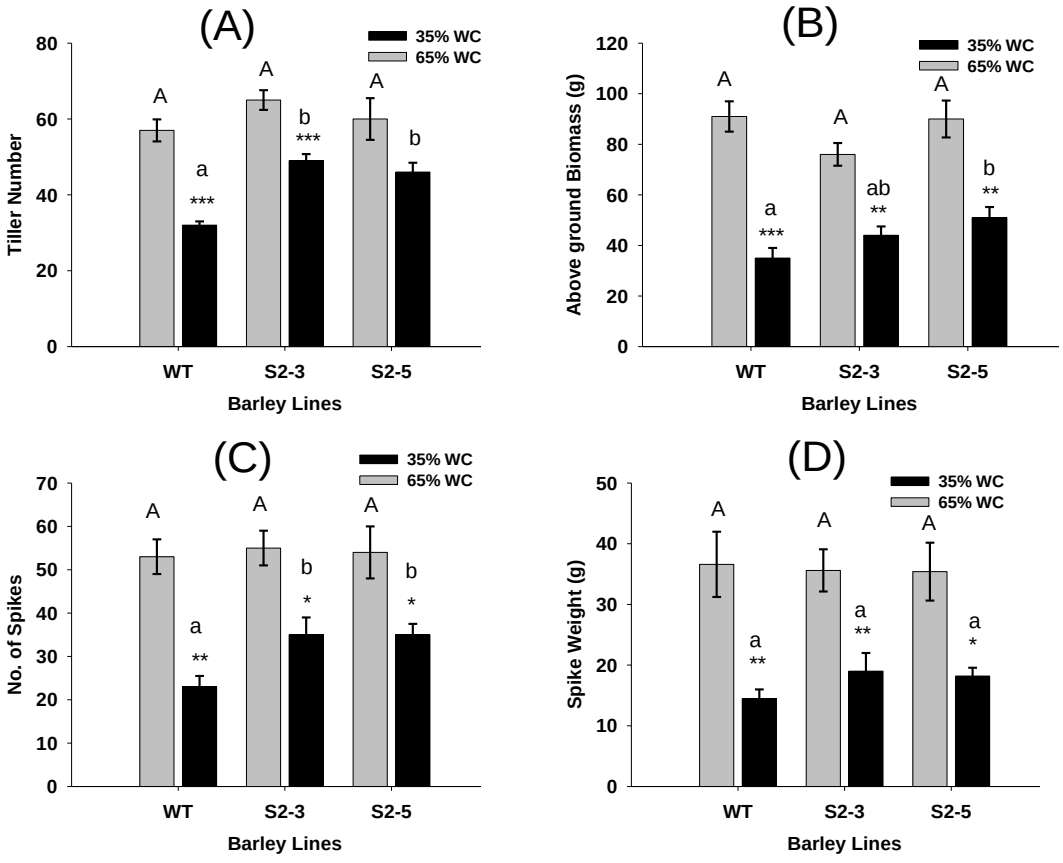
Biomass of the above-ground plant was also not significantly different between wild-type and overexpressor lines under control conditions (Fig. 4.15 b). Under drought stress, biomass of the wild-type plants was reduced by 62%, whereas the drought-induced reduction of the biomass of both overexpressor lines was only 42.3% (S2-3) and 41.7% (S2-5). Under drought, line S2-5 had significantly higher biomass than the wild-type.

The number of spikes showed no significant differences between wild type and overexpressors when plants were cultivated under control conditions (Fig. 4.15 c). However, a considerable decrease in the number of spikes was found under drought conditions in plants of the wild-type. This decrease was significantly less pronounced in the overexpressors, resulting in a higher spike number than in the wild type under drought stress.

The weight of the unharvested spikes was also comparable in all lines under control conditions (Fig. 4.15 d). However, a slightly, but not significantly higher weight was evident in the overexpressor lines than in the wild type under drought stress.

Under control conditions, the height of the overexpressor line S2-5 was not significantly different from that of the wild-type plants, whereas line S2-3 showed was significantly shorter (Fig. 4.15 e). A significant decrease in plant height was recorded in wild-type under drought stress, which was similar to the height of line S2-3, whereas both overexpressor lines were not affected by drought.

The harvest index was slightly, but not significantly higher in overexpressors compared to the wild-type under control conditions (Fig. 4.15 F). Drought stress did not significantly affect the harvest indices.



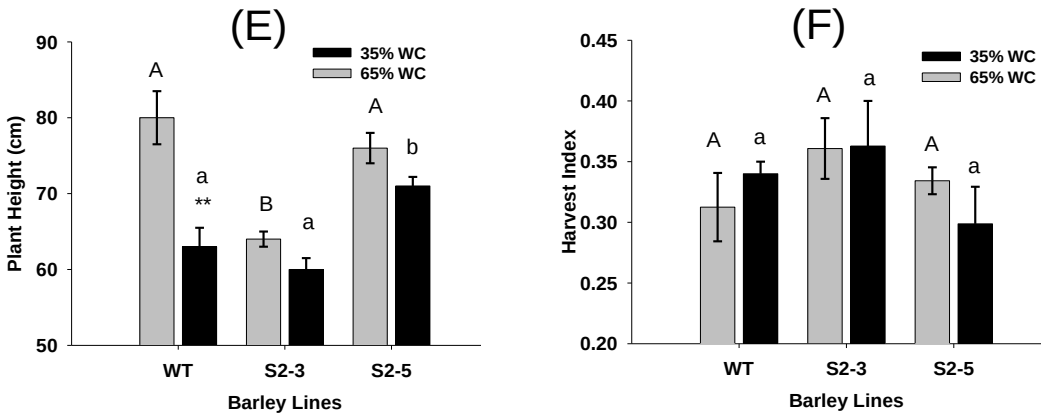


Fig. 4.15: Post-harvest parameters in overexpressor transgenic lines compared to wild-type determined in long-term drought experiment. (a) Tiller number, (b) plant biomass, (c) spike number, (d) spike weight, (e) plant height height, (f) harvest index. Significant differences between the control line and transgenic lines are indicated with upper-case letters (65% soil water content) and lower case letters (35% soil water content) at $P < 0.05$. Within a line, significant differences between treatments (control VS stress) are indicated with asterisks, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$. Data are the mean of five replicates ± SE.

4.4 Discussion

Different species of plants have different level of drought tolerance (Erdei *et al.*, 2002). However, plants have developed mechanisms to survive un the stress environments created by salinity (Munns and Tester, 2008) and drought (Vaseva *et al.*, 2012). Drought adaptation strategies of plants involve different mechanisms which contribute to avoidance of water scarcity, stabilization of damaged proteins, and accumulation of antioxidant molecules and enzymes. Abiotic stresses caused low transpiration rate and reduced photosynthesisis followed by decrease in plant growth and development which leads to several affected biochemical processes and eventual decline in crop production (Shannon, 1997, Tiwari *et al.*, 2009). Increased frequency of extreme temperatures and drought cause oxidative stress which negatively influences crop plant productivity. ROS act as a signal molecule under drought stress conditions as plants commence antioxidant mechanism for protection against ROS (Khan *et al.*, 2003).

Polyamines, in particular spermine, have been observed to play a role in stomatal closure and hence drought tolerance. After investigating the potential role of spermine in drought tolerance (Chapter 2), transgenic barley lines were generated in which the expression of *SPMS* gene was increased or decreased (Chapter 3). The response to drought and osmotic stress of those lines, in particular *SPMS2* overexpressor lines, was analysed in this part of the project.

4.4.1 Analysis of drought stress in a soil based-systems

Drought caused by drying-out of the soil is natural and causes soil water potential to decrease slowly due to water status declining when drought is simulated by withholding water to plants (Krizek, 1985). However, the rapidity of the onset of stress varies depends on the species, pot size, soil texture, irrigation regime, and environmental factors (Kramer, 1983).

Glasshouse experiments have been reported to affect plant yield parameters under water deficiency (Blum and Pnuel, 1990). The intensity of the response depends on the stress severity and its duration. In this study, overexpressor lines were subjected to an increasingly severe drought condition in order to study the physiological changes and adaptations as compared to wild-type. While plants were subjected to decreaseing soil moisture levels caused by lack of irrigation, all other factors like temperature, light, and air humidity were kept at a constant level.

4.4.1.1 Changes in *SPMS2* expression level

Overexpressor lines were shown to survive longer under extreme drought stress (Section 4.3.1). The relative expression of *SPMS*2 was 20 fold higher in overexpressors under control conditions which became 25 fold higher under drought stress, however this difference between control and drought is not significant (Fig. 4.1a). In drought, *SPMS* expression was increased which has been reported as *Spermine synthase* has been shown to be up regulated under drought stress in *Arabidopsis* (Alcazar *et al.*, 2006a), whereas, *spermine synthase* mutant of *Arabidopsis* was more sensitive to drought and salt stress (Yamaguchi *et al.*, 2007). As the plant developed, an increase in *SPMS2* expression was experienced in early developmental stage which started decreasing in the later phases of growth. However, under drought stress, a slight decrease in absolute expression of *SPMS2* gene was observed in wild type plants (Fig. 4.1d). Hence, an increase in relative expression of *SPMS2* was observed under control and stressed conditions in both overexpressor lines (Not shown). An increase in the absolute values of *SPMS2* expression is visible under both conditions till 30 days. Similarly, an induction of *spermine synthase* genes has been observed under different drought treatments (Do *et al.*, 2014). Three spermine genes has been identified as drought induced in rice as consititutively expressed by (Do *et al.*, 2013). Harsh drought conditions i.e. after 30 days, decrease in absolute values was observed in the wild-type under control conditions as well as under drought stress.

4.4.1.2 Changes in spermine concentration

Polyamine concentrations were increased in the *SPMS2* overexpressor lines as compared to the wild type (Fig. 4.2). Drought stress had an impact on spermine concentrations similar to the expression levels of *SPMS2*, as the concentration of spermine increased compared to control conditions. This increase was upto 2 folds but was not significant. Tolerant rice cvultivars has been known to accumulate high levels of spermine when osmotic stress is applied (Liu *et al.*, 2004a). An increase in spermine concentration was also observed during plant development. The overexpressors showed higher Spm activities and accumulated more Spm in the leaves than wild-type under both, well-watered conditions and water stress. It was observed that overexpression of SPD synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana* plants (Kasukabe *et al.*, 2004). The overexpressor lines had a higher polyamines accumulation than the wild type plants, suggesting that these have the ability to respond to drought stress through increases in polyamine levels. Increase in spermine concentration has been reported in drought tolerant cultivars when stress is applied (Do *et al.*, 2014). Significant increase in spermine has been observed in rice cultivars under drought stress indicating role of spermine in drought (Do *et al.*, 2013). It is proposed that an initial response to stress signals would help plants to adapt to stress and prevent them from being seriously damaged (Chen and Zhang, 2000).

4.4.1.3 Changes in stomatal conductance

Stomatal closure is a common drought avoidance response allowing plants to keep water in their tissues (Ludlow, 1980). A quick response is needed in order to counter drought stress with a view to increasing plant survival by losing less water through the stomata. Stomata consist of two guard cells surrounding the stomatal pore. When the stomata are open, water is transpired, and CO_2 enters the leaf through the stomatal pore. Transpiration rate, determined by the stomtal conductance, is one of the

critical parameters to identify drought tolerant cultivars. It was discovered in the inital characterization that *SPMS2* overexpressor lines show reduced stomatal conductance under control conditions (Fig. 3.7). This result was replicated in the drought experiment with plants under sufficient water supply (Fig. 4.3). However, when drought stress was applied, transpiration rate in wild-type was identical or lower as compared to the overexpressor lines. Ultimately, under harsh conditions with 10-15% soil water content after 35 days of culture, the overexpressor lines showed an increased transpiration rate compared to the wild-type. This may be a result of the conservation of water at early stages, allowing plants to utilize water in late extreme stress when soil moisture contents are reduced to less than 20% (Fig. 5). (Nerkar *et al.*, 1981) also described considerable differences in water loss and transpiration in different genotypes of faba bean (*Vicia faba*) and suggested that the lower transpiration rate would contribute to drought tolerance. (Anjum *et al.*, 2011) indicated that drought stress in maize led to a considerable decline in net photosynthesis (33%), transpiration rate (38%) and stomatal conductance (25%). Lower stomatal conductance under drought likely resulted in higher water retention. Transpiration rate is linked with biomass as photosynthesis is directly affected by stomatal aperture and leaf area of the plants (Collins *et al.*, 2008). It was observed that spermine is responsible for the closure of stomata. Overexpression of spermine in our transgenic lines led to partial closure of stomata which reduced stomatal conductance (Fig. 4.13a) . Spermine accumulation endogenously has been reported under drought and helped in prevention of tissue damage by effective ROS scavenging and lower water loss which was attained by closed stomata (Shi *et al.*, 2010). This response may be effective in increasing drought tolerance of the plants.

4.4.1.4 Change in leaf temperature

Drought and salinity has been known to cause stomatal closure, a decreased transpiration rate, and elevated plant leaf temperatures (Halim *et al.*, 1990). Under drought, the water uptake rate cannot match the

potential transpiration rate and stomata close to maintaiyning the plant water balance. As a consequence of the reduction in transpiration rate of leaves, leaf temperature increases. Therefore leaves are subjected to both heat and water deficiency stress during drought stress (Clarke *et al.*, 1993). Rising leaf temperature causes the destruction of cell membranes. Accordingly, (Wahid *et al.*, 2007) reported high temperature to alter the permeability of membranes, so the integrity and functions of membranes are sensitive to heat stress. (Idso *et al.*, 1981) used the increase in average canopy temperature following drought stress as a key component of a crop water stress index, and (O'Neill *et al.*, 2006) considered leaf temperature as a potential indicator of plant water stress.

In the present study, under normal water conditions the leaf temperatures showed no significant differences between genotypes, though slightly increased leaf temperature were observed in overexpressor lines. This was due to lower stomatal conductance resulting in slightly warmer leaf temperatures. Drought conditions led to an increased leaf temperature in wild-type, which was likely due to low transpiration rate in the leaves. Initially, wild type plants had wider open stomata and were transpiring more water which in turn decreased water availability at later stages of the stress. When there is little available water from the soil under drought stress, the plants were forced to consume the water stored in the cells which in turn lead to the wilting of those plants. *SPMS2* overexpressor lines showed a decreased leaf temperature during stress likely due to lower stomatal conductance in earlier stages which helps the plant to transpire less and conserve water in the soil as well as the plant. Overexpressor transgenic lines showed prolonged transpiration through leaves during severe drought, which is evident from the cooler than ambient leaf temperatures. This physiological response might increase the drought tolerance of those transgenic lines and might lead to higher grain yield under severe drought.

4.4.1.5 Change in relative water content

Leaf relative water content (RWC) is an important indicator of water status in plants; it reflects the balance between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca, 2011). For example, (Ramos *et al.*, 2003) showed that the RWC of bean leaves was significantly decreased under drought stress.(Schonfeld *et al.*, 1988) showed that wheat cultivars having a high RWC are more resistant to drought stress. Longer survival of plants is dependent on water stored in the plant cells. All barley lines had around 90% relative moisture under normal watering conditions. Drought conditions significantly decreased the proportion of water in the leaves of wild-type plants, whereas the mutant lines conserved water. The latter showed an RWC of 75% in leaves even under extreme drought conditions while the wild-type showed an RWC of only 29%. This may be explained by the fact that the overexpressor lines had a reduced stomatal conductance, probably due to smaller stomatal apertures. A smaller transpiration rate helped to maintain higher water contents in the leaves, which again helped the plant to perform its functions more efficiently and to survive for longer period of time in a water-limited environment. Low relative water content under drought has been shown to accumulate polyamines which act as signal molecules and trigger negative drought effects caused by different survival and adaptive mechansims (Kubis *et al.*, 2014). Overexpressor lines which accumulate spermine, were able to accumulate water in the leaf, hence delaying the drought effects. It has been reported that pretreatment with polyamines observed in reduction of drought effects as it helped to adjust osmotic potential due to the increase of solutes, enabling cells to maintain turgor during drought stress conditions (Kubis *et al.*, 2014).

4.4.1.6 Change in chlorophyll concentration

It has been reported that the degradation of chlorophyll (Chl) and carotenoids (Car) is observed in drought-stressed plants, which was presumably associated with photo-oxidative processes in chloroplasts (Munné-Bosch and Peñuelas, 2004). Hence, considerable reduction in

chlorophyll content has been reported in most crop species due to water deficit (Ashraf *et al.*, 1994, Kyparissis *et al.*, 1995). Chlorophyll content is decreased in under drought stress which is caused by the production of ROS which damages the chloroplasts (Smirnoff, 1995). Water deficit can not only destroy the chlorophyll, but also also prevent its synthesis. Chlorophyll is an important drought parameter as its decrease was reported in drought susceptible genotypes whereas, drought tolerant genotypes showcased lower decrease when compared in chlorophyll content (Kraus *et al.*, 1995, Sairam *et al.*, 1997a, Sairam *et al.*, 1997b).

In the present study, chlorophyll measurements were performed by SPAD meter to investigate the effect of drought and *SPMS2* expression on chlorophyll contents. Plants of all lines used in this study showed no changes in chlorophyll contents under standard watering regime. However, under drought conditions, a decrease in chlorophyll contents of the wild-type could be observed when plants were subjected to extreme stress after 35 days. In contrast, overexpressor lines showed only a slight, or even no, reduction in chlorophyll contents, indicating an improved ability of the plants to adapt to and survive in this harsh environment. Hydrogen peroxide production is the main reason for the decrease in chlorophyll content during drought stress which can lead to lipid peroxidation and eventually destruction of chlorophyll (Foyer *et al.*, 1994). ROS is responsible for the oxidative damage resulting in chlorophyll degradation and senescence. It is produced by oxidative stress which inducted physiological disorders in plants (Yamauchi, 2015). Higher production of polyamines leads to scavenging of ROS which in turn prevents oxidative stress and lead to drought tolerance (Shi *et al.*, 2010). It has been previously reported that pretreatment of spermine leads to prevention of ROS formation (Zhao and Yang, 2008, Yiu *et al.*, 2009).

4.4.1.7 Change in chlorophyll fluorescence parameters

Photosynthesis is one of the primary metabolic processes affected by drought stress. Stomatal movement is an early physiological response to drought, after which loss in chlorophyll fluorescence is observed

eventually. This has been explained as it is due to the reduction in PSII efficiency which was calculated by measuring low quantum yields of PSII (Giardi *et al.*, 1996). Many environmental stresses are responsible for the photoinhibtion damage in the plants which is also one of the reasons for decrease in photosynthesis (Maxwell and Johnson, 2000).

Pulse amplitude modulation (PAM) imaging technique was used to measure the PSII efficiency of the plants, which indicates the ability to use the light and convert it into energy. Chlorophyll within a leaf exists as pigment–protein complexes in PSII and PSI complexes. Light energy is absorbed by chlorophyll molecules, driving three processes, i.e. photosynthesis, emission as heat (non-photochemical quenching) and re-emission as light (chlorophyll fluorescence). These three processes are in competition with each other. PAM imaging measures the chlorophyll fluorescence emission. At room temperature, the fluorescence signal only arises from PSII (Baker, 2008). This is essential for measuring plant photosynthesis, as photochemistry is used to provide energy for different mechanisms in the plant cell. In the present study, PSII efficiency, as the chlorophyll content, was similar in all tested genotypes under well-watered conditions. Wild-type plants showed a decrease in PSII efficiency with increasing drought stress, whereas overexpressor transgenic lines showed stable values (Fig. 4.7). This indicated the increased energy production and photosynthesis in transgenic lines under stress environment which helped plants to survive even harsh conditions. Several publications showed that the value of Fv/Fm is not sensitive to drought stress up to 60 % of relative water content (RWC) in the plant (Matoušková *et al.*, 1999). The decline in PSII efficiency was a consequence of the increase in the thermal dissipation of energy from PSII as it is known that drought stress leads to an increase in NPQ (Tezara *et al.*, 2005). It was also found that the NPQ values in wild-type were increased under drought stress which contributes to reduced PSII efficiency (not shown).

4.4.2 Induction of osmotic Stress by PEG 6000

Hydroponic culture systems have been used for research purposes for a long time. Since high molecular weight (MW) polyethylene glycol (PEG) provides an osmotic stress that is more homogeneous and precise than soil drought (Krizek, 1985), this greatly facilitates experimentation. Hence, high MW PEG is widely used for simulating drought (Zwiazek and Blake, 1990). In contrast to lower MW PEGs, high MW PEGs were judged more suitable since they were thought not to be quickly absorbed by healthy roots in short-term experiments, and PEG 6000 was considered to be used for drought experiments (Michel and Kaufmann, 1973, Michel, 1983). There are other PEGs, usually with a molecular weight of more than 4000 g mol^{-1}, used to induce osmotic stressin plants, e.g. PEG4000 (Wu *et al.*, 2015), PEG 8000 (Xu *et al.*, 2013), and PEG10000 (Hamayun *et al.*, 2010).The reason for using PEGs with a large molecular weight is that it is not taken up by the plant, compared to smaller PEGs that can diffuse through the cell wall and can be distributed through the plant, which may influence the results.

4.4.2.1 Physiological parameters

It was determined in soil experiments that *SPMS2* overexpressor lines generated are drought more tolerant and are likely to have the ability to survive for longer periods than wild-type under drought stress. However, parameters related to roots are difficult to be studied in the soil-based system. The concentration of PEG6000 needed to induce moderate and severe stress was calculated by determining the osmotic potential of PEG. 14% PEG 6000 was used for mild stress (300 mOsm/kg) and 18% for severe stress treatments (600 mOsm/kg). *SPMS2* overexpressors and RNAi knockdown plants were compared to the wild-type. Stomatal conductance of overexpressor lines was reduced compared to the wild-type under unstressed conditions, but increased under osmotic stress (Fig. 4.8), similar to what was found before on soil. Knockdown lines showed increased stomatal conductances than the wild type under control

conditions, but no alteration if plants were osmotically stressed. They perform like the wild type under higher stress.

4.4.2.2 Changes in plant biomass and root-shoot ratio

Drought stress leads to growth reduction, which was reflected in plant height, leaf area, dry weight, and other growth parameters. Plant biomass is primarily a product of photosynthesis, a process needing carbon dioxide and light as the energy source, and determines the overall yield of the plant. *SPMS2* overexpressor plants were shown to have similar values of combined biomass i.e. fresh weight of shoot and root, under unstressed conditions, but their decrease in biomass was less than in wild-type plants under osmotic stress (Fig 4.19). It was also apparent that transgenic plants were more turgent that the wild type under stress. Conversely, osmotic stress also had a stronger effect on the RNAi knockdown lines than the wild-type. Up to 85% biomass reduction was observed in RNAi lines under drought, as compared to a 25% decrease observed in overexpressor lines.

Root and shoot biomass when measured separately were lower than wild type plants, during different treatments (not shown). However, the decrease in biomass of wild type plants was higher than overexpressor lines, indicated by increased biomass (root + shoot) values under high stress (Fig. 4.9). This may be due to less prominent decrease in root biomass in overexpressor lines compared to wild type as evident in root-shoot ratio calculation as well (Fig. 4.10). It was explained that when plants are stressed by a lack of resources, relative allocation below ground may increase while total biomass production declines (Poorter and Remkes, 1990). The decrease in output occurs through all or some combination of reductions in leaf-level photosynthesis, in leaf area, and greater relative allocation to roots, even if absolute root growth is decreased (Pace *et al.*, 1990, Kage *et al.*, 2004). Such biomass allocation is often associated with improved survival under drought stress (Espinoza *et al.*, 2014).

The root-shoot ratio indicates the relative effect of osmotic stress on roots and shoots of the plants. Plants can survive extreme conditions with the help of developing secondary roots to absorb more water from the surrounding media. Hence, the root to shoot ratio increases as an adaptive mechanism of the plants towards drought stress. Researchers considered the increase in root growth as an indicator of the ability of plants to withstand water stress, as well as to screen plant cultivars for drought tolerance.

SPMS2 overexpressing lines displayed an increased root to shoot ratio when high osmotic stress was experienced by the roots. Significant increase ($P<0.05$) was observed in S2-5 overexpressor line while the ratio did not significantly change in the wild-type (Fig. 4.11). Contrarily, RNAi knockdown lines experienced severe effects of stress which showed no significant change in their root to shoot ratio compared to wild-type. This was due to the reduced shoot biomass of transgenic plants under drought conditions. (Fernández *et al.*, 1996) also found that drought affected shoot growth in young cotton plants before the root growth. Root growth was not decreased in the drought-treated plants, compared with the controls, until the end of the drought period. For the survival of plants in drought conditions, a large root to shoot ratios will be advantageous, perhaps in stress environments (Gedroc *et al.*, 1996). Extensive root systems achieved deeper soil levels in which water remains available throughout the summer (Canadell and Zedler, 1995). Increased root to shoot ratios were observed under drought stress, providing sufficient water to maintain photosynthesis comparable to control conditions (Edwards *et al.*, 2016) which also helped the plants to survive better.

4.4.2.3 Changes in leaf area and relative water content

The leaf area is an indicator of crop growth, development, and plant health in crops (Aase, 1978). It varies widely among species due to nutrient and moisture availability, light intensity, and temperature (Poorter *et al.*, 2009). Furthermore, the leaf area is considered to be an essential trait in plant growth, plant breeding and influences the crops' responses to different

growth conditions. For example, (Witkowski and Lamont, 1991) reported that leaves are smaller under drought stress conditions. The leaf area was also measured in this study. A higher plant leaf area can indicate more overall stomata, more transpiration of water and higher photosynthesis. The *SPMS2* overexpressor lines showed a comparable leaf area to the wild type under control conditions. However, osmotic stress led to a decrease in leaf area in the wild-type, mainly due to leaf wilting in response to water shortage, whereas one of the overexpressors was less affected (Fig. 4.12). This result coupled with the fact that relative water content was also higher in overexpressor lines, determined the transgenic plant's ability to conserve water under drought conditions. It was also noticed that the relative water content was decreased in knockdown lines, similar to the wild-type, when osmotic stress became higher, leaving very little water in the leaves for the survival of plants. Reduced transpiration rates helped increase the relative water content and also maintain the turgor pressure of the cell which in tuen help to sustain the leaf areas of the plant (Kramer and Boyer, 1995).

With regard to relative water content, plants maintain their water uptake by osmotic adjustment mechanism to adapt to drought conditions by increasing solutes, improving cell hydration and maintaining turgor pressure to help plants to survive longer. However, it is a slow process and can be influenced by intensity of stress (Sanders and Arndt, 2012). Below 80% relative water content have 1.5MPa water potential and can start the drought tolerance processes such as osmotic adjustment (González and González-Vilar, 2001). Overexpressor lines were grown in glass house with gradual stress and illustrated increased relative water content in the leaves showing plants survival under drought and might not need osmotic adjustment to counter drought. Though the level of osmotic adjustment to enhance drought tolerance is still not known.

Drought-tolerant plant species keep a high RWC compared to drought-sensitive species (Stoyanov, 2005). Similarly, to the present study (Tambussi *et al.*, 2000)reported that under water stress, plants showed a decrease in the RWC which affected the plant leaf area. Plant leaf area

has been known to reduce under drought stress environment which is explained by the mechanism which leads to reduction in cell size (Schuppler *et al.*, 1998). (Rasmusson, 1987) suggested that a larger leaf area produces more and larger spikes and kernels, and a close relationship between leaf area, grain number, and grain yield at the time point of harvest was found.

Similar to the previously performed drought experiment, the *SPMS2* overexpressor lines were found to perform better with increased root to shoot ratio, having higher chlorophyll contents with a higher PSII efficiency and an increased biomass.

4.4.3 Long-term water limitation

Water stress is considered as less detrimental to grain yield when occurring early in the growth cycle (Blum, 1996). In addition, for commercially viable crops, it is necessary to study the effect of genetic manipulations and stress treatments on postharvest parameters. Drought-tolerant lines need to perform equally or better than the wild-type when considering plant yield, biomass and/or harvest index. In the present work, plants were grown under control conditions (65% soil moisture) and moderate drought conditions (35% soil moisture). If grown under well-watered conditions, the tiller number was comparable in all lines (Fig. 4.15a). However, under drought stress, an increased number of tillers could be observed in overexpressors as compared to the wild type. Similar differences, significant or tendentially, were found for the number of spikes, spike weight and above ground plant biomass. Hence, drought conditions reduced spike number, spike weight, and biomass of wild-type, while all those parameters were less affected in overexpressors. Although this showed the potential ability of the transgenic plants to have higher yields than wild-type, the difference in spike weight was not significantly different here. A larger leaf area potentially leads to a higher accumulation of biomass due to increased photosynthetic rates, which in turn increases spike dry matter. Harvest index describes the plant's capacity to allocate biomass to grains, which makes it an important trait for plant breeding

(Wnuk *et al.*, 2013). Its relationships with biomass and grain yield follow the multiplicative yield component model, in which grain yield is a product of harvest index and biomass yield. In the present work, harvest index was not significantly different between genotypes and between treatments. Significant reduction in harvest index is known to occur under drought stress due to a higher reduction in grain yield than in biomass production (Shafazadeh *et al.*, 2004). However, this was not obvious in the present work, likely because the harvest index was low under well-watered conditions, so that plants could not attain their full yield potential.

The limitation of shoot growth by a decreased number of tillers in response to water limitation is considered as a strategy to reduce water use under stress (El Soda *et al.*, 2010). (Talebi *et al.*, 2009) showed that different genotypes of wheat had significant differences in plant height, spike length, seed number per spike, and grain yield. Drought stress is known to also reduce 1000-seed weight by shortening the grain-filling period (Mamnouie *et al.*, 2010). Unfortunately, this parameter could not be studied in the present work due to time constraints. (Mirbahar *et al.*, 2009) also found that water stress significantly reduced the spike length, spikelets per spike, grains per spike of wheat varieties. With up to 54% grain yield was reduced most strongly, followed by biomass (45%), grains per spike (36%), and harvest index (18%).

Polyamines have been reported to help in the growth and development of plants (Kusano *et al.*, 2007a). Spermine contents have been positively correlated with grain filling rate and grain weight (Tan *et al.*, 2009). Overexpressor lines having higher quantities of spermine were responsible for longer survival of these lines and hence, were able to attasin greater yield potential. It was similar to the results reported when pretreatment with external spermine enabled plants to increase grain weight in wheat (Liu *et al.*, 2013). Exogenous application of spermine also promoted tillering rates to affect plant growth and enhance stress tolerance (Shukla *et al.*, 2015).

4.4.4 Stomatal measurements in transgenic barley lines

Stomatal size, frequency, and aperture have been used as an indicator of water loss potential by many investigators (Wang and Clarke, 1993, Singh and Sethi, 1995). It has been suggested that wheat cultivars having wider stomatal aperture produce higher yields without consuming more water (Shimshi and Ephrat, 1975). (Miskin *et al.*, 1972) found that a decrease in frequency of stomata of 25% reduced transpiration rates by about 24%. They found that stomatal frequency did not influence the rate of photosynthesis. (Wang and Clarke, 1993) reported that stomatal frequency positively correlated with the rate of water loss.

In order to further elucidate the mechanism of the differential water relationships in the transgenic lines and the overexpressor found in the previous experiments, stomatal bioassays were performed. One aim was to determine whether the lower stomatal conductance of the overexpressors under unstressed conditions was due to lower stomatal aperture or the presence of a smaller number of stomata per cm^2 of leaf. The length, width, and area of stomata was also measured to determine possible differences in guard cell size between the lines. It was found that a similar density of stomata was present on the leaf surface in all lines. In contrast, stomatal aperture was reduced in lines that overexpressed *SPMS2.* Overexpression of this gene leads to a higher production of spermine, which eventually leads to reduced stomatal aperture, confirming the response of stomata to externally applied spermine (Chapter 2). Knockdown lines showed no change in stomatal aperture in comparison to the wild-type, which is in line with the similar stomatal conductance observed in RNAi lines compared to wild-type (Fig. 4.8). The area of stomata, including the guard cells, was slightly smaller in overexpressor lines, which was due to a reduced length and width caused by the partial stomatal closure. Correlations between stomatal aperture with yield and photosynthetic rate under stress conditions have been found before. It was suggested by (Singh and Sethi, 1995)) that stomatal frequency and size influence water loss in durum wheat, which was also apparent in the

present study. (Venora and Calcagno, 1991) found a negative association between water loss and stomatal pore size. This indicates that breeding for bigger and fewer stomata may lead to a reduction in water loss and an increase of yield in water limitation condition.

5 General Discussion

5.1.1 A proposed role of spermine in drought-stressed plants

Taken together, we conclude a potential role of spermine during drought stress. Polyamine biosynthesis is enhanced in drought-stressed plants (Bouchereau *et al.*, 1999), and the spermine content also increases. Previous studies have pointed to a relevance of spermine during salt and drought stress. It has been known and reported that increased spermine biosynthesis or application of spermine stimulate various responses in the cell. Fast Vacuolar (FV) channels are cation channels for monovalent ions, such as K^+, whereas slow vacuolar (SV) channels are less specific channels for both monovalent and divalent ions, including Ca^{2+}. Spermine was found to increase the cytosolic pH, which has been reported to block the FV and partially block SV channels. FV and SV channels from red beet taproots and from *Vicia faba* also show a similar response to spermine (Dobrovinskaya *et al.*, 1999, Dobrovinskaya *et al.*, 1999 b). Spermine has been known to show stronger inhibition of FV channels compared to SV channels, which may limits the K^+ concentration of ions in the cytoplasm (Barkla and Pantoja, 1996, Bruggemann *et al.*, 1999). Inhibition of FV channels may allow fewer K^+ ions to enter the cytosol, whereas spermine application also changes intracellular pH, which may trigger an increase of cytosolic calcium concentration through calcium-permeable channels. Changes of free Ca^{2+} in the cytoplasm of guard cells are involved in the stomatal movement (Peiter *et al.*, 2005). Spermine production likely modulates Ca^{2+}-permeable channels in a pH-independent way, resulting in the increase of cytoplasmic Ca^{2+} concentration to trigger stomatal closure (Maathuis *et al.*, 1997). Spermine leading to ROS production has also been known to activate Ca^{2+} channels (Gorlach *et al.*, 2015). Spermine application results in increase of cytosolic free calcium in the cells leads to the inactivation of inward K ions which contributes to the final closing of stomata (Liu *et al.*, 2000). The spermine-deficient mutant plant has been

hypersensitive to drought stress (Yamaguchi *et al.*, 2007). This phenotype was recovered by the pretreatment with spermine. When exposed to drought condition, the mutant plant lost more water than the wild-type plant as the plant was not able to close stomata quickly enough. This phenomenon can be explained by the impairment in Ca^{2+} homeostasis indicating a link between spermine, calcium signals, and stomatal regulation.

Current study confirmed the role of polyamines i.e. spermine is responsible to induce stomatal closure under drought conditions. It was also observed that Ca^{2+} played a pivotal role in stomatal closure where extracellular Ca^{2+} as well as vacuolar Ca^{2+} from internal stores facilitated this process. Ca^{2+} signals generated by spermine were concentration dependent and also tend to increase cytosolic pH which play a role upstream of stomatal movement.

5.1.2 Drought tolerance responses in barley

Stomatal regulation is one of the main factor in plant drought tolerance. Therefore, it was deduced in the present study that stomatal closure is caused by water stress due to overexpression of *SPMS* gene, which resulted in reduced water loss and increase in leaf temperature. Leaf temperature variation in the leaves maybe damaged the cells. The damage in the cell membrane caused by drought, does not allow the plant to continue normal metabolic processes, and for the reason the plant growth period get reduced.

It has been determined that biomass reduction is the earliest response of the drought stress experienced by plants even under mild stress environment where it had a minor effect on the photochemical efficiency of PSII and photosynthetic rates were not affected (Lu and Zhang, 1999, Shangguan *et al.*, 2000, Verelst *et al.*, 2013). This response was further explained when the plant biomass reduction was observed as an adaption response to stress rather than as a secondary result of resource limitations (Rollins *et al.*, 2013). Reduced plant size or decreased stomatal

conductance are the protective mechanisms against stress which are responsible for reduced yield in plants (Deikman *et al.*, 2012). It was observed in present study that under extreme stress conditions, photosynthetic rate was decreased while leaf temperature was increased due to reduced stomatal conductance.

The loss of water lead to dehydration in plants, wherein, success of the plants is dependent on their ability to adjust stomatal aperture (Savvides *et al.*, 2012). Stomata may be able to remain open (Matthews *et al.*, 1990) or partially open (O'Toole and Cruz, 1980) in stressed leaves. High number of stomata had a positive effect where they resulted in decreased leaf temperature, which encouraged photosynthesis (Hetherington and Woodward, 2003). It was found out in this study that under drought overexpressor lines led to reduced stomatal conductance which was due to significantly decreased stomatal aperture. Partial stomatal closure helped to conserve water in the leaves which was confirmed by decrease in plant leaf temperature and increased photosynthetic efficiency.

(Schonfeld *et al.*, 1988) observed a decline in relative water content in wheat due to drought stress and reported the highest RWC in a tolerant genotype. Decreased relative water content reduced the leaf turgor potential which is directly responsible to affect different physiological processes such as stomatal opening, photosynthesis and leaf expansion (Jones and Turner, 1978). These researchers reported that with a decrease in RWC, leaf osmolality increased and the slow development of water deficits resulted not only in osmotic adjustment but also in a reduction in leaf tissue elasticity.

It has been shown that reductions in both leaf and cell wall extensibility can occur within minutes of root exposure to PEG based osmotic stress in cultivars of maize and wheat (Lu and Neumann, 1998). Under water deficit, cell membrane subjects to changes such as penetrability and decrease in sustainability (Blokhina *et al.*, 2003). Microscopic investigations of dehydrated cells revealed damages including cleavage in the membrane and sedimentation of cytoplasm content. Probably, in these

conditions, the ability to osmotic adjustment is reduced (Meyer and Boyer, 1981). Current study suggested that overexpressor transgenic barley lines remain hydrated under drought stress which enhanced biomass accumulation and increased photosynthetic efficiency.

In conclusion to this study, several effects of spermine and of *Spermine synthase* overexpression in development and adaptation to stress were revealed, but it is not yet known how the calcium signaling responses are mechanistically regulated by spermine and which downstream target genes are regulated. One more aspect to study can be the analysis of plants being rewatered after being under harsh drought because the *SPMS2* overexpressors showed a better performance particularly under extreme conditions. More research is also needed to elucidate the genetic and biochemical pathways to understand the molecular mechanisms at organ and single cell level underlying drought tolerance as influenced by *SPMS*. In the past decades, the understanding about molecular mechanisms of spermine has improved substantially. However, there are still many aspects to be discovered, and it is crucial to reveal how plant signalling and changes in gene expression are integrated into phenotype and specific traits. More investigations will be needed to clearly address if *SPMS* interfere in drought responses other than stomatal regulation, such as the transcriptional regulation of drought-responsive genes.

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Acknowledgements

A decade ago, my father had a dream for me to be recognized as a Doctor. Although he meant some other doctor, but I am sure he would be the most happiest and proud person of my title of PhD. Many people deserve appreciation and acknowledgements in one way or the other way to make this thesis possible. It is my pleasure to thank them all but my apologies in advance if I miss anybody.

All the praises, thanks and acknowledgements are for the Creator **Allah Almighty**, the most beneficent, the most merciful, who gave me strength and enabled me to undertake and execute this research task. Countless salutations upon the Holy Prophet **Hazrat Muhammad (S.A.W)**, source of knowledge for enlightening with the essence of faith in Allah and guiding the mankind, the true path of life. In accordance of Almighty Allah's order his creature must also be acknowledged.

I cannot but thank my supervisor and mentor, **Prof. Dr. EDGAR PEITER** for accepting me as a PhD student. I still remember when I met him for the first time and immediately felt his warmth and friendliness. I always admire you for your immense experience, knowledge, enthusiasm and vision about the science that matters. My heartfelt thanks for your kind supervision, scholastic guidance, critical remarks on my drafts, constructive suggestions for the improvement of writing skills and above all your patience. I offer my sincere thanks for your scientific and personal support over the past four years. It was a great honour and pleasure to work with you, which shaped not only this project but also the course of my future career.

I would also gratefully acknowledge the financial, academic and technical support of the Higher Education Commission (HEC) of Pakistan, who initiated such a nice project for young people to get a PhD from abroad. I am also thankful to all staff from the Prof. Peiter Group for nice gestures, cheerful company and social gatherings. I offer my sincerest gratitude to my lab colleagues for their valuable input, kind and supportive attitude and

amusing conservations. I obliged **Anja Janssen, Liane Freitag, Kristin and Tina Peiter-Volk** for technical support. I always liked to talk to you and you were always very helpful to me. Miss Muller´s help to solve financial and administrative problems is greatly acknowledged.

It would not have been possible to submit this thesis without the help and support of my colleagues and friends, **Tasmia Bashir**, **Bastian Meier** and **Dr. Steffanie Holler**. I would especially thank them for their suggestions, inputs and time to help me in completion of my work. Special mention for my lab colleague, **Ricardo Happeck**. I am most grateful for his advice and knowledge of Molecular techniques and intellectual support during my research work. His enthusiasm in research had always motivated me and he was always accessible and willing to share bright thoughts with me.

To my Pakistani friend in the Halle, **Ali Nawaz and his Family**. Thanks for keeping me sane. Thanks for nice and cheerful company during the past three years. Thanks for being co-sharer of my struggle, arranging dinners, lunch time company, travelling together around Europe, nice jokes, discussions on petty issues, your help in thesis formatting, and endless encouragement. My sincere thanks for everything you have done for me.

Where would I be without my family? I owe my affectionate gratitude to my family back home in Pakistan who has been continuous support to me in the form of prayers and best wishes. No knowledge could ever adequately express my obligation and indebtedness to my great father **Dr. Nasrullah Khan** who encouraged me to be hard-working from the life of my childhood. My Mother, **Ms. Fozia Nasrullah**, is the one who sincerely raised me with her caring and gently love. I just cannot write the word “Thanks” for her as it can never express my feelings for her. My grandmother, whose prayers have been with me. My Beloved Brother **Junaid Khan**, and my sister **Muqeeta Omer** thanks for being supportive and caring siblings. They made this journey a lot easier with words of encouragement, friendship and fun. I just simply wish everybody to be with me healthier and happier ever.

Nufaid Khan

Appendix

1. Chemicals

Substance	Formula	Company	Catalog Nr.
1-Brom 3-Chlorpropane	C_3H_6BrCl		
Acetic Acid	$C_2H_4O_2$	Apolda	579-94-2
Acetonitrile	C_2H_3N	Promega	
Agarose		Biozym Scientific	840004
Ammonium heptamolybdate	(NH4)6Mo7O24		
Ammonium chloride	NH_4Cl		
Ampicillin	$C_{16}H_{18}N_3O_4SNa$	Duchefa	A0104
BAPTA	$C_{22}H_{24}N_2O_{10}$		
BCECF-AM			
Boric acid	BH_3O_3	Fluka	15665
Calcium chloride	$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	31307
Aldrich C2786			
Coelenterazine	$C_{26}H_{21}N_3O_2$		
Copper sulphate	$CuSO_4$		
D(+)-Glucose	$C_6H_{12}O_6$	Carl Roth	HN06.2
Dansyl chloride	$C_{12}H_{12}ClNO_2S$		
Dimethly sulphoxide	$C_2H_6O_3$	Duchefa	D1370
D-Mannitol	$C_6H_4O_6$	Sigma-Aldrich	M1902
EDTA	$H_{14}N_2NaO_3 .2H_2O$	Carl Roth	8043.1

Fe-Na-EDTA	$C_{10}H_{12}FeN_2NaO_8$		
Formaldehyde	CH_2O	Sigma-Aldrich	F8775
Isopropanol	C_3H_8O	Carl Roth	T910.1
Lithium chloride	LiCl		
Magnesium chloride	$MgCl_2$	Fluka	63068
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	Fluka	63140
Manganese sulphate	$MnSO_4$		
MES	$C_6H_{13}NO_4S \cdot xH_2O$	Sigma-Aldrich	M2933
MS Salts+Vitamin		Duchefa	M0231
PEG6000 Poly (ethylene glycol)		Sigma-Aldrich	
Potassium Dihydrogen phosphate	KH_2PO_4	Fluka	60220
Potassium chloride	KCl	Duchefa	PO515
Potassium nitrate	KNO_3		
Sodium dodecyl sulphate	$C_{12}H_{25}NaO_4S$	Carl Roth	2326.2
Sodium chloride	NaCl	Carl Roth	3957.1
Sodium hypochlorite	NaOCl		
Putrescine	$C_4H_{12}N_2$	Sigma	
Saccharose	$C_{12}H_{22}O_{11}$	Carl Roth	4621.1
Spermine	$C_{10}H_{26}N_4$	Sigma	
Spermine tetrahydrochloride	$C_{10}H_{30}Cl_4N_4$		
Spermidine	$C_7H_{19}N_3$	Sigma	
Wuxal			

Zinc Sulphate	$ZnSO_4$

2. Company Address

Company	**Address**
Apolda	Apolda, Germany
Biozym	Oldendorf, Germany
Carl Roth	Karlsruhe, Germany
Duchefa	Haarlem, The Netherlands
Fluka	Buchs, Switzerland
Invitrogen	Darmstadt, Germany
Macherey-Nagel	Duren, Germany
MBI Fermentas	Waltham, MA U.S.A
New England Biolabs (NEB)	Ipswich, U.K
Sigma-Aldrich	St. Louis, USA
Promega	Madison, WI U.S.A
Qiagen	Venlo, The Netherlands

3. **Buffers**

B1: DNA Lysis Buffer

4.5 M guanidinium thiocyanate

2% (w / v) N-lauroylsarcosine

(Reset before use) 1% (v / v) β-mercaptoethanol

50 mM Hepes-KOH

pH 7.5

B2: DNA extraction buffer

150 mM NaCl

5 mM EDTA

50 mM Tris-HCl

pH 8.0

B3: TAE-buffer

20 mM sodium acetate

2 mM EDTA

40 mM Tris-HCl

pH 8.3

B4: 6 x DNA loading buffer

30% (v / v) glycerol

60% 10x TAE buffer (Buffer 5)

0.25% (w/v) bromophenol

B5: Transfer buffer 20 x SSC

3 M NaCl

0.3 M sodium citrate

pH 7.5

B6: Hybridization buffer

5x SSC (Buffer 5)

0.1% (w/v) N-lauroyl sarcosine

0.02% (w/v) SDS

1% (w/v) blocking reagent (Roche Diagnostics, Mannheim, Germany)

Autoclaved

B7: 2 x Wash buffer I

2x SSC (Buffer 5)

0.1% (w/v) SDS

B8: 0.5 x Wash buffer I

0.5 x SSC (Buffer 5)

0.1% (w/v) SDS

B9: 0.25 x Wash buffer I

0.25 x SSC (Buffer 5)

0.1% (w/v) SDS

B10: Blocking buffer

1% (w/v) blocking reagent (Roche Diagnostics, Mannheim)

in wash buffer I and autoclaved

B11: Detection buffer

100 mM NaCl

100 mM Tris-HCl

pH 9.5 and autoclaved

B12: Stripping solution

0.05% (w/v) SDS

3 M NaCl

0.3 M sodium citrate

pH 7.5

B13: Solution M

100mM Maleic Acid

150mM NaCl

pH 7,5

0.3 % Tween 20

The buffers mentioned above, when indicated, were autoclaved for 20 minutes at 121 °C and 2 bar.

B14: Denaturing solution

Stock solution: Mix in a 50-ml-Falcon tube: 29.3 ml Water + 1.76 ml 0.75 M sodium citrate, pH 7.0, and 2.64 ml of 10% (w/v) N-lauroylsarcosine (Sarkosyl). Add 25 g guanidine thiocyanate and dissolve at 60° to 65°C. Store up to 3 months at room temperature.

Working solution: Add 7 µl 2-mercaptoethanol (2-ME) to 1 ml of stock solution.

Final concentrations are 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.1 M 2-ME.

B15: Sodium acetate, 2 M

Add 16.42 g sodium acetate (anhydrous) to 40 ml water and 35 ml glacial acetic acid. Adjust solution to pH 4 with glacial acetic acid and dilute to 100 ml final with water. (The solution is 2 M with respect to sodium ions.) Store up to 1 year at room temperature.

B16: Home-Made Trizol

500 µl Working!!! Denaturation Solution (B14)

50 µl Sodium acetate Solution (B15)

500 µl Aqua-Phenol (Roti-P/C/I)

Curriculum Vitae

Personal data

Name	Nufaid Khan
Date of birth	10th November 1986
Place of birth	Rawalpindi, Pakistan
Address	House 517-D, Askari X, Lahore Cantt (In Pakistan)
	Room No: 11-01, Richard Paulick Str. 13, 06124 Halle (Saale) (In Germany)

Education

September, 2005 - June, 2009	Bachelor of Science in Agriculture, B.Sc (Hons.) Major in Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan
September, 2009 - November, 2011	Masters of Philosophy (M.Phil) in Biotechnology, Forman Christian College, A Chartered University, Lahore, Pakistan
Since 1st April, 2013	PhD studies at the Institut fur Agar- und Ernahrungswissenschaften, Naturwissenschaftliche Fakultat III, Martin-Luther-Universitat Halle-Wittenberg, Germany

Occupations

January, 2012 – July, 2012	Teaching Assistant in Molecular Biology Lab at FCC University, Lahore, Pakistan

Halle (Saale), 30 June 2017 Signature:_____

List of Publications and/or Abstracts

Meeting abstracts / poster presentations

1. **Khan, N.,** Nowak, N., Freitag, L., Peiter E**.**.

Cross-talk of polyamines and calcium signalling in stress tolerance of barley and *Arabidopsis*

International meeting of Plant Calcium Signaling, Münster 2014

2. **Khan, N.,** Nowak, N., Freitag, L., Peiter E**.**.

Cross-talk of polyamines and calcium signalling in stress tolerance of barley.

International Conference of the German Society of Plant Nutrition, Halle 2014

3. **Khan, N.,** Nowak, N., Freitag, L., Peiter E**.**.

Cross-talk of polyamines and calcium signalling in stress tolerance of barley and *Arabidopsis*.

International Conference on Emerging Trends for Life Sciences for Sustainable Development, Lahore 2014

4. **Khan, N.,** Nowak, N., Peiter E**.**.

The role of spermine in drought tolerance of barley (*Hordeum vulgare*)

International Conference of the German Society of Plant Nutrition, Hohenheim 2016

5. **Khan, N.,** Nowak, N., Peiter E**.**.

Cross-talk of polyamine and calcium signalling in stress tolerance of barley (*Hordeum vulgare*)

Annual meeting of Interdisciplinary Center for Crop Plant Research (IZN), Halle 2015

Eidesstattliche Erklarung / *Declaration under Oath*

Ich erklare an Eides statt, dass ich die Arbeit selbststandig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wortlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

_____ _____

Date Signature of Applicant