Altering ABA levels in leaf and seed tissue of barley to study the role of ABA on plant performance under post-anthesis drought stress using the transgenic approach

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

vorgelegt der

Naturwissenschaftlichen Fakultät I

Biowissenschaften

der Martin-Luther-Universität Halle-Wittenberg

von

Vokkaliga T. Harshavardhan

geb. am: 14.12.1981 in: Madikeri, Karnataka, India.

Gutachter /in:

1.Prof. Dr. Ulrich Wobus

2.Prof. Dr. Klaus Humbeck

3.Prof. Dr. Gerhard Leubner

Halle (Saale): 12.07.2012

Verteidigungsdatum: 11.02.2013

Contents

Abbreviation i

1 Introduction 1

1.1 Barley seed development: 1

1.2 Terminal drought and its impact on developing seeds: 2

1.3 Functional role of ABA in seed development: 3

1.4 Pronounced effects of ABA under drought stress: 4

1.5 ABA perception and signalling: 6

1.6 ABA metabolism and transport: 7

1.7 ABA biosynthesis, catabolism and de-conjugation pathway genes in barley: 10

1.8 ABA manipulation using transgenic strategy: 11

1.9 Aim of the present study: 15

2 Materials and methods 16

2.1 Materials 16

2.1.1 Plant materials 16

2.1.2 Bacterial strains 16

2.1.3 Plasmids and Vectors 16

2.1.4 Primers 16

2.1.5 Chemicals 16

2.1.6 Enzymes and kits 17

2.1.7 Special instruments 17

2.1.8 Media 17

2.2 Methods 18

2.2.1 Plant material and growth conditions for LP110 breeding line: 18

2.2.2 RNA isolation from leaf and developing seed: 20

2.2.3 Quality checking of the RNA using NanoDrop and the Agilent 2100 bioanalyzer: 20

2.2.4 Complementary DNA (cDNA) synthesis and quality of the cDNA: 21

2.2.5 Quantitative real time polymerase chain reaction (qRT-PCR): 22

2.2.6 Barley seed cDNA macroarray (12K) analysis: differential expression of starch biosynthesis and degradation pathway genes 22

2.2.7 *In-silic*o Promoter Analysis: 23

2.2.8 Extraction and analysis of ABA (LC-MS/MS), ABA catabolites and other hormones: 23

2.2.9 Starch measurement: 23

2.2.10 Enzyme activity: 24

2.2.11 Basic cloning methods and sequencing: 25

2.2.12 Cloning of *AtNCED6* under *SalT* promoter: 25

2.2.13 Cloning of *AtNCED6* under *1AX1* promoter: 26

2.2.14 Stable transformation of barley and generation of double haploid plants: 28

2.2.15 Genomic DNA extraction: 30

2.2.16 Southern hybridization: 30

2.2.17 Plant growing condition for the wild type (WT) and transgenic plants: 31

2.2.18 Drought stress imposition, sample collection and phenotyping: 32

2.2.19 Parameters analyzed: 32

2.2.20 Branched spike characterization: 34

2.2.21 Physiological characterization of plants under drought stress: 34

2.2.22 Screening of the transgenic plants in the rain shelter: 35

2.2.23 Measurement of endo-polyploidy: 35

3 Results 36

3.1 ABA accumulation and catabolism in source (flag leaf) and sink (seed) tissues under terminal drought stress: 36

3.1.1 ABA biosynthesis: 36

3.1.2 ABA degradation: 37

3.1.3 ABA de-conjugation: 38

3.2 Impact of ABA on seed filling under drought: 39

3.3 Characterization of transgenic plants altered for ABA homeostasis: 42

3.4 Characterization of *OsSalT*:: *AtNCED6* (SN) transgenic plants: 43

3.4.1 Phenotypic alterations observed in the SN transgenic plants: 43

3.4.2 Transgene expression in SN transgenic under post-anthesis drought: 46

3.4.3 ABA levels in leaf and seeds of WT and SN transgenic plants under post-anthesis drought stress: 46

3.4.4 Regulation of *HvNCED* and *HvABA8′*OH family genes in WT and SN transgenics: 47

3.4.5 Regulation of ABA catabolic products in leaves and seeds of WT and SN410_8 plants under post-anthesis drought stress: 50

3.4.6 Hormonal levels altered in WT and SN410_8 in leaf and seed under post-anthesis drought: 52

3.4.7 Performance of WT and SN transgenic under stress: 53

3.4.8 Stress and endoreduplication 58

3.5 Characterization of *Ta1AX1*::*AtNCED6* transgenic plants: 59

3.5.1 ABA content in flag leaf and developing seed of XN transgenic plants: 61

3.5.2 Regulation of *HvNCED* and *HvABA8′OH* family of genes in leaf and seeds of XN26_5 transgenic plant: 62

3.5.3 Physiological performance of XN26_5 transgenic plants: 63

3.5.3 TGW and yield related parameter of XN26_5 transgenic plants: 64

3.6 Exploring the role of altered ABA homeostasis, at different time points of grain filling period, on seed starch accumulation: 66

3.6.1 Estimation of starch content and starch accumulation rate in seeds: 66

3.7 Expression of starch metabolism genes under control and stress in the developing seed under post anthesis drought: 68

3.7.1 Starch biosynthesis...68
3.7.2 Starch degradation: 72
3.8 Performance of green house grown WT and transgenic plants (SN410_8 and XN26_5) under later stages of drought stress (12- 20 DAS):.......................................74
3.8.1 TGW 74
3.8.2 Starch and moisture content:...74
3.8.3 Regulation of starch metabolism genes: 76
3.8.4 Regulation of *AtNCED6*, *HvNCEDs* and *HvABA8'OHs* genes and ABA content:..78
3.9 Screening of WT and transgenic lines (SN410_8 and XN26_5) in rain shelter:.....79
4 Discussion 82
4.1 Feed-back and –forward regulation by ABA regulates its homeostasis:.................83
4.1.1 Regulation of ABA biosynthesis: 83
4.1.2 Regulation of ABA catabolism:...84
4.2 Disturbances in ABA homeostasis affect endosperm polyploidization:..................85
4.3 De-regulation of ABA homeostasis in seeds affects starch metabolism: 86
4.4 Conclusion: how altered ABA homeostasis affects starch metabolism, TGW and yield: 89
5 Summary 92
6 Zusammenfassung...95
7 References 98
8 Supplementary figures and tables: 120
9 Acknowledgement 133
10 Appendix..136

Abbreviation

Chemicals (e.g. NaCl), restriction enzymes (e.g. *EcoR* I), units (e.g. mL, s, min, h,) as well as media and buffers are explained in the text and they are included in the abbreviation list.

2ip	Isopentenyladenine
A	Assimilation rate
AAO	Abscisic aldehyde oxidase
ABA	Abscisic acid
ABA1	ABA deficient 1; Zeaxanthin epoxidase
ABA2	ABA deficient 2; Short chain dehydrogenase reductase
ABA3	ABA deficient 3; Molybdenum co-factor sulfurase
ABA8'OH	ABA 8' hydroxylase
ABA-GE	Abscisic acid glucose ester
ABI1	ABA-insensitive 1
ABRE	ABA responsive element
AGP-L	ADP-glucose-pyrophosphorylase large subunit
AGP-S	ADP-glucose-pyrophosphorylase small subunit
Ah	*Arachis hypogea*
Amp^r	Amppicillin resistance
AMY	Amylase
ANOVA	Analysis of variance
AREB	ABA responsive element binding protein
At	*Arabidopsis thaliana*
AtABCG	Arabidopsis thaliana ATP binding cassette transporter gene
BG	Beta glucosidase
bZIP	Basic leucine zipper
CCD	Carotenoid cleveage deoxygenase
cDNA	Complementary DNA
Ci	Internal CO_2
cv.	cultivar
CYP707A	Cytochrome p450
DAF	Days after flowering
DAS	Days after stress
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribo nucleic acid
DNase	deoxyribonucleases
DPA	Dihydro phasc acid
DW	Dry weight
E	Transpiration rate
EST	Expressed sequence tag
FW	Fresh weight
GA	Gibberlins

GBSS	Granular bound starch synthase
g_s	Stomatal conductance
Hv	*Hordeum vulgare*
IAA	Indole-3-acetic acid (Auxin)
IAA-Asp	IAA- aspartic acid
IRGA	Infra red gas analyser
ISA	Isoamylase
MoCo	Molybdenum co-factor
MCSU	Molybdenum co-factor sulfurase
NCED	9-*cis*-epoxycarotenoid dioxygenase
Os	*Oryza sativa*
PA	Phaseic acid
PCR	Polymerase chain reaction
PP2C	Type 2C protein phosphatase
PPM	Parts per million
PYL	PYR-like protein
PYR	Pyrabactin resistance protein
qRT PCR	Quantitative real time polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
RCAR	Regulatory component of ABA receptor
RNA	Ribo nucleic acid
RNase	Ribonuclease
RT	Room temperature
RT	Room temperature
RWC	Relative leaf water content
SBE	Starch branching enzyme
SDR	Short chain dehydrogenase
SE	Standard error
SM200	Soil moisture sensor 200
SN	*OsSalT* promoter driving the expression of *AtNCED6* (SN)
SN23_3	Homozygous T2 line of SN transgenics
SN410_8	Double haploid plant in second generation
SnRK	SNF- related protein kinase
$Spec^r$	Spectinomycin resistance
SS	Starch synthase
SUS	Sucrose synthase enzyme
T_0	Primary transgenic
T_1	Progeny of the T_0 plants
T_2	Progeny of the T_1 plants
Ta	*Triticum aestivum*
TGW	Thousand grain weight
t-Z	Trans-zeatin
t-ZR	Trans-zeatin riboside
WT	Wildtype (*Hordeum vulgare* cv.Golden promise)

WUE	Water use efficiency
XN	*Ta1AX1* promoter driving the expression of *AtNCED6* (XN)
XN17_2 XN26_5	Homozygous T_2 line of XN transgenics
ZEP	Zeaxanthin epoxidase

1 Introduction

1.1 Barley seed development:

Cereals are important staple food crops which are central to food security as they are used for feeding the human population worldwide and used as fodder for cattle. In today's worldwide production, barley ranks fourth among cereals. Since barley is used as a model crop of triticeace, any genetics and genomic resources generated in barley will help to study and explore important crops such as wheat (Sreenivasulu et al., 2008). Barley seeds have traditionally been used in malting and brewing industry and therefore also are of commercial importance.

Seed development is a dynamic complex phenomenon which involves cell division, followed by cell differentiation, storage accumulation and maturation (Sreenivasulu et al., 2010a; Weber et al., 1997). Seeds are complex organs containing diploid embryo and triploid endosperm. In barley, endosperm is the predominant filial sink organ that mainly stores starch and storage proteins. These filial organs are surrounded by maternal tissue (pericarp), which possesses a main vascular bundle in the ventral region and two lateral vascular bundles. The vascular bundles download nutrients into specialized maternal cells (nucellar projection), where these are further mobilized into the underlying filial tissues *i.e.* endosperm and embryo via active and passive transport mechanisms (Patrick, 1997; Patrick and Offler 2001; Weber et al., 1997). Major events of barley endosperm development include three main stages: prestorage stage (syncytial stage: 0-4 DAF), Intermediate stage (cellularization stage: 5-8 DAF) and differentiation stage covering mainly seed storage and maturation events (8-20 DAF) (Sreenivasulu et al., 2010a).

During syncytial stage, mitotic division without cytokinesis dominates resulting in a multinucleate endosperm. During 5 to 6 DAF, vacuolation of the evenly spaced nuclei in cytoplasm takes places that help in deposition of the first anticlinal walls of the endospermal cells resulting in the onset of cellularization of endosperm. By 8 DAF, the cellularization of endosperm is completed and the frequency of mitotic division diminishes. The endosperm cells then enter the maturation phase, with formation of elongated polyhedral starchy endosperm cells and differentiation of aleurone during early phase. In the later phase of the maturation, mitotic activity stops and storage product deposition initiates first at central regions and proceeds slowly towards the periphery of

starchy endosperm (Olsen et al., 1992; Weschke et al., 2000). In barley, the endosperm stores complex carbohydrates and proteins which are major sources of nutrients. At the end of the seed filling stage, starchy endosperm undergoes programmed cell death and embryo attains desiccation tolerance. The vast majority of published research has been devoted to the study of endosperm development under ambient conditions (for review, see e.g. Ritchie et al., 2000), but only little is known about the influence of terminal drought on seed re-programming events that affect grain weight and seed filling events during the onset of storage phase. Besides assimilates, hormones are also known to be imported into the developing seed from maternal tissue. Drought can alter the dynamics of growth promoting and growth retarding hormones dramatically in seeds. Also, in particular, we lack comprehensive understanding of hormonal homeostasis within developing seeds and its alteration under challenging environments.

1.2 Terminal drought and its impact on developing seeds:

Plants are exposed to various environmental stresses because of their sessile nature. Among the abiotic stresses, drought is the most wide spread and most stringent factor limiting plant growth and productivity, resulting in reduced yield in several species including barley, maize, rice, sorghum and other grasses, for decades (Heyne and Brunson, 1940; Boyer, 1982; Araus et al., 2002). Besides the importance of severity of drought stress, the timing of drought stress has a larger impact on yield. If drought stress occurs early in the season that coincides with sowing, it impairs germination and results in poor crop stand (Harris et al., 2002); and if it occurs during the vegetative phase of plant development, it results in reduction of leaf area, resulting in reduction of both fresh and dry mass production. This is basically due to its effect on leaf expansion and development (cell division and expansion), gas exchange and photosynthesis (Rucker et al., 1995; Nam et al., 1998). Occurrence of drought during later stages of crop development (flowering and grain filling) results in barren sinks and if sinks are set, it results in reduced assimilate flux mobilization to developing sinks (assimilate partitioning or phloem loading) as well as reduced starch synthesis (Yadav et al., 2004; Farooq et al., 2009).

The combined effect is reduction in not only the source tissues, but also impacting the reprogramming of reproductive tissues. The occurrence of drought in particular during flowering, at the time of fertilization, endosperm establishment and seed filling leads to

severe yield loss and decline in seed quality (Sreenivasulu et al., 2007; Barnabás et al., 2008). Typical physiological mechanisms occurring during drought leads to close down of stomata lowering photosynthesis rate, high respiration with activation of mitochondrial related energy release, production of reactive oxygen species, cell damage and activation of senescence and cell death. Strategies to improve yield stability under harsh environmental conditions are dependent on the synchrony between source (leaf) and sink (reproductive tissue/developing seed) organs by either supplying required assimilate (sucrose) by optimizing photosynthesis or by triggering remobilization events from stem and leaf (Yang and Zhang, 2006; Yang et al., 2006a and 2006b) to meet the required seed demand. It is known that decrease in photosynthetic efficiency and shortage of assimilates under drought stress results in termination of seed set and drastic reduction in kernel number (Zinselmeier et al., 1999).

1.3 Functional role of ABA in seed development:

Abscisic acid (ABA) is a sesquiterpene plant hormone (Addicott, 1983) that was discovered during the early 1960's (Wareing et al., 1964; Ohkuma et al., 1963; Cornforth et al., 1965; Addicott et al., 1968). This hormone is ubiquitously found in all vascular and non-vascular plants (Milborrow, 1967; Addicott and Lyon, 1969; Knight et al., 1995). ABA at its basal levels influence many important plant growth and development processes, such as embryo and seed development, promotion of seed desiccation tolerance and dormancy, germination, seedling establishment, vegetative development, general growth, reproduction, senescence and adaptive responses to environmental stress (extensive reviewed in Cutler et al., 2010; Finkelstein et al., 2002; Hubbard et al., 2010; Nambara and Marion-Poll 2005; Raghavendra et al., 2010; Wasilewska et al., 2008; Weiner et al., 2010). ABA biosynthesis and signaling genes have been well studied in non-seed tissues, but little is known about the situation in seeds (Nambara and Marion-Poll, 2003). Analysis of the wilting mutants and exogenous treatment of ABA revealed its importance in maintaining water relations in plants by regulating stomatal closure and opening that in turn also affected the photosynthetic capacity of plants (Imber et al., 1970; Jones and Mansfield, 1970; Tal et al., 1970).

In many species, two peaks of ABA accumulation during seed development have been reported. The first peak of ABA in seeds is contributed from maternally derived ABA while the second peak of ABA depends on its *de-novo* biosynthesis in the seed itself. The

first peak of ABA contributed by maternal tissue (Frey et al., 2004) helps in maintaining the embryos in an embryonic state (Raz et al., 2001; Taiz and Zeiger, 2006). However, after which its levels are maintained low and are found to increase during the maturation phase. The second peak of ABA is required for the induction of storage protein accumulation and generally for seed maturation, dormancy and desiccation tolerance as seeds lose around 90% of its water (Karssen et al., 1983; McCarty, 1995; Hopkins, 1999; Leung and Giraudat, 1998). This phase is further characterized by the synthesis of late-embryogenesis abundant proteins (LEA) that are induced by ABA that provide desiccation tolerance to seeds (Delseny et al., 2001; Rock and Quatrano, 1995). In addition, ABA antagonizes the GA effects there by avoids precocious seed germination by maintaining seed dormancy (Thomas et al., 1965; Finkelstein et al 2002; Raz et al., 2001; White and Rivan, 2000; Seo et al., 2006). In barley too, two ABA peaks during seed development have been reported (Jacobsen et al., 2002; Millar et al., 2006).

Temporal patterns of ABA biosynthetic gene expression related to seed tissues have been described for *ZEP* (Audran et al., 2001), *NCED* (Lefebvre et al., 2006) and *AO* (Seo et al., 2004) in *Arabidopsis*, but little is known for cereal grains with exception of the aleurone tissue (Ritchie et al., 2000). According to our previous work in barley, endogenous production of ABA is likely to take place both in endosperm and embryo during seed maturation as reasoned by the activation of ABA biosynthesis genes (Sreenivasulu et al., 2006; Sreenivasulu et al., 2008; Sreenivasulu et al., 2010a). However, a comprehensive knowledge of ABA biosynthesis and catabolism events involved in the regulation of ABA levels in different grain tissues and its regulation of homeostasis under drought is still lacking.

1.4 Pronounced effects of ABA under drought stress:

Plants have evolved escape, avoidance and tolerance mechanisms to overcome drought stress (Levitt, 1980; Price et al., 2002). Most of the breeding programs to improve performance of plants under drought have concentrated on producing varieties that exhibit drought avoidance rather than drought tolerance mechanism (Blum, 2005). With the growing unpredictable occurrence and distribution of drought across plant development, it necessitates the generation of plants that exhibit drought tolerance mechanisms. Plants use multiple drought tolerance strategies and these components need to be dissected by exploring genetic diversity. The tolerance mechanisms exhibited by

plants for these stresses is complex as they usually face multiple stresses during their development. However, plants perceive and respond to abiotic stress with adaptive processes that is mainly controlled by the plant phytohormone abscisic acid (ABA), which basically acts as a messenger in regulating plant water status.

Among the plant hormones, ABA that is synthesized in response to drought coordinates plant growth and development with its environment to protect plants from drought (Hubbard et al., 2010). Levels of ABA in a given tissue depend on the rate of its biosynthesis, catabolism and transport to and from other tissues. In general, ABA is known for its negative effect on growth. It is involved in regulating processes such as affecting germination, stomatal closure (minimize transpiration), accelerate abscission and senescence (Finkelstein et al., 2002; Fujita et al., 2005). In spite of ABA's negative role, its production under drought stress is found to protect plants against drought stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein et al., 2002; Xiong et al., 2002a and b; Fujita et al., 2005). In addition, many of the genes that are induced under drought stress are also activated by ABA (Ingram and Bartels, 1996; Seki et al., 2002). Moreover, transcriptome analysis of Arabidopsis and rice using microarrays revealed that nearly half of the genes in Arabidopsis that were induced by drought were also responsive to ABA, and all the ABA inducible genes were also induced by drought in rice (Yamaguchi-Shinozaki and Shinozaki, 2006).

Arabidopsis is known to regulate its gene expression under drought stress both by ABA-dependent and –independent pathway (Shinozaki and Yamaguchi-Shinozaki, 2000). However, ABA is shown to regulate a large subset of genes compared to any other plant hormone (Goda et al. 2008; Mizuno and Yamashino 2008; Nemhauser et al. 2006). Numerous transcriptomic studies in Arabidopsis have provided a global picture of the ABA-regulated genes (Cutler et al. 2010; Fujita et al. 2009; Goda et al. 2008; Kilian et al. 2007; Nemhauser et al. 2006; Seki et al. 2002; Shinozaki et al. 2003; Urano et al. 2009), which along with metabolomic studies have identified that nearly 10% of Arabidopsis protein coding genes are regulated by ABA (Nemhauser et al. 2006). Drought responsive mechanisms and the role of ABA in drought tolerance have been extensively studied in Arabidopsis (Bray, 1997; Shinozaki et al., 2003). Recent efforts need to be in the direction of using this knowledge to manipulate plants that can produce and respond to

ABA as this hormone controls many of the adaptive responses such as regulating stomatal closure, canopy area, root biomass that would help improve the crop water use efficiency.

The ABA content is also known to increase in response to water deficits; and ABA in coordination with other hormones regulates root growth that plays as one of the key adaptive response of plants to maintain adequate water supply during drought (Sharp et al., 2004). By its regulated expression of stress-responsive genes it also helps in accumulation of compatible osmolytes by the regulated synthesis of LEA proteins, dehydrins and other protective proteins that help in maintaining cellular water status and protecting cellular organelles and proteins (Ingrams and Bartels, 1996; Verslues et al., 2006). In addition, ABA is also known to be involved in biotic stress affecting pathogen responses. It is shown to play role in promoting resistance to pathogens by regulating pathogens entry into plant system and regulating plants defense response (review by Ton et al., 2009). The ABA mediated responses depend on ABA levels and tissues sensitivity to ABA that is achieved by the regulated biosynthesis or catabolism, its transport and signalling.

1.5 ABA perception and signalling:

ABA recognition receptors were recently identified, *viz.,* the plasma membrane localized two GPCR-type G proteins, a plastid localized Mg-chelatase, and cytosol and nucleus localized soluble START or pyrabactin resistant (PYR)/PYR-like (PYL)/regulatory component of ABA receptor 1 (RCAR) (Ma et al., 2009; Pandey et al., 2009; Park et al., 2009; Shen et al., 2006). However, only PYR/PYL/RCAR class of receptor was shown to bind and regulate the activity of protein phosphatase 2C (PP2C), involved in ABA signalling, in presence of ABA agonist pyrabactin. Arabidopsis has 14 members that represent the PYR/PYL/RCAR receptor proteins (Park et al. 2009), 9 members that code for type A PP2Cs (Schweighofer et al. 2004) and 10 members for *SnRK2* subfamily. All identified receptor members are proposed to be involved in ABA signalling while only 6 of the negative regulators, PP2Cs, are involved in ABA signalling and only 5 of the SnRK2s were activated by ABA (Boudsocq et al. 2004, 2007; Park et al., 2009; Szostkiewicz et al., 2010). The various combinations of interaction suggest that a highly complex signalling mechanism exists for ABA. Binding of ABA or its agonist is proposed to induce conformational changes in the receptor that then interacts with active site of PP2Cs inhibiting their phosphatase activity of de-phosphorylating the SnRK

proteins involved in various ABA-induced physiological processes (Fujii et al., 2009; Hao et al., 2010; Peterson et al., 2010; Umezawa et al., 2009; Vald et al., 2009).

Under low ABA levels, SnRK2s are maintained in an inactive state by the dephosphorylation of its serine residue, which is mediated by binding of a PP2C member. However, elevated levels of ABA result in the binding of PYR/PYL/RCAR receptor to PP2C and there by releasing the SnRK2s that by auto-phosphorylation enter into an active state. The active SnRK2s are able to phosphorylate various down-stream targets, like the bZIP transcription factors and ion channels. The bZIP transcription factors can bind to the ABA-responsive elements (ABRE) in the promoter of ABA responsive genes, *AREB1* (ABRE binding protein 1), *AREB2*, *AREB3* and *ABI5* (Uno et al. 2000; Fujita et al. 2005; Furihata et al. 2006; Fujii et al. 2009; Yoshida et al. 2010) that further activate ABA responsive genes (Furihata et al. 2006; Fujii et al. 2009). The ion channels, *SLAC1* (slow anion channel 1) are activated and *KAT* (inward rectifying K^+ channel) is deactivated by SnRK2s that mediate ABA-regulated stomatal closure (Pilot et al. 2001; Vahisalu et al. 2008).

1.6 ABA metabolism and transport:

Most of the enzymes involved in ABA biosynthesis were identified (for extensive review see Finkelstein and Rock, 2002; Nambara and Marion-Poll, 2005) by characterization of ABA auxotrophic mutants of Arabidopsis and corn (Koorneef et al., 1982; Schwartz et al., 1997a and b; Tan et al., 1997; Koorneef et al.,1998). ABA biosynthesis occurs in plastids with the exception of the last two steps where xanthoxin is converted to ABA in the cytosol (Marin et al., 1996; Tan et al., 1997; Seo and Koshiba, 2002). The first biosynthetic step in ABA biosynthesis is the conversion of zeaxanthin a C40 carotenoid precursor to all-trans-violaxanthin, a two-step epoxidation process catalyzed by zeaxanthin epoxidase (*ZEP*/*AtABA1*) (Marin et al., 1996; Audran et al., 2001). The enzyme(s) involved in the conversion of all-trans-violaxanthin to 9-cis-violaxanthin or 9-cis-neoxanthin have not yet been identified. 9-cis-epoxycarotenoid dioxygenase (*NCED*) catalyzes the next step, the oxidative cleavage of 9-cis-violaxanthin and/or 9-cis-neoxanthin to produce xanthoxin, considered to be the rate limiting step (Tan et al., 1997; Burbidge et al., 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002). Xanthoxin is eventually exported to the cytosol and converted to abscisic aldehyde by a short-chain dehydrogenase/reductase (*SDR*/*AtABA2*) (Cheng et al., 2002; Gonzalez-

Guzman et al., 2002). Abscisic aldehyde is then oxidized to ABA by aldehyde oxidase (*AAO*/*AO*) (Seo et al., 2004). AO needs the sulfurylated form of a molybdenum cofactor (*MoCo*/*AtABA3*) for its activity (Bittner et al., 2001).

Most of these ABA biosynthesis genes are transcriptionally up-regulated under abiotic stress (Bray, 2007). Like most biosynthetic pathways, ABA biosynthesis is also regulated by its end product where in ABA negatively regulates its own accumulation by activating its catabolic enzymes (Cutler and Krochko, 1999). Using antibodies that are specific for AtNCED3, AtABA2 and AAO3 it was shown that leaf vascular parenchyma cells are the primary sites of ABA biosynthesis both under control and drought stress treatments (Endo et al. 2008; Koiwai et al. 2004). Based on this, it was proposed that ABA and its precursors produced in the vascular tissue are transported to guard cells leading to accumulation of ABA in stomata. However, it was later shown that ABA is also synthesised in guard cells. The evidence came from the reporter gene analysis of *AtNCED2*, *AtNCED3* and *AAO3* in guard cells (Tan et al., 2003; Koiwai et al., 2004). Apart from this, it was further confirmed by the analysis of localization of ABA catabolic enzyme, *CYP707A1* and *3* which are induced in vascular tissue and guard cells (Seo and Koshiba, 2011), suggesting to attain hormonal homeostasis.

In contrast to ABA biosynthesis, very little is known about its catabolism leading to the production of less active and inactive forms (Nambara and Marion-Poll, 2005). Reduction of elevated ABA levels is regulated by two mechanisms: (i) catabolism of ABA and (ii) inactivation of ABA. Higher levels of ABA are known to trigger the major catabolic route, which leads to the formation of 8'-hydroxy ABA catalyzed by the cytochrome P450 monooxygenase, ABA 8'-hydroxylase. The 8'-hydroxy ABA is subsequently converted to phaseic acid by spontaneous isomerization (Kushiro et al., 2004). Except the gene for this first step of ABA catabolism, other genes involved in ABA breakdown are not known. Hydroxylation of ABA occurs in three different ways to form hydroxylated ABA that retains substantial biological activity (Zhou et al., 2004; Zou et al., 1995). The spontaneous cyclization of the hydroxylated ABA results in phaseic acid (PA) which is further reduced to dihydro phaseic acid (DPA) by a soluble reductase (Gillard and Walton 1976).

This spontaneous cyclization results in significant reduction of the biological activity of ABA in PA form and null activity of the ABA in its DPA form (Balsevich et al., 1994; Gusta et al., 1992; Walton and Li 1995; Zou et al., 1995). Majority of ABA is converted to its inactive form via hydroxylation at 8' which is carried out by a cytochrome p450, ABA 8'-hydroxyalse (Kushiro et al., 2004; Saito et al., 2004). Although these catabolic enzymes are regulated under non-stressful condition, they are also shown to be up-regulated under stress (Saito et al., 2004). A different form of ABA inactivation is the conjugation with glucose by ABA glucosyltransferase to form the inactive ABA glucose ester (ABA-GE) (Xu et al., 2002). This reaction is reversible, and the de-conjugation of ABA-GE is catalyzed by ß-glucosidase (Lee et al., 2006). ABA conjugates with glucose to form ABA-GE which is regarded as the major transport form of ABA (Sauter and Hartung, 2002; Wilkinson and Hartung, 2009), which also functions in root to shoot signalling under stress (for review see Sauter et al., 2002). This conjugated form was recently shown to be hydrolysed to its active ABA form by a β-glucosidase (Lee et al., 2006).

It is not only the endogenous synthesis of ABA which determines its levels in a tissue, but also the transport of ABA determines the concentration of ABA at the site of action (Jiang and Hartung, 2008; Sauter et al., 2001; Wilkinson and Davies, 2002). The mobile nature of ABA is shown by crossing and grafting experiments. Grafting between wild type and ABA deficient mutants proved that ABA found in seeds is due to its transport from vegetative tissues (Frey et al., 2004). Although there were several indications of the presence of carriers that play a role in ABA uptake by cells (Bianco-Colomas et al., 1991; Perras et al., 1994; Windsor et al., 1992), only recently ABA transporters were identified. Based on mutant analysis, two ABA transporters, AtABCG25 and AtABCG40, were identified that play role in mediating ABA export (from inside to outside the cell) and import (from outside to inside the cell), respectively. The promoter of *AtABCG25* was also found to be active in vascular tissue and that of *AtABCG40* was active in guard cell, which was consistent with the hypothesis that the primary site of ABA synthesis is vascular tissue from which it is transported to guard cells (Kuromori et al., 2010; Kang et al., 2010).

However, the mutants defective in both these transporters were not similar in phenotype to typical ABA deficient mutants. This gives rise to the possible existence of redundant

transporters or passive transport mechanism mediated by pH gradient. Under soil drying conditions, the pH of xylem sap and the apoplast increase that mediate the transport of ABA from root to shoot, and by transpiration stream help in its movement into guard cells. In addition, pH changes of the cell could help in either the export or import of ABA according to pH gradient without presence of specific transporters (Wilkinson, 1999; Wilkinson and Davies, 2002). Nevertheless, cytosol usually has a pH of 7.2 -7.4 and the apoplast has a pH of 5.0 - 6.0, indicating the need for a specific transporter of ABA.

Using a modified yeast two-hybrid system that used ABA receptor complex (PYR/PYL/RCAR) as sensors, functional screen was carried out to identify ABA transporters. One of the candidates of low-affinity nitrate transporter NRT1.2 was found to also function as an ABA-importing transporter (AIT1). This ABA importer probably functions in regulation of stomatal aperture in inflorescence stems. This was further confirmed by the promoter activity of AIT1 which was highly active in vascular tissues of inflorescence stems, leaves and roots (Kanno et al., 2012).

1.7 ABA biosynthesis, catabolism and de-conjugation pathway genes in barley:

In total 41 sequences that code for proteins involved in ABA biosynthesis, catabolism and de-conjugation genes in barley were identified using Arabidopsis and rice genes to query the "HarvEST assembly" which is the source of barley *ESTs*.

Putative gene family members of ABA biosynthesis genes in barley have been annotated. Barley has five *ZEP* (*HvZEP1, 2, 3, 4* and *5*) members. Except for *HvZEP1* all the others are partial clones. The NCED enzyme (known to play role in ABA biosynthesis) and carotenoid cleavage dioxygenases (*CCDs*: differ slightly from *NCED*) are collectively grouped as NCEDs. Apart from the already known *NCED* genes in barley (*HvNCED1* and *HvNCED2*) three putative *CCD*/*NCED* sequences of which two coded for full-length (*HvCCD3* and *HvCCD4*) protein and one coded for partial (*HvCCD5*) protein were identified. Although only one *SDR* coding gene is identified in Arabidopsis and rice, barley was found to have seven putative *SDR* coding genes. Eight putative *AO* coding sequences were identified of which only *HvAO2* and *HvAO3* was full-length and the rest were partial. Oxidation of abscisic aldehyde to ABA, in Arabidopsis, is also catalyzed by AO isoform which needs the sulfurylated form of molybdenum cofactor (MoCo) catalyzed by molybdenum cofactor sulfurase (*MCSU*). Three putative *HvMCSU,* were identified all of which were only partial length (Seiler et al., 2011).

The most common conjugation of ABA is the formation of ABA-glucose ester (ABA-GE), catalyzed by ABA glucosyltransferase. ABA can be released from ABA-GE via the action of β-D-glucosidase (BG). However, because of the broad substrate specificity of BG it is difficult to identify similar gene in barley that can specifically deconjugate ABA. Only AtBG1 is functionally proven to be involved in de-conjugation process in Arabidopsis. Using AtBG1 as query, ten putative *HvBG* genes (7 were full-length cDNAs) were identified. Although the catabolites formed by hydroxylation at C-7′, C-8′ , or C-9′ are identified; only the genes coding for ABA8′ hydroxylase (*ABA8′*OH) have been widely characterized. Using the Arabidopsis and rice CYP707A sequences, *HvABA8′OH3* in addition to the two already known *HvABA8′OH1* and *HvABA8′OH2* genes in barley was identified (Seiler et al., 2011).

1.8 ABA manipulation using transgenic strategy:

There are few transgenic studies reported to enhance drought tolerance by overexpression of ABA responsive genes (Jeanneau et al., 2002; Ding et al., 2009). Drought tolerance is a complex trait. Usually the transgenic plants are said to be drought tolerant if they perform better than the untransformed plants under drought stress in terms of yield, plant biomass, assimilation, WUE, reduced water loss etc. However, it is the thousand grain weight or yield that is the best parameter that defines the overall performance of the plant under stress. A detailed list of transgenic plants produced to alter ABA levels by the manipulated expression of either ABA biosynthetic or catabolic genes is described in Table 1.1. Although in most of the cases an increase or decrease in ABA was found based on the manipulation of ABA biosynthetic or catabolic gene, none of them were able to relate it to yield. Out of the 6 independent studies that have over expressed *ZEP* gene, ABA increase was observed only in 2 studies (Frey et al., 1999; Park et al., 2008); and increased tolerance to drought stress (decreased stomatal aperture and increased expression of stress responsive genes) was observed only by the plants that had increased ABA (Park et al., 2008). However, the effect of it on yield was not studied. Similarly, the key rate limiting enzyme of ABA biosynthesis, *NCED*, has also been over-expressed to alter ABA levels. There are 14 independent studies that have tried to regulate ABA via *NCED* expression. In most cases it is over-expressed under constitutive promoter (35S) resulting in retarded growth under control conditions (Table 1.1). In these studies ABA was found to increase under control conditions and in some cases it was either found to be increased or decreased on induction of stress. In nearly 50 % of the studies, stress

tolerance of the plants was increased that could be correlated to altered ABA levels. Nevertheless, only in two studies, increase in ABA correlated to increase in biomass (Zhang et al., 2008, 2009; Aswath et al., 2005). Apart from regulating these two key enzymes of ABA biosynthesis, there are three studies that have over-expressed *AAO3*, *AtABA2* (SDR) and *AtABA3*, respectively (Melhorn et al., 2008; Lin et al., 2007; Yue et al., 2011). Only over-expression of *AtABA2* and *AtABA3* resulted in increased ABA levels, and the transgenic plants also displayed increased tolerance to salinity and drought stress (decreased water loss, improved survival, increased anti-oxidative enzymes). Besides, there are four studies that have tried to over-express the ABA catabolic enzyme, CYP707A or ABA8′OH, which resulted in decreased ABA levels and increase in PA levels, as expected. In one of the study the *ABA8′OH* was down-regulated by RNAi approach and this resulted in increased ABA levels (Gubler et al., 2008).

None of the transgenic studies that regulated ABA levels studied the effect of ABA on yield performance of plants, and therefore it is difficult to conclude if altering ABA levels under stress is beneficial to the plant. ABA is known as an inhibitor that shortens the grain filling period and grain filling rate. Grain filling is mainly the process that is closely associated with starch biosynthesis and its accumulation in sink tissue that contributes up to 65 – 75 % of the final grain dry weight. There are four major key enzymes that play role in starch biosynthesis (sucrose to starch conversion) viz. sucrose synthase, ADP glucose pyrophosphorylase, starch synthase, and starch branching enzyme that are also closely associated with the sink strength (Hawker and Jenner, 1993; Ahmadi and Baker, 2001; Hurkman et al., 2003). Under water stress, ABA content was found to significantly and positively correlate with the activity of these enzymes that also partially explains the role of ABA in enhancing sink strength. However, how ABA regulates or activates these enzymes is not clear (Yang et al., 2004). The other probable effects of ABA is the regulation of ATPase activity of phloem cells in sink that regulate the metabolism of assimilates in sink cells (Peng et al., 2003). ABA also affects gene regulation in sink (Rock and Quatrano, 1995) that could substantially contribute in enhancing sink strength.

Table1.1 Transgenic plants produced to alter ABA levels by the manipulated expression of ABA metabolism genes OE: over expression; AS: anti sense; NA: not available

ABA hydroxylase gene altered transgenic

Plant	Gene	Promoter/Construct	Stress	ABA	ABA catabolites	Stress tolerance	Yield	Reference
Arabidopsis	*CYP707A1*	35s-OE	NA	↓	NA	NA	NA	Millar et al., 2006
Arabidopsis	*CYP707A3*	35s-OE	Drought	↓	↑ PA & DPA	NA	NA	Umezava et al., 2006
Nicotiana sylvestris	*PvCYP707A1-A3*	35s-OE	NA	↓	↑ PA	NA	NA	Yang and Zeevaart 2006
Rice	*TaABA8'OH1*	Os6GB tapetum-OE	Cold	↓	NA	↑	NA	Ji et al., 2011
Barely	*HvABA8'OH1*	UBQ-RNAi	NA	↑ in seed	NA	NA	NA	Gubler et al., 2008

ZEP, SDR and MCSU genes altered transgenic

Plant	Gene	Promoter/Construct	Stress	ABA	ABA catabolites	Stress tolerance	Yield	Reference
Tomato (notabilis mutant)	*LeZEP1*	35s-AS-OE	NA	NA	NA	NA	NA	Thompson et al., 2000a
N.Plumbaginifolia	*NpZEP*	35s-OE & 35s-AS-OE	NA	↑ in OE and ↓ in AS-OE in seeds	NA	NA	NA	Frey et al., 1999
N.Plumbaginifolia	*AtZEP1*	Isocitrate lyase promoter,pABI3 & pAT2S2	NA	No significant difference	NA	NA	NA	Frey et al., 2006
Tomato	*LeZE*	35s-OE	High light & temperature	NA	NA	↓	NA	Wang et al., 2008
Arabidopsis	*AtZEP*	35s-OE	Salinity, mannitol & drought	↑	NA	↑	NA	Park et al., 2008
N.Plumbaginifolia	*ZEP*	35s-OE	Drought	Un altered	NA	NA	NA	Borel et al., 2001a and b
Arabidopsis	*AtABA2/SDR*	35s-OE	Salinity	↑	NA	↑	NA	Lin et al., 2007
Tobacco	*AtABA3/Molybdenum cofactor sulfurase*	Super promoter	Drought	↑ under 12h after 30% PEG treatment	NA	↑	NA	Yue et al., 2011

Table1.1 Transgenic plants produced to alter ABA levels by the manipulated expression of ABA metabolism genes

NCED gene altered transgenic								
Plant	**Gene**	**Promoter/Construct**	**Stress**	**ABA**	**ABA catabolites**	**Stress tolerance**	**Yield**	**Reference**
Arabidopsis	*OsNCED3*	35s-OE	Drought	↑under control and ↑ under salt and glucose stress at one time point.	NA	↑	NA	Hwang et al., 2010
Arabidopsis	*AtNCED6*	35s-OE	Drought	↑ in transgenic at 100% RWC but ↓ at 80% RWC compared to WT.	NA	NA	NA	Lefebvre et al.,2006
Arabidopsis	*AtNCED3*	35s-OE and 35s-AS-OE	Drought	↑ in OE, ↓ in AS under control at one time point.	NA	↑ in OE, ↓in AS	NA	Iuchi et al., 2001
Arabidopsis	*AtNCED6* & *AtNCED9*	Ecdysone receptor and methoxyfenozide		↑ under induction	NA	NA	NA	Martinez Andujar et al., 2011
Arabidopsis	*AhNCED1*	35s-OE	Drought	↑under stress at one time point	NA	↑	NA	Wan and Li 2006
Tobacco	*SgNCED1*	35s-OE	Salinity & Mannitol	↑ under control at one time point	NA	↑	↑Total biomass	Zhang et al., 2008, 2009
N.Plumbagi-nifolia	*PvNCED1*	35s-OE & DEX inducible 35s-OE	Drought	↑ under control for 35s-OE and on indcution by DEX .	↑	↑	NA	Qin & Zeevart 2002
Tobacco	*LeNCED1*	Tetracycline inducible-OE	NA	↑under induction	NA	NA	NA	Thompson et al., 2000b
Tomato	*LeNCED1*	35s-OE and super promoter-OE	Drought	↑ under control in leaf , root and seed and in root under stress	NA		No difference in biomass	Thompson et al., 2000b,2007a,2007b
Tomato	*LeNCED1*	rbcS3C-OE	NA	↑	NA	NA	↓ biomass under control .	Tung et al.,2008
Tomato	*SINCED*	E8 promoter-RNAi	NA	Decreased ABA in fruit	NA	NA	NA	Sun et al., 2012
Creeping bent grass	*VuNCED1*	35s-OE	Salinity & drought	Increased	NA	Improved	↑ shoot and root biomass	Aswath et al., 2005
Broad bean	*AtNCED3 & AtAAO3*	35s -OE (transient in guard cell)	NA	NA	NA	NA	NA	Melhorn et al.,2008
Tobacco	*GlNCED1* & *GlNCED2*	35s-OE	Drought	Un altered	NA	Un altered	NA	Zhu et al., 2007
Maize	*NCED/VP14*	Constitutive sense and antisense OE	Drought	Decreased in AS-OE under stress	NA	NA	NA	Voisin et al., 2006

1.9 Aim of the present study:

The response of agricultural crops to drought stress and the role of ABA in conferring drought tolerance among crops have not been extensively studied, during seed set and grain filling. ABA's role in grain filling is complex. There are reports of both positive and negative role of ABA in altering grain filling. However, it is the concentration of ABA in plants or seeds that determines its role. Barley is considered as a model for triticeae family that include wheat, rye and their wild relatives (Schulte et al., 2009). Hence, we used barley to explore the role of ABA in grain filling and starch metabolism under control and post-anthesis drought stress. The role of ABA during grain filling was explored by:

1. Studying the regulation of ABA homeostasis in flag leaves and seeds under control and drought stress, in elite LP line.

a. Monitoring expression of ABA biosynthetic and catabolic pathway genes.
b. ABA and its metabolites were measured to correlate with expression of ABA metabolism genes.
c. Exogenous treatment of developing spikes with ABA and its inhibitor (fluridone) to study effect on TGW and yield.

2. Studying the regulation of ABA homeostasis in transgenic plants that are altered for its homeostasis in leaves and developing grains.

a. Developing transgenic plants (homozygous T_2 and or double haploid-DH) overexpressing *AtNCED6* under *OsSalT*, stress inducible promoter, and *Ta1AX1*, seed specific promoter.
b. Molecular characterization of transgenic plants for expression of transgene and endogenous genes of ABA biosynthesis and catabolism.
c. Screening the ABA altered transgenic plants for physiological and yield performance under control and post-anthesis drought stress.
d. Biochemical characterization of transgenic for ABA and its metabolites along with other phytohormones.
e. Studying regulation of starch and it metabolism genes in seeds at various developmental time points under control and drought stress.
f. Testing the performance of transgenic plants under rain shelters for drought tolerance.

2 Materials and methods

2.1 Materials

2.1.1 Plant materials

Hordeum vulgare cv Golden promise, the two-rowed barley spring cultivar obtained from Gene bank department (IPK, Gatersleben, Germany) was used throughout this study as wildtype plant and along with the transgenic plants produced in this background.

2.1.2 Bacterial strains

Escherichia coli DH5α	F-, f80d/lacZ_M15, recA1, endA1, gyrA96, thi-1, hsdR17(rK-, mK+), supE44, relA1, deoR, _(lacZY AargF) U169; (Grant et al, 1990)
One Shot TOP10	F - *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G(Invitrogen, Karlsruhe)
Agrobacterium. tumefaciens	Strain AGL-1 (Hellens et al., 2000)

2.1.3 Plasmids and Vectors

pCR2.1-TOPO	Ampr (Invitrogen, Karlsruhe)
pCR4-TOPO	Amp^r (Invitrogen, Karlsruhe)
pAXi-pNOS-Ab-M	Amp^r 1AX1 promoter cloned from wheat
pNOS-AB-M	Amp^r (DNA cloning service Hamburg)
p6U	$Spec^r$ (DNA cloning service Hamburg)

2.1.4 Primers

The primers for PCR and qPCR were designed using primer 3 software and were synthesized by Metabion (Germany) for details refer supplementary table 1 and 2.

2.1.5 Chemicals

Amersham, Germany	[γ 32P]ATP, Nylon membrane Hybond N^+
Difco, USA	Bacto®-Agar, Bacto®-Trypton, Yeast extract, Beef extract, Peptone.
Duchefa, Belgium	Murashige-Skoog, hormones, vitamins, antibiotics.
Fluka Germany	DEPC
Gibco-BRL, USA	Agarose,

Merck, Germany	Ethidium bromide, Benzamidine, DTT, Formamide, Iodine, Magnesium chloride, MOPS, Potassium iodide Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium hypochloride, Sodium phosphate, Sucrose, Tris.
Pall, Germany	Nylon filter Biodyne
Roth, Germany	Acetic acid, Acetone, Ethanol, Chloroform, Formaldehyde, Glycerol, Isoamylalcohol, Isopropanol, Lithium chloride, Phenol
Roche, Basel,	ATP, BSA, dNTPs, SDS
Schleicher & Schuell, Germany	Blotting paper GB 002
Serva, Heidelberg, Germany	EDTA, EGTA
Sigma-aldrich	DMSO
Whatman Ltd., UK	Phosphocellulose P81 paper, Anopore® inorganic membrane
Invitrogen	Trizol

2.1.6 Enzymes and kits

Amersham, Germany	Megaprime DNA Labeling Kit, Sure Cloning Kit, RediPrimeII Labeling System
Qiagen, Germany	Plasmid isolation Kit, QIAquick PCR purification Kit, RNA isolation kit, RNase free DNAase set
Stratagene GmbH, Germany	Pfu polymerase, Salmon sperm DNA
Fermentas	GeneRuler™DNA Ladder Mix,Rapid DNA Ligation Kit,Restriction enzymes
Agilent	RNA nano kit
Applied Biosystems	SYBR-GREEN-PCR master mix
Invitrogen	Superscript III,TA-cloning kit

2.1.7 Special instruments

Agilent	Bioanalyzer
Applied Biosystems	qPCR machine
Peqlab	Nanodrop
Delta T service	Moisture sensor (SM200) and moisture meter reader (HH2)
GTA sensorik GmbH	Marvin

2.1.8 Media

Bacterial media

LB	10g NaCl, 5g Tryptone, 5g Yeast extract for 1L (pH 7.4-8.0)
SOC	0.58g NaCl, 0.186g KCl, 10mM $MgCl_2$, 20g Tryptone, 5g Yeast extract, 20mM Glucose for 1L (pH 7.4-8.0)

All solidified media contain 1.5% Difco-agar.

Plant growth media

The co-culture media (CCM) was prepared according to Tingay et al., 1997. The callus induction media (CIM) and plant regeneration media (PRM) was prepared according to Hensel and Kumlehn 2004. The pH of the media maintained at 5.8.

Components		CCM	CIM	PRM
Macroelements (mg/L)	NH_4NO_3	1650	1650	320
	KNO_3	1900	1900	3640
	KH_2PO_4	170	170	340
	$CaCl_2·2H_2O$	441	441	441
	$MgSO_4·7H_2O$	331	331	246
Microelements (mg/L)	H_3BO_3	6.2	6.2	3.1
	$MnSO_4·4H_2O$	22.4	22.4	11.2
	$ZnSO_4·7H_2O$	8.6	8.6	7.2
	KI	0.83	0.83	0.17
	$Na_2MoO_4·2H_2O$	0.25	0.25	0.12
	$CuSO_4·5H_2O$	0.025	1.275	0.13
	$CoCl_2·6H_2O$	0.025	0.025	0.024
	$Na_2FeEDTA$	36.7	36.7	36.7
Vitamins (mg/L)	B5 Vitamins			112
	Thiamine-HCl	1	1	10
Amino acids (mg/L)	L-Cysteine	800		
	L-Glutamine			146
	L-Proline	690	690	
Sugars (g/L)	Maltose monohydrate	30	30	36
Growth regulators (mg/L)	DICAMBA	2.5	2.5	
	6-BAP			0.225
Miscellaneous (g/L)	Acetosyringone	0.098		
	Casein Hydrolysate	1	1	
	Myo-Inositol	0.25	0.25	
	Timentin		0.15	0.15
	Phytagel		3	3

2.2 Methods

2.2.1 Plant material and growth conditions for LP110 breeding line:

Barley plants (*Hordeum vulgare*, LP110 breeding line provided by KWS Lochow GmbH) were cultivated in phytochamber chamber on substrate consisting of compost, growing substrate (Klasmann) and sand (2:2:1). Two seeds were directly sown per pot and later thinned to maintain 1plant/pot. The phytochamber was maintained at 11°C day and 7°C night temperature along with light period of 12 hour (h) from germination till end of 8 weeks of vernalization. After vernalization, the plants were subjected to four phases of growing conditions each lasting for 2 weeks. The day/night temperature and the durations

of light period for each phase are as follows, phase 1: 14°C/ 9°C, 12 h light; phase 2: 16°C /9°C, 14 h light; phase 3: 20°C/12°C, 16 h light; and phase 4: 20°C/14°C, 16 h light. The plants were fertilized at regular intervals and once in a week during spike development with either osmocote Pro or 2 - 4 % of Hakaphos Rot. The developmental stage of the plant was assessed by analyzing the caryopses in the mid-region of the ear as described by Weschke et al. (2000). All the spikes emerged were tagged manually with the date of its fertilization, so that only spikes at a given developmental stage could be used for collection of flag leaf and grain samples for further analysis.

Drought stress was imposed by maintaining a batch of plants at 20 – 25 % field capacity (corresponds to 10% soil moisture content) when maximum number of plants were at 7 - 8 days after flowering (DAF) stage. Stress was maintained until maturity or seed harvest, and control plants were maintained at 100 % field capacity (found to be 40 % soil moisture content). The soil moisture content was monitored daily with the help of soil moisture sensor (SM200) inserted in each pot that is connected to soil moisture meter (HH2). The pots were manually irrigated to maintain plants at either 40 % or 10 % soil moisture content level. The developing seeds (mid-region of the ear) and the flag leaf were harvested at 12, 16, 20 and 25 days after flowering (DAF) from stress and control plants. Two replications were maintained by growing them independently. The stages 12, 16, 20 and 25 DAF were chosen to cover middle and late stages of seed development. These stages cover the start (12 DAF), peak (16 and 20 DAF) and end (25DAF: seed maturation phase) of seed storage phase. According to the Zadoks system for barley, these stages are related to late milk, soft dough and hard dough physiological stages (Tottman 1987).

For field studies, random block design was followed and 50 plants per plot were maintained. A rain shelter was installed at anthesis stage to protect plants from natural rainfall. Control plots were continuously watered and watering of stress plots was stopped at 7- 8 days after anthesis. Two sets of drought stress plots were sprayed with 200 µM ABA and 50 µM fluridone, respectively, at two time points (one week after anthesis and 10 days after first spray). After maturity seeds were collected from control, drought stress alone or and ABA or fluridone treated plots to asses grain yield and thousand grain weight (TGW) per plot.

2.2.2 RNA isolation from leaf and developing seed:

Total RNA was isolated from developing seeds and flag leaves using the TRIZOL reagent and RNAeasy columns. Tissue was ground in liquid nitrogen and 100 mg of the homogenized powder was added to 1 mL TRIZOL and incubated for 5 minutes (min) at room temperature for leaf samples and at 60°C for seed samples. Samples were centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to a new tube. 200 µL of chloroform was added and incubated at room temperature for 2 – 3 min. Samples were again centrifuged as described above and the supernatant was transferred to Qia shredder column and centrifuged for 30 s at 10,000 rpm. 350 µL of RLT buffer (plus beta-mercaptoethanol 10 µL /mL of RLT buffer) and 250 µL of absolute ethanol were added to the flow-through and passed through an RNAeasy spin column. All the following steps were performed as described in the manufacturer's protocol followed by in-column DNAse digestion.

2.2.3 Quality checking of the RNA using NanoDrop and the Agilent 2100 bioanalyzer:

RNA concentration was measured using the NanoDrop photometer according to the manufacturer's instructions. The purity of the RNA was also determined based on the absorbance value at 230 nm, 260 nm, and 280 nm recorded using the NanoDrop. Nucleic acid absorb light at a wave length of 260 nm, the organic contaminants like phenols and other aromatic compounds used during the process of RNA extraction also absorbs light at 230 nm and 280 nm. If the RNA samples are not contaminated with the organic compounds or any other impurities then the 260/230 and 260/280 absorbance value should be more than 1.8. RNA quality was also checked on a 1 % (w/v) agarose gel to check for the contamination of the DNA and the decay of the RNA. The absence of the genomic DNA in the RNA samples was confirmed by checking the PCR with the primers designed from the intron region of the control gene using the RNA samples as template.

The quality of the RNA was also checked using Agilent 2100 bioanalyzer instrument using Agilent RNA 6000 nano kit as per the manufactures instruction and protocol. Depending on the tissue used for RNA isolation we will be able to observe the RNA peak (18s and 28s in the seed sample and in addition to the 18s and 28s there will be additional 16s and 23s from the chloroplast in the leaf samples) in the electropherogram. Depending on the electrophoresis and the RNA integrity number the quality of the RNA was determined (Figure A).

2.2.4 Complementary DNA (cDNA) synthesis and quality of the cDNA:

The first strand cDNA was synthesized from RNA which was free from any DNA contamination using the SuperScript III (Invitrogen GmbH). 2 µg of total RNA, 1 µL of 50 µM oligo (dT-20mer) primer and 1 µL of 10 mM dNTP mix and water were added to each tube to obtain a total volume of 10 µL and the reaction mixture was incubated at 65°C for 5 minutes, and then rapidly cooled on ice. 10 µL of cDNA master mix consisting of 2 µL of 10X RT buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT (40 units/ µL) and super script III RT (200 units/ µL) was added and incubated at 50°C for 50 minutes. The reaction was terminated by incubating at 85°C for 5min and chilled on ice. 1 µL of the RNase H was added to each reaction tube and incubated at 37°C for 20 min to remove the RNA and the synthesized cDNA was stored at -20°C for further use.

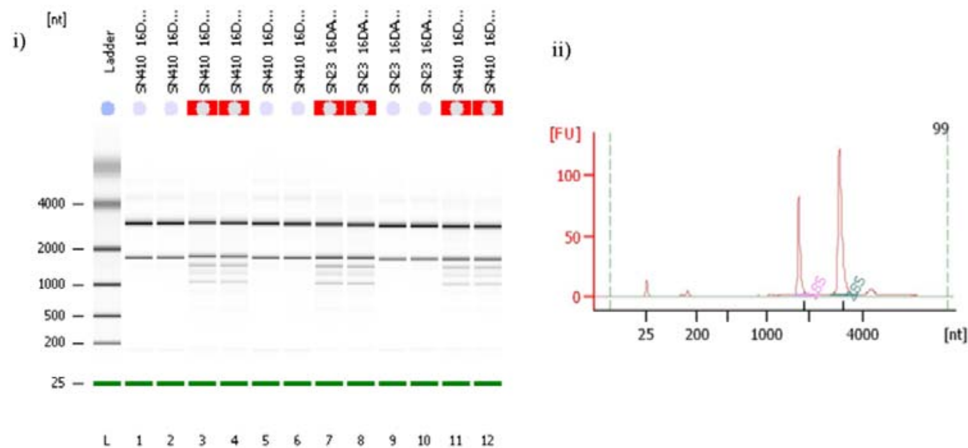


Figure A: Representative i) gel image and ii) electropherogram summary of RNA depicting the quality of RNA analyzed in Agilent bioanalyzer using Agilent nano kit.

To test cDNA yield, qPCR was performed using primers of a reference gene (serine/threonine protein phosphatase PP2A-4, catalytic subunit, EST clone HZ44D03). The reference gene is stably expressed under all the experimental conditions tested. The quality of cDNA was assessed by using two primer pairs for a reference gene (elongation factor 1a, EST clone HZ42K12) from the 5’ and 3’ regions. The C_T value of the 5’-end primer did not exceed that of the 3’-end primer by more than one C_T, indicating a uniform synthesis of cDNA.

2.2.5 Quantitative real time polymerase chain reaction (qRT-PCR):

qRT-PCR reactions was carried out using power SYBR Green mastermix reagent and the of double stranded DNA (dsDNA) synthesis was monitored with an ABI PRISM 7900 HT sequence detection system. The reaction was carried out in an optical 384-well plates, each reaction well consist of 5 µL of Power SYBR Green mastermix reagent, 1 µL of cDNA and 200 nM of each gene-specific primer in a final volume of 10 µL. The following standard thermal profile was used for all PCR reactions: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves, i.e. melting curves, were recorded after 45th cycle by heating from 60°C to 95°C with a ramp speed of 1.9°C per minute. For internal control, three reference genes (RG) were checked for their expression pattern in different tissue. We found that serine/threonine protein phosphatase PP2A-4, catalytic subunit, EST clone HZ44D03 was stably expressed in different tissue across developmental stages, and different treatments condition and it was used as reference gene for further studies. Expression level of the gene of interest (GOI) was calculated using comparative C_T method as described by Schmittgen and Livak, 2008. All the data were analyzed using the SDS2.2.1 software (Applied Biosystems).

Expression level of the gene of interest (GOI) relative to the reference gene (RG) was calculated using the equation $2^{-\Delta CT} = 2^{-(CT(GOI)-\ CT(RG))}$. To express the gene of interest in terms of fold change $2^{-\Delta\Delta CT}$ was used, where $^{\Delta\Delta CT}$ = Stress $^{(CT(GOI)-\ CT(RG))}$- Control $_{(CT(GOI)-\ CT(RG))}$.

2.2.6 Barley seed cDNA macroarray (12K) analysis: differential expression of starch biosynthesis and degradation pathway genes

Total RNA isolated from developing seed (12, 16 and 20 DAF) from control and post-anthesis drought stressed plants was used for the macroarray hybridisation. The $poly(A)^+$ RNA was extracted from total RNA (35 µg) using oligo (dT) coated magnetic beads (Invitrogen). First strand cDNA was synthesised using superscript II following the manufactures protocol. ^{33}P-labelled second strand cDNA obtained by random priming reaction using the megaprime labelling kit was used for hybridisation. The hybridisation was carried out at 65°C for at least 16-18 h. After 6h of exposure of hybridised cDNA array to phospo image screens they were developed using a Fuji FLA 5100 phosphor scanner. Quantile normalisation was carried out and normalised expression value for starch metabolism genes was extracted.

2.2.7 *In-silico* Promoter Analysis:

Using BLAST search, the upstream region of beta-amylase (*HvBAM1*), ADP-glucose pyrophosphorylase small subunit (*HvAGP-S1*), and sucrose synthase (*HvSUS1*) genes were identified. The identified promoters were amplified from the genomic DNA and confirmed by sequencing. The presence of *cis*- regulatory elements in the promoter region was identified using PLACE (Higo et al., 1999) and plantCARE (Lescot et al., 2002) databases as well as motifs extracted from the literature.

2.2.8 Extraction and analysis of ABA (LC-MS/MS), ABA catabolites and other hormones:

ABA was extracted from fresh plant material (flag leaf and developing seed) using 100% ethyl acetate containing isotopically labelled D6-ABA, internal standard. Extraction was carried out twice with 1 mL of ethyl acetate at 4°C for 30 min and by centrifuging at 13,000 rpm for 10 min at 4°C. The supernatant collected was evaporated to dryness using vacuum concentrator set to room temperature. The dried sample was re-dissolved in 200 µL of acetonitrile-methanol (1:1) mix and filtered using 0.8 µm filters (vivaclear). 10 µL of filtrate was quantified by LC-MS/MS (Dionex Summit coupled to Varian 1200L). A C18 column (4 µm, 100 mm; GENESIS; Vydac/USA) was used for chromatographic separation. MRM and quantification was done using the mass traces 263/153 for ABA and 269/159 for D6-ABA. Chromatogram acquisition and data processing was accomplished with the Varian software, "Work station". The validity of the extraction and measurement procedure was checked by recovery experiments (approx. 82 - 95 %). ABA standards and individual recovery rates was used to quantify ABA in samples. Freeze dried samples were used for quantification of ABA catabolites, ABA-GE and other hormones. The analysis was performed at the National Research Council of Canada Plant Biotechnology Institute (Saskatoon, Canada) (http://www.pbi.nrc.gc.ca/). Quantification was carried out using high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ES-MS/MS) and deuterated internal standards were used, as described in Chiwocha et al., (2003) and Kong et al., (2008).

2.2.9 Starch measurement:

NADH generated during the conversion of glucose-6-phosphate to 6-phosphogluconate is used to measure sugars and starch in developing seeds. Around 15- 20 mg of powdered seed was incubated in 80 % ethanol for 30 min at 60°C with continuous shaking at 500rpm in thermomixer. The sample was centrifuged at 13,000 rpm for 8 min and the

pellet was re-washed following same protocol. Pellet obtained was dissolved in 2N HCl (1.5 mL) and incubated for 1 h at 95°C (Kozloski et al., 1999). Supernatant obtained after centrifugation (13,000 rpm for 5 min) was used for glucose estimation, the hydrolyzed product of starch. Around 5 - 10 µL of supernatant is incubated with 750 µL imidazole buffer (2 mM NAD and 1 mM ATP, pH 6.9) and 2 µL of glucose-6-phosphate dehydrogenase (2 units) at room temperature and initial absorbance is recorded immediately at 340 nm to measure NADH. Absorbance at 340 nm is recorded after incubation with 10 µL of hexokinase (8 units) for 25 min. The difference in the absorbance for the NADH is proportional to the total amount of glucose formed by the hydrolysis of starch.

2.2.10 Enzyme activity:

β-glucosidase assay

The activity of β-glucosidase is estimated by measuring the amount of p-nitrophenol released from synthetic substrate p-nitrophenyl-β-D-glucopyranoside (p-NPG) (Simos and Georgatsos, 1988). Crude enzyme is extracted by incubating finely ground seed material at 4°C in ammonium acetate extraction buffer, pH 6.0, in a sample to buffer ratio of 1:3. The sample is centrifuged at 10,000 rpm for 10 min 4°C and the supernatant obtained is used for enzyme assay. For the assay, 100 µL of the crude enzyme extract is incubated with 450 µL of ammonium acetate extraction buffer, pH 6.0 and 250 µL of 7 mM p-NPG at 37°C for 15 min. The reaction is ended by addition of 800 µL of 0.2 M sodium carbonate. The p-nitrophenol released (yellow color formation) was estimated by recording the absorbance at 400 nm. The activity of β-glucosidase is expressed as µmoles of p-nitrophenol produced per gram fresh weight tissue per minute (µmol g^{-1} min^{-1}).

Enzyme activities of AGPase and sucrose synthase:

The activity of AGP is estimated by the reverse reaction of pyrophosphate and ADP-Glucose to ATP and glucose 1-phosphate (Smith et al., 1990). The synthesis of glucose-1-phosphate is estimated by recording absorbance at 340 nm. Around 20 mg of finely ground seed powder was extracted with 50 mM sodium phosphate buffer in presence of 5 mM DTT and 5 mM EDTA. Nearly 30 µL of supernatant after centrifugation of the mix at 13,000 rpm for 15 min at 4°C was incubated with 700 µL of determination buffer (100 mM HEPES, 5 mM NaCl2, 2 mM ADP-glucose, 0.5 mM NAD, 2 units

Phosphoglucomutase and 5 units NAD-linked glucose-6-phosphate). The reaction was started by addition of 2 mM sodium pyrophosphate, and after 5 min the absorbance was recorded at 340 nm (Weber et al., 1995, 1996).

Sucrose synthase (SUS) activity is estimated based on measurement of NADH formation. Sucrose and UDP is converted to fructose and UDP-glucose by SUS. UDP-glucose is converted to UDP-glucuronic acid during which NAD^+ is reduced to NADH by UDP-glucose dehydrogenase. The amount of NADH formed is recorded by estimating absorbance at 340 nm. Around 20 mg of finely ground seed powder was extracted with 5 volumes of extraction buffer (100 mM MOPS pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, and mixed) and centrifuged at 13,000 rpm for 10 min at 4°C. Nearly 10 µL of supernatant is incubated with 150 µL determination solution 1(75mM HEPES pH 7.5; 7 mM $MgCl_2.6H_2O$, 4 mM UDP and 200 mM sucrose), two batches were maintained. The batch is immediately boiled for 5 min at 95°C and to stop the reaction. The second batch is incubated for 10 min at 25°C and then the reaction stopped by boiling for 5 min at 95°C. To both batches, 750 µL of determination solution II (200 mM glycine, 5 mM and 1 mM $MgCl_2.6H_2O$ $NAD.3H_2O$) and UDP-Glc-DH 8 µL is added and incubated for 30 min at 37°C with continuous shaking. The sample is then centrifuged (10 min, 13000 rpm, 4°C) and the absorbance of supernatant are recorded at 340 nm (Weber et al., 1995, 1996).

2.2.11 Basic cloning methods and sequencing:

The basic molecular cloning methods such as polymerase chain reaction, DNA electrophoreses and enzymatic digestion was performed according to the standard protocols described by Sambrook and Russell (2001). DNA fragments were isolated and purified from agarose gel by QIA-quick gel extraction kit.

Plasmid extractions were done using Qiagen Plasmid kit according to the protocol as prescribed by the manufactures. The sequences were confirmed by sequencing at AGOWA (Germany).

2.2.12 Cloning of *AtNCED6* under *SalT* promoter:

SalT promoter was obtained from ICGEB (International centre for genetic engineering and biotechnology, India) where in it was amplified from rice and cloned into Topo-TA vector. Using the *EcoR*1 site present at both ends of the promoter we further sub-cloned it

into pNos-AB-M vector. The cloning and orientation of the promoter was confirmed by digestion and sequencing; the vector was named as pSalT - pNos-AB-M.

For sub-cloning *AtNCED6* under SalT promoter, it was amplified from Arabidopsis genomic DNA as template using gene specific primers (*NCED* gene family members lack introns) and the product obtained was purified and cloned into Topo-TA vector. The cloning was confirmed by sequencing and checked for errors during PCR reaction. The *AtNCED6* topo clone was further amplified with gene specific primers having *Hind*III sites (forward and reverse primers). The purified PCR product digested with *Hind*III was ligated to linerarized SalT-pNos-AB-M to obtain SalT::*AtNCED6*-pNos vector. The SalT::*AtNCED6*-pNos was then sub-cloned into p6U binary vector for plant transformation. This was achieved by releasing the whole cassette (SalT promoter: *AtNCED6* gene: pNos terminator) using *Sfi* enzyme that is compatible between both vectors. The obtained positive clones were sequenced to register the orientation of the cassette in p6U vector (Figure B).

2.2.13 Cloning of *AtNCED6* under *1AX1* promoter:

The pAXi (RNAi construct) vector (modified form of pNos-AB-M) that has the wheat 1AX1 promoter and the pNos terminator was used to clone AtNCED6 by replacing the intron with it. *AtNCED6* was amplified from genomic DNA with gene specific primers designed with *BamH*1 and *Pst*1 sites in forward and reverse primers. The purified linerazied vector lacking the intron after digestion with *BamH*1 and *Pst*1 was ligated with *AtNCED6* purified PCR product after digestion with similar enzyme to obtain 1AX1:: *AtNCED6*-pNos vector. The cloning strategy used to transfer the whole cassette of 1AX1:: *AtNCED6*-pNos into p6U binary vector was similar to that used for SalT:: *AtNCED6*-pNos mentioned above. The obtained positive clones were sequenced to register the orientation of the cassette in p6U vector (Figure C).

Transformations of vector to *E. coli* and *A. tumefaciens* was carried out using heat shock (Sambrook and Russell, 2001) and freeze-thaw method (Weigel and Glazebrook, 2002), respectively.

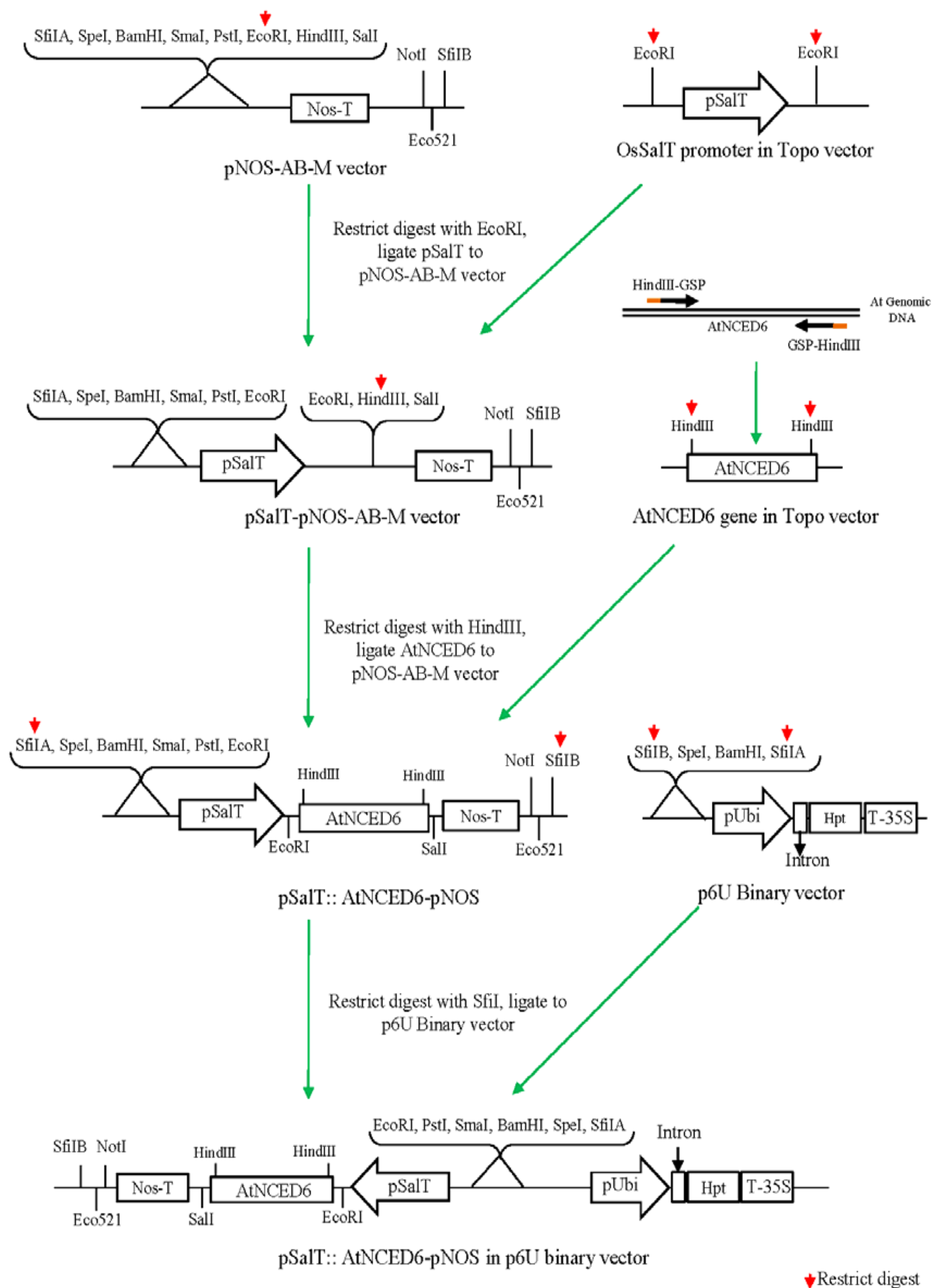


Figure B: Overview of the strategy used for cloning *AtNCED6* under the control of *OsSalT* promoter in the p6U binary vector, for barley transformation. Restriction digestion is indicated by red downward arrow.

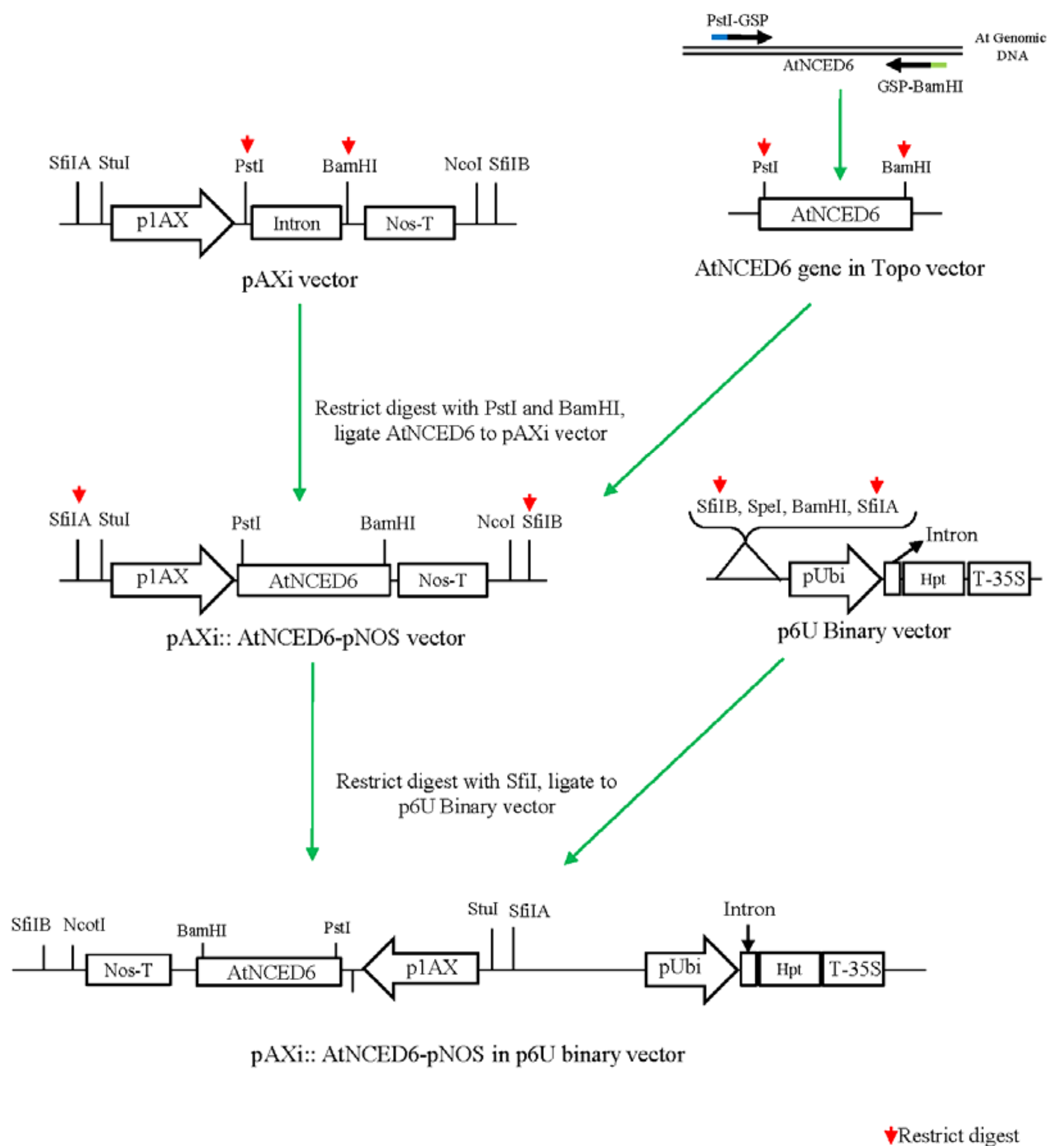


Figure C: Overview of strategy used for cloning *AtNCED6* under the control of *Ta1AX1* promoter in the p6U binary vector, for barley transformation. Restriction digestion is indicated by red downward arrow.

2.2.14 Stable transformation of barley and generation of double haploid plants:

The transgenic lines (T_2 and DH plants) used in this study were produced by the plant reproductive biology group at IPK. Stable transformants were created by transforming barley embryos. Embryo isolated from seeds, of cv. Golden promise, at developmental stage of approximately 12 days after fertilization was used as explants for agrobacterium

(AGL-1 strain) mediated transformation following Goetz et al. (2009) protocol. The steps followed are described below:

1. Seeds were surface sterilized by washing for 3 min with 70 % ethanol followed by 20 min wash with 5 % sodium hypochloride and 4 - 5 washes with distilled water each lasting for 5 - 10 min.
2. Immature embryos were isolated by dissecting the seeds under a stereo microscope using forceps and lanzette needle by maintaining sterile conditions (Tingay et al., 1997).
3. The embryonic axes was dissected from embryos (30 embryos) which was incubated with 2.5 mL co-culture media (CCM) supplemented with acetosyringone (9.8 mg/l) in each well of a 6 well plate (Greiner Bio-One Gmbh, Austria).
4. The CCM media was then replaced by 600 μL of agrobacterium, harboring the construct to be transformed (OD range 0.20 - 0.25) and vacuum infiltrated for 1 min at 500 mbar (diaphragm pump MP201E from Ilmvac, Ilmenau, Germany). After further 10 min incubation (covered) the embryos were washed with CCM medium (2.5 mL) along with incubation for 15 min. second wash with CCM (2.5 ml) was carried out at 21°C in the dark and incubated for 60 h.
5. After which the developing calli were transferred to callus induction media (CIM) supplemented with 20, later 50 mg/L Hygromycin B and 150 mg/L timentin and incubated at 25°C in dark.
6. Later the calli were transferred to plates containing plant regeneration medium (PRM) supplemented with 25 mg/L Hygromycin B and incubated at 24°C (with 16/8 h light/dark photoperiod and light intensity of 10,000 lux) for production of plantlets.
7. The primary shoot-lets obtained were transferred to glass tubes, height 100mm and outer diameter 25 mm, (Schutt, Germany) filled with solid PRM media supplemented with Hygromycin B (25 mg/L). Once the roots appeared, the small barley plants were transferred to small pots (6 cm diameter) and kept in greenhouse (14/12°C day/night, 12 h photoperiod, 20,000 lux, and ca. 80 % RH). Later transferred to big pots (16 cm diameter) and grown until maturation and seed collection.

T_1 seeds of SN410_8 transgenic plants were used for generation of double haploid plants of SN410_8 following protocol described by Coronado et al. (2005) and was carried out by plant reproductive biology group at IPK.

2.2.15 Genomic DNA extraction:

The genomic DNA was extracted following the protocol described by Palotta et al. (2000). Leaf tissue (100 - 200 mg) was finely ground in liquid N_2 and incubated with 800 µL of extraction buffer (100 mM Tris-HCl, 10 mM ETDA, 100 mM NaCl and 1 % N-lauryl sarkosin, pH8) for 5 min at room temperature (RT) with intermittent vortexing subsequently 800 µL Phenol/Chloroform/Isoamylalcohol (25:24:1) mix was added and further the sample was vortexed for 2 min at RT. The sample was centrifuged at 5000 rpm for 3 min at RT. The obtained supernatant (700 µL) was transferred to fresh eppendorf tube and incubated on ice for 15 min after addition of 1 volume of isopropanol (700 µL) and 1/10th volume of 3M Sodium acetate, pH5.2 (70 µL). DNA was precipitated by centrifugation of samples at 13000 rpm for 10 min (4°C). The DNA pellet is washed in 70 % ethanol by re-suspending the pellet and centrifuging the samples at 7500 rpm for 10 min (4°C). The pellet is air dried to remove any trances of alcohol and then re-suspended in water or TE buffer (100 µL). The DNA is freed of RNA by adding 2 µL of RNase (40 µg/mL) and incubating for 30 min at 37°C. DNA is then quantified using Nanodrop at 260 nm.

2.2.16 Southern hybridization:

Genomic DNA (10 µg) is digested overnight at 37°C with appropriate restriction enzymes (100 U) in 40 µL reaction volume consisting of 1X restriction buffer (4 µL of 10x buffer) and 40 mM Spermidin. The digested DNA is electrophoresed overnight (30V) or for 4 h (90V) on 1 x TAE with 1 % agarose. In addition to samples of genomic DNA, positive control plasmid (2 µL of 1:1000 times diluted plasmid is mixed with 2 µL of similarly diluted Salmon Sperm DNA, 11 µL water and 2 µL of loading dye) and DNA ladder (2 µL of 1:100 times diluted 1kb ladder or DNA ladder mix is mixed with 15 µL of water and 2 µL of loading buffer) are also electrophoresed on the same gel. After electrophoresis the gel is documented and transferred to glass tray where in it is treated with solution I (0.25 M HCl) for 5 min (on shaker) and then rinsed with water. Next, the gel is treated with solution II (1.5 M NaCl and 0.5 M NaOH) two times each with an incubation for 15 min on shaker and lastly rinsed with water and treated with solution III (0.5 M Tris-HCl and 3 M NaCl) for two times (each round for 15 min). The DNA is

transferred from the treated gel to Hybond N^+ membrane by capillary transfer in presence of 0.4 M NaOH. The membrane is then hybridized with radioactively labelled (P^{32}) cDNA fragment of choice. The labelling is carried out using RediPrimeII Labeling System that uses P^{32}dCTP. The hybridization is carried out at 65°C in Church buffer (250 mM sodium phosphate, 7 % SDS, and 1 % BSA) (Church and Gilbert, 1984) according to the procedure described by Sambrook and Russel, 2001.

2.2.17 Plant growing condition for the wild type (WT) and transgenic plants:

The WT and transgenic plants were grown both in phytochamber and green houses. The results obtained under both conditions were similar and reproducible. The growing conditions in phytochamber and green house were largely similar. However, they differed in the substrate used (pot mix), duration of vernalization, and kind of fertilizer used. In phytochamber, the pot mix used consisted of compost, growing substrate (Klasmann) and sand in 2:2:1 ratio. The same pot mix components were also used in green house but the proportions were 4:2:1.6 with additional 0.8 parts of white turf. Under both growing conditions, two seeds were directly sown per pot and later thinned to maintain 1plant/pot. In phytochamber, the chamber was maintained at 11°C day and 7°C night temperature along with light period of 12 h during germination and 8 weeks of vernalization. However in green house, the seeds were allowed to germinate at RT and 2 weeks after germination they were subjected to vernalization for 2 weeks by transferring them to cold chamber (4°C and 12 h light period).

In both phytochamber and green house, after vernalization the plants were subjected to four phases of growing conditions each lasting for 2 weeks. The only difference was, the duration of first phase lasted for 4 weeks and the last phase (phase 4) lasted until maturity in green house. The day/night temperature and the durations of light period for each phase are as follows, phase 1: 14°C/ 9°C, 12h light; phase 2: 16°C /9°C, 14h light; phase 3: 20°C/12°C, 16 h light; and phase 4: 20°C/14°C, 16 h light. The plants fertilized at regular intervals and once in a week during spike development with either osmocote Pro or 2 - 4 % of Hakaphos Rot. However, in green house the control of temperature is not so accurate as phytochamber and the source of light usually is natural light (when available) or artificial source hence we would expect fluctuation in light intensity while in phytochamber plants are exposed to a more constant source of light (artificial source).

2.2.18 Drought stress imposition, sample collection and phenotyping:

Drought stress was imposed when maximum numbers of plants were at 4 days after flowering (DAF) stage. Stress was imposed by maintaining a batch of plants at 20 – 25 % field capacity (corresponds to 10% soil moisture content), and maintained until maturity or seed harvest. The control plants were maintained at 100 % field capacity (found to be 40 % soil moisture content). The soil moisture content was monitored daily, with the help of soil moisture sensor (SM200) inserted in each pot that is connected to soil moisture meter (HH2), and manually irrigated to maintain plants at either 40 % or 10 % soil moisture content level. The developmental stage of the plant was assessed by analyzing the caryopses in the mid-region of the ear as described by Weschke et al. (2000). All the emerged spikes were tagged manually with the date of its fertilization, so that only spikes belonging to the specified developmental stage could be used for collection of flag leaf and grain samples for further analysis.

Samples (flag leaf and developing grains) were collected for physiological, biochemical and molecular analysis from plants that were exposed to different durations of stress along with sample collection form control plants at similar developmental stage. Samples were collected at 0.5, 1, 2, 8, 12, 16, and 20 days after stress (DAS) that corresponds to 4, 5, 6, 8, 12, 16, 20, and 24 DAF stages of development. All the sample collection was carried out during morning hours (8:00 am onwards) (Figure D).

2.2.19 Parameters analyzed:

Phenotypic parameters (15 - 18 individual plants as replications): plant height, tiller number, flowering time, number of primary spikes and primary spikes with branching, scanning electron microscopic analysis of spikes (62, 69, 80 and 87 days after sowing from control plants).

Yield parameters (15 - 18 individual plants as replications): TGW, yield (15 - 18 plants), seed area, and seed length and seed breadth was analysed using Marvin instrument.

Physiological parameters (5 - 6 replications): rate of water loss (control plants) relative leaf water content (RWC) at 4 DAS, gas exchange (stomatal conductance, assimilation, transpiration and WUE) parameters were analyzed at 4 DAS. The endoreduplication patterns were measured at (4 and 12 DAS: 6 and 16 DAF).

Biochemical parameters (3 replications, each replication is a pool of 3 plants): ABA (all duration of stress), ABA metabolites and other hormones (4 and 12 DAS: 6 and 16 DAF), metabolites of starch biosynthesis (all duration of stress).

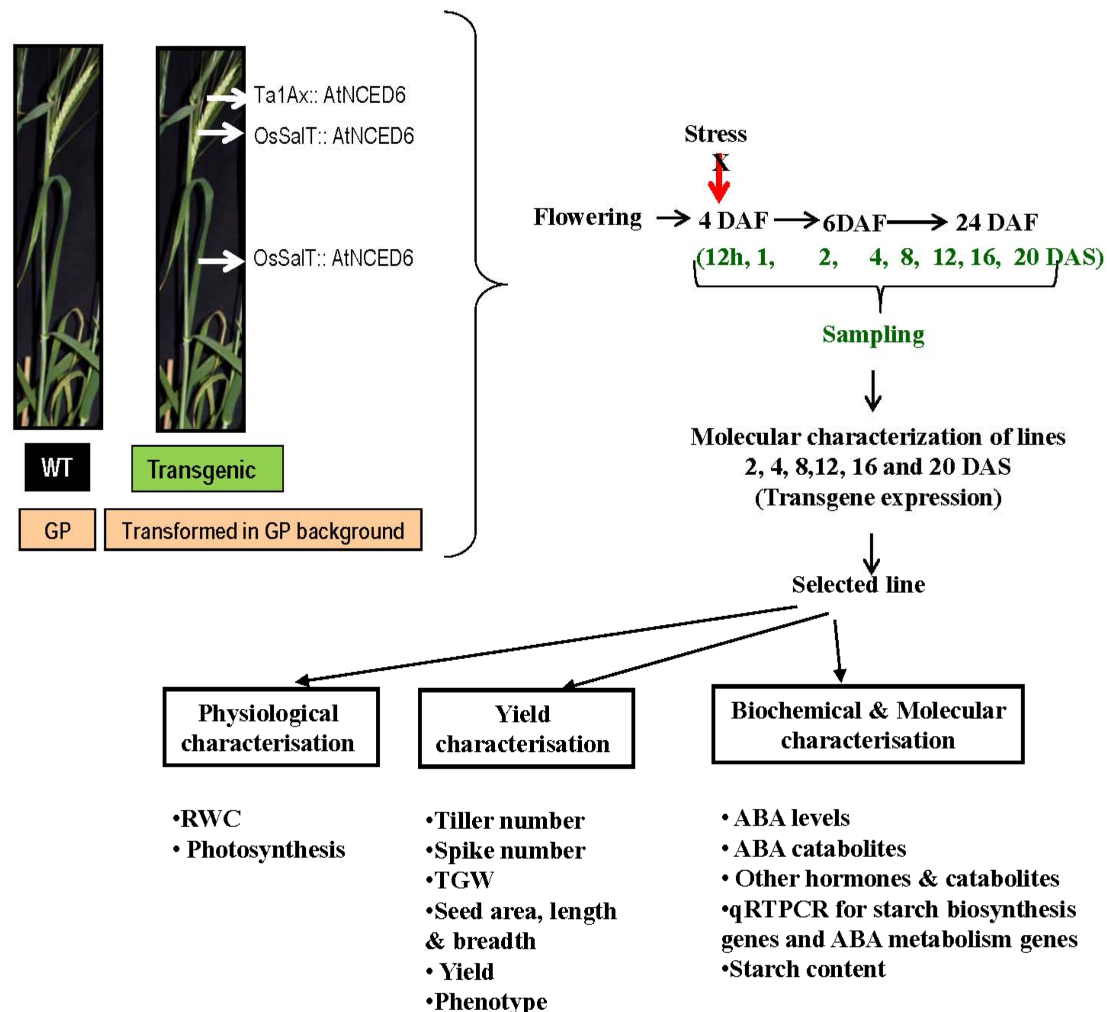


Figure D: Over view of the methodology followed to screen wild type (Golden promise) and transgenic plants for performance under post anthesis drought stress, grown in phytochamber and green house.

Molecular analysis (3 replications, each replication is a pool of 3 plants): qRT-PCR analysis (all durations of stress) of ABA biosynthesis and catabolism related genes, and genes involved in starch biosynthesis.

The data collected was subjected to statistical analysis of Ttest, one- or two-way ANOVA. Significant differences at P value of 0.05, 0.01 and 0.001 were indicated by *, ** and ***, respectively.

2.2.20 Branched spike characterization:

Wild type and transgenic line SN410_8 grown in phytochamber under control conditions were analyzed using scanning electron microscopy to record the difference in the growth and development of primary and branched spike. Length of primary and branched spikes (secondary and tertiary) spikes was recorded at 62, 69 80 and 87 days after sowing using facility at structural cell biology group, IPK, Gatersleben with the help of Dr. Twan Rutten.

2.2.21 Physiological characterization of plants under drought stress:

Rate of water loss: Flag leaves from wild type and transgenics (SN23_3 and SN410_8) plants was detached from plants just before flowering to perform the rate of water loss. The detached leaves were kept at RT and weighted on regular basis to record the change in fresh weight which was used to calculate the amount of water loss.

Relative leaf water content (RWC): The water status of the plants which are subjected to drought stress can be measured accurately by RWC. It estimates the current water content of the sampled leaf tissue (stressed) relative to the maximum water content it can hold at full turgidity (control plants). The RWC was determined from the flag leaf of the main tiller from both control and stress plant. The fresh weight (FW) of the flag leaf was recorded immediately after harvest and floated in a petriplate containing distilled water and the petriplate was kept at 4°C over night. The turgid weight (TW) was recorded next day after gently wiping the sample with tissue paper to remove the water adhering to the sample. Finally the samples were oven dried at 70°C for 24 h and the dry weight (DW) was recorded. The RWC was calculated using the formula RWC (%) = [(FW-DW) / (TW-DW)] x 100.

Photosynthetic parameters: The principle of gas exchange is based on the infra-red gas analysis (IRGA) of hetero-atomic molecules such as CO_2, H_2O, NO, and NH_3 that absorb infra-red radiation at particular infra-red wave length. The photosynthesis parameters were recorded in the flag leaf of the main tiller during post-anthesis period using an infra-red gas analyser ADC-LCPro+ system (The Analytical Development Company Ltd, UK). All the parameters were recorded in the morning hours starting from 10:00 am to 13:00

pm. The instrument was stabilized for 30 min in the green house or the phytochamber where measurements are taken. A constant supply of 400 ppm of CO_2 with a flow rate of 200 µmol/sec was obtained using a CO_2 cartridge inserted in the main console of the instrument and a photon flux density of 900 µmol/m^2 /sec was obtained through a detachable mixed red/blue LED light source mounted over the leaf chamber head. Different parameters like net assimilation rate (A), stomatal conductance (g_s), internal CO_2 concentration (C_i), transpiration rate (E), instantaneous water use efficiency (A/E), leaf temperature etc. were recorded in 3 - 6 different plants with 2 - 3 readings per plant for each treatment. All measurements were recorded once a stable C_i was obtained (2 - 3 min. after mounting the leaf to leaf chamber head).

2.2.22 Screening of the transgenic plants in the rain shelter:

The WT (golden promise), transgenic lines SN410_8 and XN26_5 were grown in the rain shelter in spring 2011. Around 200 plants per plot were maintained. The plants were grown with four replications for each line in a randomized block design (2 plots each for control and stress treatment). The plants were irrigated regularly till flowering and the irrigation was stopped for the stress plots at 4 days after flowering and continued till maturity. The plants were phenotyped for the tiller number and the spike number per plot before the imposition of the drought stress and also at maturity. Yield and TGW per plot has been determined.

2.2.23 Measurement of endo-polyploidy:

Flow cytometer was used to measure endoreduplication. Seed samples from control and stress plants of wild type and transgenic plants grown in rain shelter were used for flow cytometer. At a particular developmental stage the seeds were collected from the centre of the spike. After removal of outer husk, the seeds were used for nuclei suspension preparation. The seeds were chopped using the razor blade in 1 mL of ice cold nuclei isolation buffer supplemented with 50 µg/ mL propidium iodide and 50 µg/ mL DNase free RNase (Galbraith et al., 1983). The suspension obtained was passed through 35 µm mesh and analysed using a FACStarPLUS flow cytometer equipped with an argon ion laser INNOVA 90C. The fluorescence of the propidium iodide was excited by using 500 mW at 514 nm and measured using 630 nm band-pass filters in FL1 channel. Usually, 10^4 nuclei were measured for each analysis. The number of endo-reduplication cycles per nucleus was calculated (Barow and Meister, 2003). The above measurement has been carried out by the Karyotype evolution group at IPK.

3 Results

The expression pattern of ABA metabolism genes in flag leaf and seeds during seed-filling period were investigated to understand the source-sink relationships during seed filling. Plants were subjected to drought stress at 8 DAF by maintaining soil water status at 10 % moisture content throughout seed filling period. Flag leaf material (16 and 20 DAF that corresponds to 8 and 12 DAS, respectively) and developing seeds (12, 16, 20 and 25 DAF that corresponds to 4, 8, 12 and 17 DAS, respectively) were collected for analysis. Overall, out of the 41 putative ABA metabolism genes identified in barley only 19 of them were found to be differentially regulated under terminal drought stress; and most of them were up-regulated rather than down-regulated. In addition, at least one member of each gene family of ABA biosynthesis was up-regulated under stress conditions, in flag leaves and developing seeds. However, the regulated member was not always the same in these tissues (Figure 1). We describe the 19 differentially expressed ABA metabolism genes.

3.1 ABA accumulation and catabolism in source (flag leaf) and sink (seed) tissues under terminal drought stress:

3.1.1 ABA biosynthesis:

Of the ABA biosynthetic pathway genes, *HvZEP2* and *HvNCED2* are significantly up-regulated in flag leaves (16 DAF) while they were only slightly up-regulated in developing seeds under terminal drought stress. At later stages of drought stress, these two genes were maintained relatively high in both flag leaves and developing seeds except for *HvNCED2* levels in flag leaves (Figure 1a and 1b). Although not influenced by drought, expression of *HvZEP1* is 60 folds higher in flag leaves than in seeds (Supplementary Table 3). Expression pattern of *HvCCD5* is similar to *HvCCD3* in flag leaves, but only *HvCCD3* was significantly up-regulated in developing seeds (20 DAF) (Figure 1a and 1b). Of the *HvSDRs*, *HvSDR3, 6* and *7* are up-regulated only in flag leaves at 16 DAF and 20 DAF under drought stress expect for *HvSDR6* at 16 DAF (Figure 1a). In seeds, different members of the SDR family (*HvSDR2* and *HvSDR4*) are regulated by drought; *HvSDR2* (significantly) and *HvSDR4* are up-regulated under stress at 16 DAF and 20 DAF, respectively (Figure 1b). Of the drought stress regulated *HvAOs* (*HvAO1*, *HvAO4* and *HvAO5a*), only *HvAO1* and *HvAO5a* are significantly up-regulated in developing seeds at 16 DAF and 20 DAF, respectively; while *HvAO4* had a

trend of up-regulation under drought stress in flag leaves (20 DAF) (Figure 1a and 1b). Expression of *HvMCSU2* was similar to *HvAO4* with additional up-regulation also observed at 16 DAF in flag leaves under drought stress (Figure 1a).

Under stress, ABA levels increased by 10 folds and 6 folds in flag leaves and developing seeds compared to control plants at 16 DAF, respectively (Figure 1d). In flag leaves, the up-regulation of ABA biosynthesis genes at 16 DAF could be correlated with increased ABA levels observed under drought stress. However in developing seeds, the increase in ABA levels could not be correlated to regulation of ABA biosynthesis genes at 16 DAF. In addition, the regulation of ABA catabolism genes and ABA catabolic products under drought stress was analyzed.

3.1.2 ABA degradation:

Of the three members of *HvABA8'OH*, only *HvABA8'OH1* is up-regulated by drought stress in both flag leaf and developing seeds. In flag leaves, *HvABA8'OH1* was up-regulated at both 16 DAF and 20 DAF while in developing seeds it was up-regulated at 16 DAF and later at 25 DAF (Figure 1a and 1b). The up-regulation of *HvABA8'OH1* transcripts could be correlated to the increased levels of PA and DPA observed in both flag leaves and developing seeds at 16 DAF. Under drought stress compared to control plants, PA levels increased by 9 folds in flag leaves while in developing seeds only moderate increase of PA is observed. On the other hand, DPA levels increased only to moderate levels in the flag leaf while it increased by 6 folds in developing seed compared to its levels in control plants. The absolute levels of PA and DPA in flag leaf under drought stress at 16 DAF corresponded to 6,212 ng/g and 5,198 ng/g dry weight of tissue, respectively. During similar stage (16 DAF), the ABA content in flag leaves was observed to be 1,054 ng/g dry weight of tissue. Suggesting, nearly 12 folds increase of catabolic products compared to ABA in flag leaves at 16 DAF. Overall, the levels of ABA, PA and DPA are much lower in developing seeds compared to its levels in flag leaves; and DPA is the major catabolic product of ABA in developing seeds (Figure 1d). ABA-GE is considered to be the inactive long-distance transport form of ABA de-conjugation of which results in enhancement of ABA pool. Therefore, we studied the expression of BG genes in flag leaves and developing seeds.

3.1.3 ABA de-conjugation:

The expression pattern of BG gene family members in barley is diverse under control and drought stress. While in flag leaves *HvBG2* is significantly down-regulated under drought stress, *HvBG8* is found to be significantly up-regulated (Figure 1a). However in developing seeds, all the three *HvBG3*, *HvBG6* and *HvBG7* were significantly up-regulated at 16 DAF and 20 DAF under drought stress (Figure 1b).

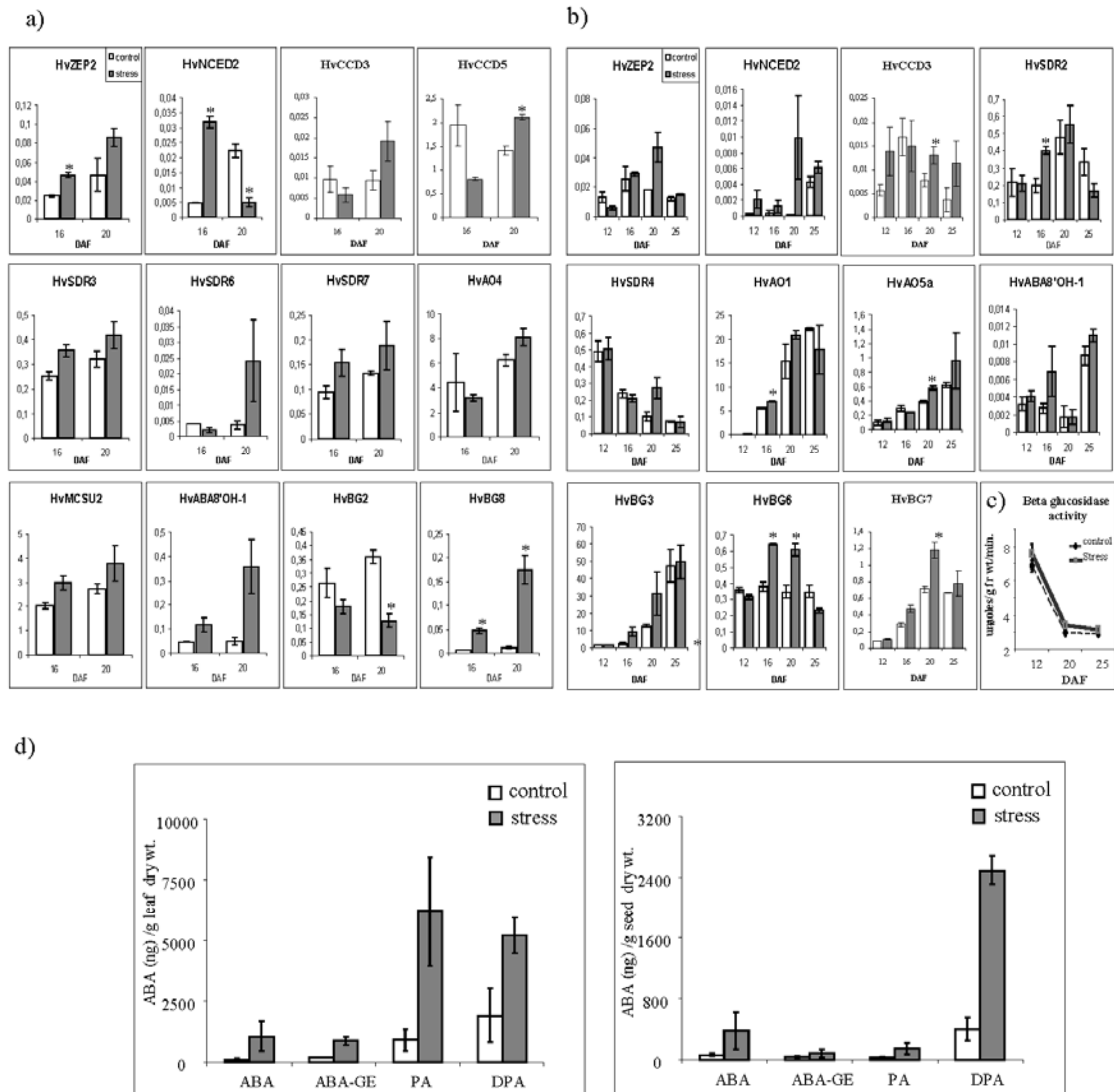


Figure 1: ABA biosynthetic , catabolic and de-conjugation genes were differentially regulated in a) flag leaves and b) seeds of elite barley genotype (LP110) under post-anthesis drought stress compared to control; β-glucosidase activity (c), and ABA and its metabolites at 16DAF (d) were analyzed.

Represented are means SD; for transcript analysis n = 2 with 2 technical replicates while for activity and metabolite measurements n = 3 with 2 technical replicates each. White and black bars represent control and stress, respectively. Asterisk (*) indicates significant differences between plants under stress and control, at $P < 0.05$.

Metabolite measurements at 16 DAF showed that the content of ABA-GE in flag leaves increased by 4.5 fold under drought stress compared to the control. However, the ratio of free ABA to ABA-GE remained nearly 1:1. This indicates that de-conjugation is not a predominant event in flag leaves to increase its ABA level rather the conjugation events seems to take place (Figure 1d). In seeds, under drought stress the levels of BG transcripts correlated well with ABA-GE levels that were nearly 5 fold lower than free ABA levels (Figure 1d). The increased expression of BG correlated with increased ß-glucosidase activity observed in developing seeds under terminal drought (Figure 1c). Overall, it indicates that ABA could be transported from flag leaves to developing seeds in conjugated form (ABA-GE) where it is de-conjugated leading to increased levels of ABA. The source of ABA in developing seeds at 16 DAF comes mainly from flag leaves and is not due to its *de-novo* synthesis; ABA biosynthesis genes were not significantly up-regulated in seeds at 16 DAF.

3.2 Impact of ABA on seed filling under drought:

Not much is known about the role of ABA in the endosperm during seed filling. Considering the gene expression data related to ABA metabolism (Figure 1a and 1b) and ABA levels (Figure 1d), we conclude that ABA biosynthesis probably takes place in both endosperm and embryo tissues during seed maturation. ABA levels were found to profoundly increase under drought stress in both flag leaves and developing seeds (Figure 2). This elevated ABA levels in seeds correlate with increased starch content especially at 12 and 16 DAF under stress (Figure 3). This indicates that ABA probably accelerates the rate of starch accumulation in addition to reducing the duration of seed filling period. Correspondingly, the transcript and enzyme activity of sucrose synthase (SUS) and ADP-glucose pyrophosphorylase (AGL) was not affected at 12 and 16 DAF under drought, but was decreased at 20 DAF onwards under drought (Figure 3). Also, the genes involved in amylopectin biosynthesis, starch branching enzyme (*SBE1*) and starch synthase (*SSIIIa*) were induced at 12 and 16 DAF under drought (Figure 3). In addition, starch break down genes, ß-amylase (*BAM1*, *BAM2*) were induced in developing seeds at 16 DAF under drought (Figure 3). The relevance of ß-amylase induction in developing grains during storage under drought on starch mobilization is unclear.

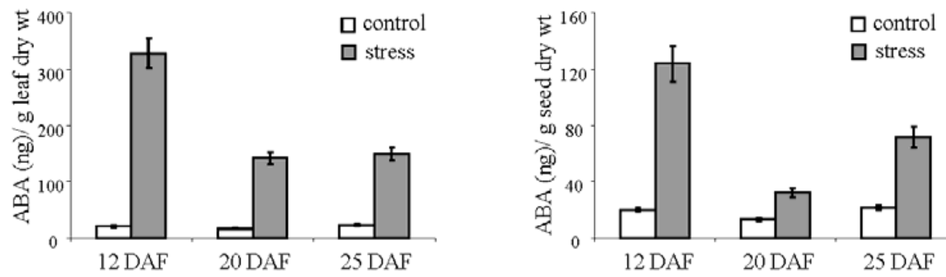


Figure 2: ABA content increased in flag leaves and seeds of elite barley line (LP110) under post-anthesis drought stress.

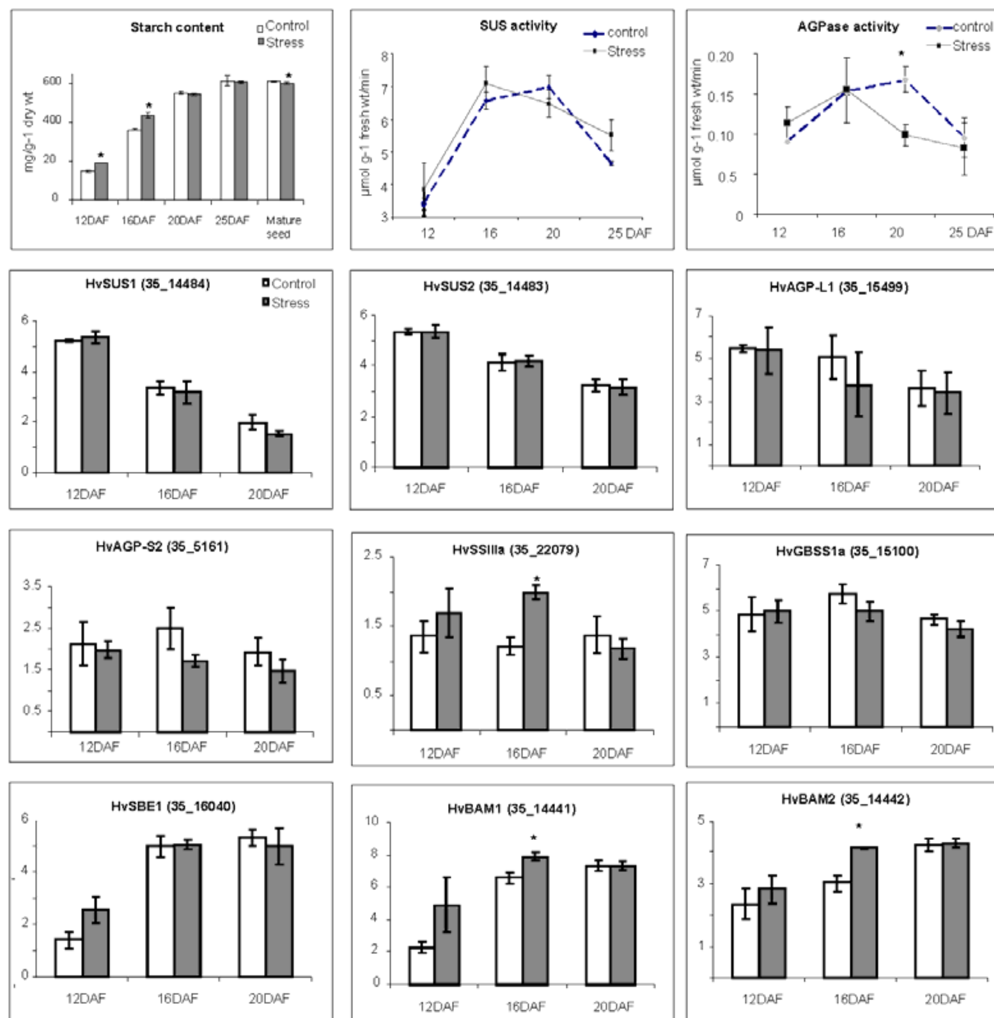


Figure 3: Few starch metabolism genes significantly expressed (macro-array data) in seeds under drought stress over control; and, AGPase activity significantly reduced under stress that probably are responsible for significant changes observed for starch content under stress.

The genes *sucrose synthase* (SUS); *ADP-glucose pyrophosphorylase* small subunit (AGP-S); *starch branching enzyme* (SBE); *starch synthase* (SS); and *β-amylase* (BAM) are analyzed and represented are the normalized expression values. Asterisk (*) indicates significant differences between plants under stress and control, at $P < 0.05$.

Furthermore, we isolated promoters of sucrose synthase1 (*HvSUS1*; D63574), ADP-glucose pyrophosphorylase small subunit (*HvAGP*-S1; AJ239130) and ß-amylase 1 (*HvBAM1*; X73221) and found several putative ABA-responsive and dehydration-responsive *cis*-elements in addition to endosperm specific and light responsive *cis*- elements (Figure 4). Based on this, we propose that elevated ABA under stress, during which the filling phase is shortened, influences faster seed filling by influencing starch biosynthesis and degradation pathway.

To further test this hypothesis, ABA or fluridone (an inhibitor of carotenoid/ABA biosynthesis) was sprayed to developing spikes of plants under drought stress in field, and analyzed its influence on TGW. Under drought stress, there was significant reduction in yield (6 %) compared to control conditions. Exogenous ABA treatment compensated for the reduction in TGW under stress, whereas exogenous fluridone treatment resulted in substantial decrease in TGW and starch content up to 20% in mature seed (Figure 5). These results clearly point out the importance of ABA in promoting starch accumulation in developing seeds.

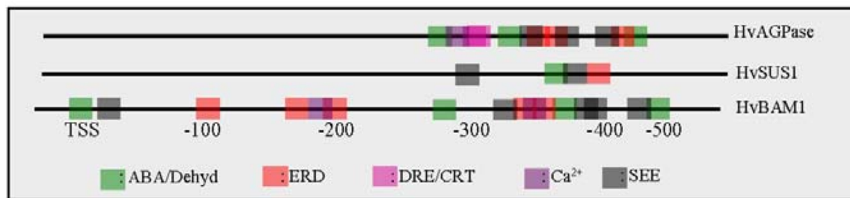


Figure 4: Results of promoter analyses of *HvAGPase-S1*, *HvSUS1* and *HvBAM1* genes; *cis*-elements enriched in 500 bp upstream of transcription start sites (TSS) of the respective gene. *cis*-elements include ABRE : ABA and dehydration responsive element; ERD : early responsive to dehydration; DRE /CRT : drought responsive element; Ca^{2+}: calcium and ABA responsive; SEE : seed/embryo/endosperm specific element.

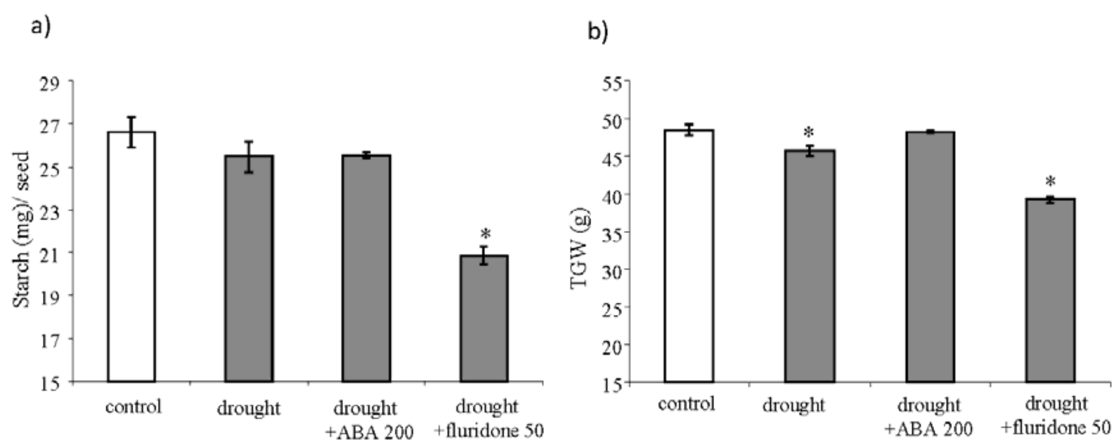


Figure 5: Drought stress and or exogenous treatment of developing spikes with ABA inhibitor under stress significantly affected (a) starch content per seed and (b) thousand grain weight. Asterisk (*) indicates significant differences between plants under stress or exogenous treatment under stress and control plants, at $P < 0.05$.

From the above study of transcript expression pattern of ABA and starch metabolism genes along with exogenous ABA treatment study, it is clear that basal levels of ABA are necessary for the plant to maintain grain filling. Under drought stress, as such ABA levels increase; however, drought stress resulted in reduction in TGW, but exogenous ABA application under drought stress compensated for yield reduction. This indicates that ABA influences grain filling, however, it is its levels that decide if it would have positive or negative effect. Based on this we hypothesise that during the grain filling phase, certain period or window of development is highly sensitive for alteration in ABA homeostasis that greatly affects starch accumulation or grain filling. To test the hypothesis, transgenic plants with altered levels of ABA in leaves and seeds (at different time point) were studied at various developmental time points to correlate ABA levels and starch metabolism under control and post-anthesis drought stress, respectively.

3.3 Characterization of transgenic plants altered for ABA homeostasis:

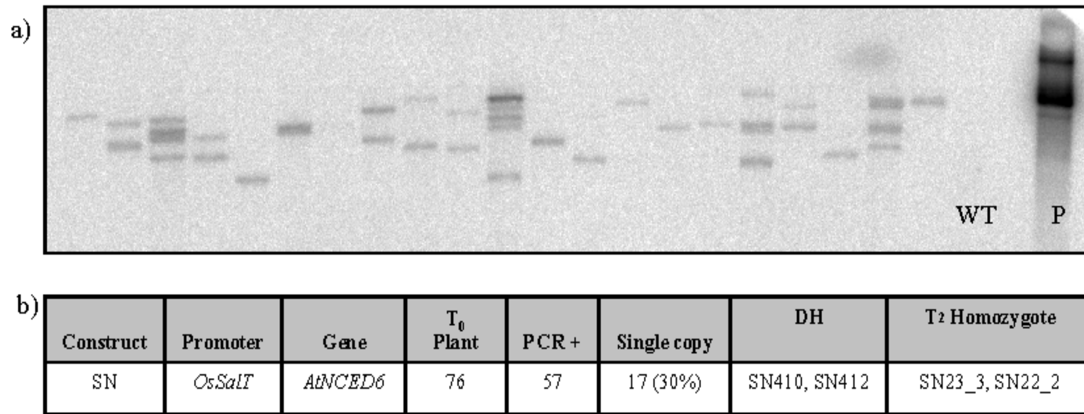
Drought stress greatly affects yield potential of plants, but to what extent ABA that significantly increases under stress contributes to reduction in yield is not clear. Altering ABA levels is the most critical component to study the role of ABA. However, regulating ABA levels is very difficult as plants have their own system of maintaining ABA

homeostasis by regulating its biosynthesis and catabolic pathway. Although there are many studies on ABA manipulation in vegetative tissue, there is no clear evidence of its impact on ABA homeostasis, grain filling and yield. In this study, transgenic plants have been generated overexpressing *AtNCED6* driven by inducible promoters, to regulate ABA levels in turn its homeostasis under stress. Stress was imposed at 4 DAF and continued throughout the grain filling period till seed maturity. The well characterized drought stress inducible promoter from rice, *OsSalT*, and endosperm specific promoter (high molecular-weight glutenin subunit) from wheat, *Ta1AX1*, driving the expression of *AtNCED6*, were used to generate SN and XN transgenic plants, respectively. These plants with altered ABA homeostasis in leaves and seed tissue would aid in studying the impact of altered ABA on yield performance of plants under stress. In addition, help in understanding the critical stages of grain development that is most sensitive to disturbances in ABA levels. An understanding of these processes would eventually help to improve drought tolerance of plants.

3.4 Characterization of *OsSalT*:: *AtNCED6* (SN) transgenic plants:

3.4.1 Phenotypic alterations observed in the SN transgenic plants:

The transgenic plants were characterized by southern hybridization to identify the number of transgene copies integrated. One of the plants with single insertion was taken ahead for generation of homozygous T_2 plants (SN23_3) and another line with 2 transgene copies was used for production of double haploid plants (SN410_8). These two lines (homozygous T_2 and DH2 generation) were used for further characterization of plant performance under drought stress (Figure 6). During early stages of plant growth, the transgenic plants had significantly lower number of tillers compared to wild type plants at similar stage of development, respectively. However, during later stages of development (115 days after sowing), when the plants have almost completed flowering, transgenic plants had tiller number similar to that of wild type (WT) plants (Figure 7a). The SN410_8 plants that had 2 copies of *AtNCED6* displayed significantly early flowering compared to both SN23_3 and WT plants, but within 5 days there were no more differences between the transgenic and WT plants (Figure 7b).



Construct	Promoter	Gene	T_0 Plant	PCR +	Single copy	DH	T_2 Homozygote
SN	*OsSalT*	*AtNCED6*	76	57	17 (30%)	SN410, SN412	SN23_3, SN22_2

Figure 6: Representative (a) southern blot of the primary transgenic plants, which was carried out to determine the copy number, and (b) summary of transgenic plants produced in barley over expressing *AtNCED6* under the control of rice *OsSalT* promoter. WT: wild type; P: positive control.

The WT plants (*Hordeum vulgare cv*. Golden promise) used for transgenic production is a mutant (gamma irradiated) that sometimes displays emergence of small secondary rudimentary spike as branch from the primary spike. This phenomena of spike branching was significantly higher only in SN410_8 plants compared to WT plants. The SN410_8 plants not only had significantly higher number of spikes with one secondary spike compared to WT plants, but also were found to have spikes with 3 to 5 secondary spikes per one primary spike. The WT plants had a maximum of only 1 secondary spike per primary spike (Figure 7c and side panel). Further, the spikes were analyzed at different time points before spike emergence and it was observed that the length of primary spike meristem of SN410_8 was significantly lengthier than that of WT plants. In addition, the length of the secondary spike (branched spike) meristem was also significantly longer than that of WT plants (Figure 7d). The scanning electronic microscopic analysis clearly displays the phenotypic differences in the length and development of spike meristem, especially secondary spikes (Figure 7d, side panel). However, at later stages (120 days after sowing) the length of the primary spike meristem was similar between SN410_8 and WT plants while length of the secondary spike meristem still significant differed between SN410_8 and WT plants (Figure 7d, inset). The plants grown under control and stress were further analyzed for the expression of transgene in flag leaves and developing seeds.

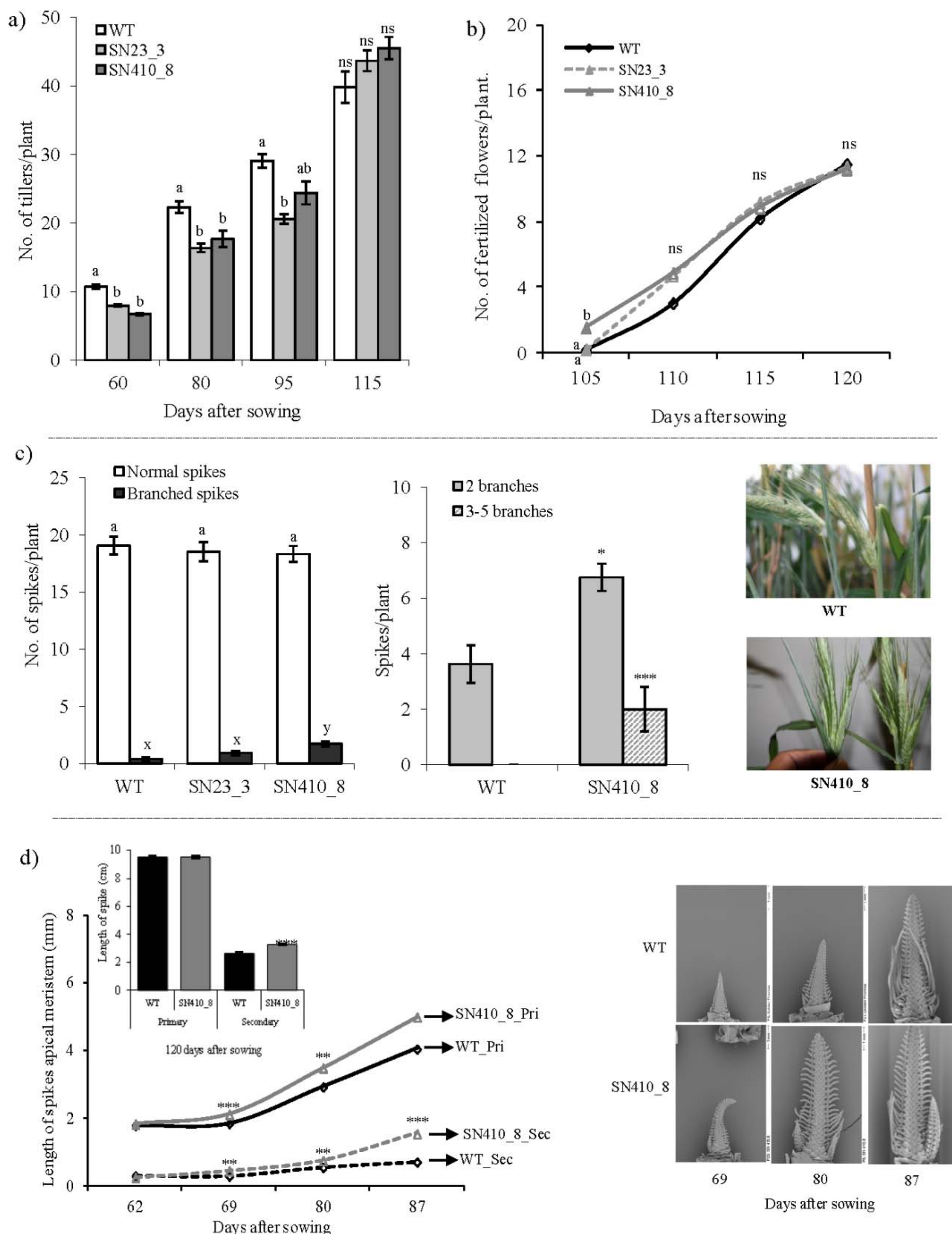


Figure 7: Phenotypic alteration observed due to over expression of AtNCED6 transgene in terms of a) tiller number, b) flowering time, c) spike characteristics and d) development of the primary and branched spikes. Letters (in a, b and c) represent significant differences between genotypes under control (a, b, c) and stress (x, y, z) condition, respectively, at $P < 0.01$. Similar letters indicate no significant differences exists between the genotypes, at $P < 0.01$; and Asterisks (in c and d) represent significant differences between genotypes, at $P < 0.05$:*, $P < 0.01$:** and $P < 0.001$:***.

3.4.2 Transgene expression in SN transgenic under post-anthesis drought:

The transcript level of transgene (*AtNCED6*) was analyzed in WT, SN23_3 and SN410_8 plants under control and different days after stress (DAS) (short term stress: 0.5 - 4 DAS; long term stress: 8 - 12 DAS) using qRT-PCR.

As expected, *AtNCED6* transcripts were undetectable in WT plants. Although both SN23_3 and SN410_8 showed significant up-regulation of *AtNCED6* expression in leaves at 4 DAS, SN410_8 plants accumulated significantly high *AtNCED6* levels within 24 h of stress imposition. However, SN23_3 and SN410_8 lines differed in the pattern of *AtNCED6* expression in seeds. *AtNCED6* levels in SN23_3 seeds were 1000 folds lower than that of SN410_8, at 8 DAS (12 DAF), but was significantly up-regulated under stress compared to its respective control. Contrastingly, expression of *AtNCED6* in seeds of SN410_8 stress plants was not significantly different from its respective control plants (Figure 8a). ABA levels were measured to study if differences in *AtNCED6* transcript levels lead to differences in ABA levels in these lines.

3.4.3 ABA levels in leaf and seeds of WT and SN transgenic plants under post-anthesis drought stress:

These two lines which had slight differences in the pattern of *AtNCED6* expression also displayed differences in ABA accumulation. Under control conditions, ABA levels in leaves and seed tissue of both transgenic lines SN410_8 and SN23_3 was significantly lower than that of WT plants, except at 2 DAS (6 DAF). Contrastingly under stress, ABA levels in leaves of SN410_8 plants was significantly higher while it was significantly lower in leaves of SN23_3 plants compared to ABA levels of WT plants at 2 DAS. At 8 DAS, leaves of both lines had significantly lower levels of ABA than WT plants. Seeds of SN410_8 plants also had significantly high levels of ABA (2 DAS: 6 DAF) compared to WT plants (no data for SN23_3, at 2 DAS). Both transgenic lines, maintained significantly low ABA in seeds at 8 DAS (12 DAF) compared to ABA in seeds of WT plants, respectively. However, with increase in duration of stress (12 DAS), ABA level in SN23_3 seeds were between that of WT and SN410_8, with ABA levels significantly lower in SN410_8 than WT plants (Figure 8b). The differences in ABA levels could not be completely explained by differences in

transgene expression. Hence, endogenous *Hv-NCEDs* and -ABA hydroxylase transcript levels were also analyzed.

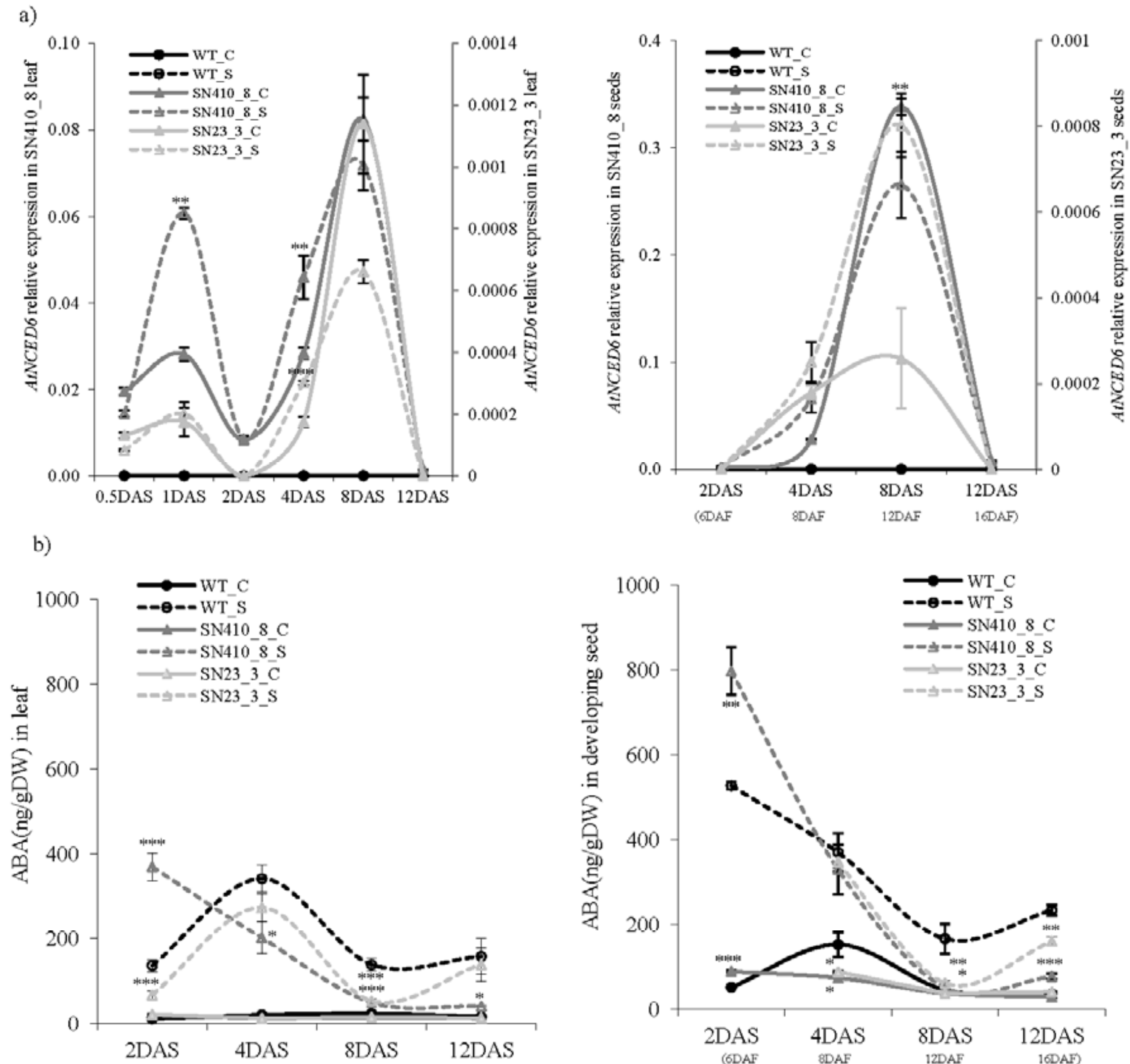


Figure 8: Transgene, *AtNCED6*, expression was found to be highly stress inducible under the regulation of *OsSalT* promoter in both flag leaf and developing seed; transgenic line accumulated high ABA in flag leaves under short term stress.
Two independent homozygous lines, SN410_8 and SN23_5 that expressed *AtNCED6* under control of *OsSalT* promoter were selected for analysis. Mean ±SE (n = 4) a) relative expression level of *AtNCED6* in comparison to housekeeping gene and b) ABA content, in the WT and SN transgenic in leaf (left panel) and seed (right panel). Solid lines represent control condition and dashed lines represent drought stress conditions, respectively. Asterisks represent significant differences between genotypes within treatment group at P < 0.05:*,P < 0.01:** and P < 0.001:***. DAS: days after stress

3.4.4 Regulation of *HvNCED* and *HvABA8′*OH family genes in WT and SN transgenics: In leaves

WT and transgenic plants that differed only by expression of transgene were found to differ in the pattern of *HvNCEDs* and *ABA8'OHs* transcript accumulation (Figure 9 and supplementary figure 1a). This indicates that expression of *AtNCED6* transgene seems to effect the regulation of the ABA biosynthesis and catabolic genes. Fold change in levels of ABA metabolites and transcript under stress compared to control condition was calculated. This helps in correlating transcripts of ABA biosynthetic and catabolic pathway to ABA levels. In both WT and transgenic plants, ABA levels observed could be explained by the changes in levels of *HvNCEDs* and *HvABA8'OH* transcripts.

In flag leaves of WT plants, *HvNCED2* majorly contributed to ABA accumulation under short term stress and the transcript pattern was similar to ABA accumulation pattern. Nevertheless, *HvNCED1* was the major contributor for ABA levels in WT plants under long term stress (Figure 9a). However in transgenic plants, ABA levels were contributed by the expression of both *HvNCED1* and *HvNCED2* apart from *AtNCED6*. The regulation of transcripts was similar to that of ABA accumulation pattern. Although fold change in the expression of *AtNCED6* was not high, it contributed in increasing ABA under short term stress (Figure 9c and 9e). Under long term stress, SN23_3 behaved similar to SN410_8 at 8 DAS. At 12 DAS the fold change in ABA levels of SN23_3 was similar to WT plants but transcript pattern was similar to SN410_8 plants. Moreover, the increased levels of ABA at 12 DAS in SN23_3 could not be explained by fold changes in *HvNCED* transcripts (Figure 9e).

Nevertheless, only slight difference in the regulation of all three *HvABA8'OH* coding genes was observed between the WT, SN23_3 and SN410_8 under both short term and long term stress. All the three hydroxylase genes were mostly down-regulated under stress (Figure 9a, 9c and 9e). Exceptionally, *HvABA8'OH1* was up-regulated in both WT and transgenic lines under stress. In SN23_3 plants, it was up-regulated under short term stress while in WT and SN410_8 plants it was up-regulated under long term stress. In addition to *HvABA8'OH1*, *HvABA8'OH3* was also up-regulated in SN410_8 under long term stress. This explains the difference in ABA levels under long term stress between SN410_8 and WT or SN23_3 lines (Figure 9a, 9c and 9e). Similar analysis was also carried out in developing seeds.

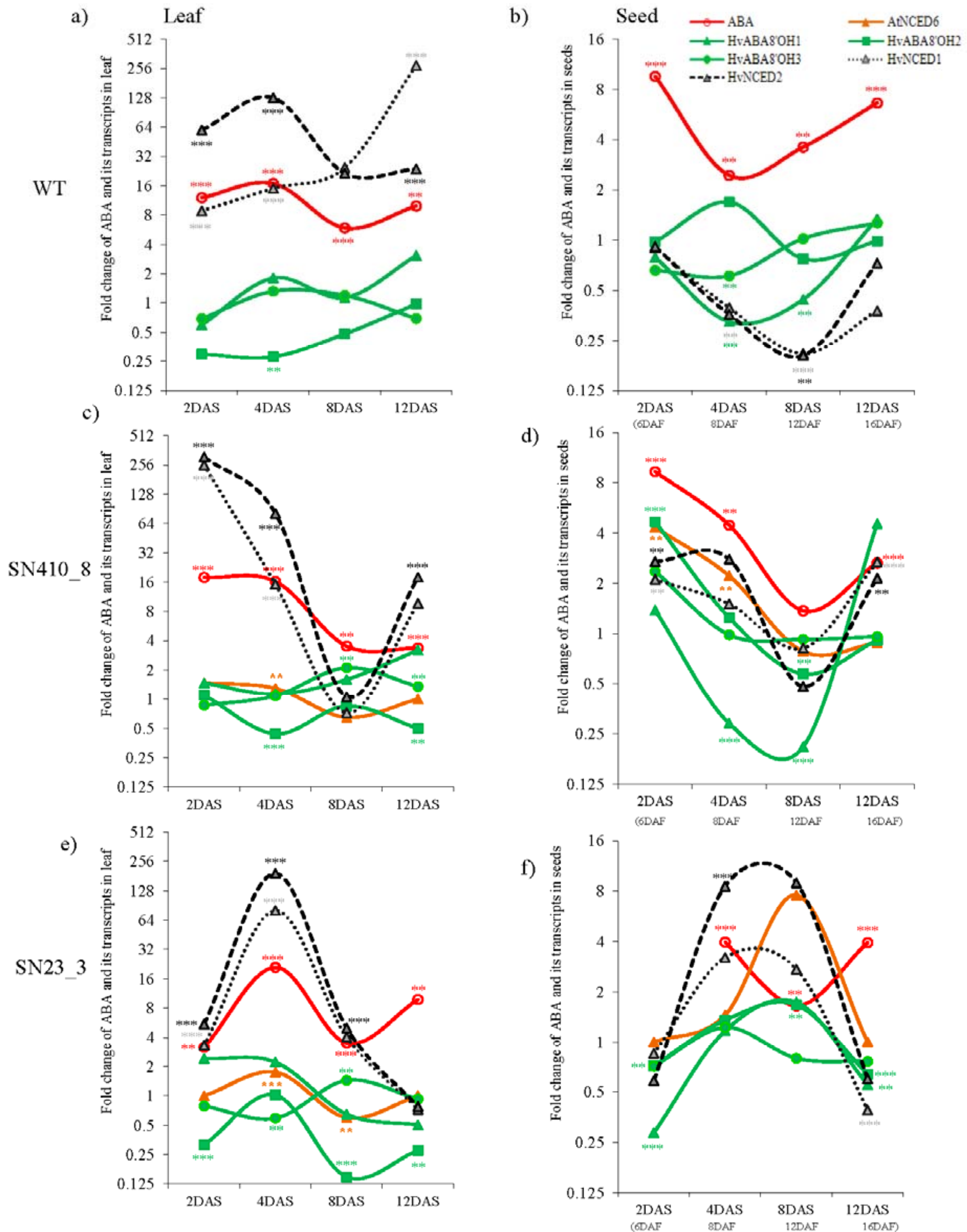


Figure 9: Change in *HvNCED* transcripts correlates with change in ABA content in flag leaves of WT, SN410_8 and SN23_3 while in seeds, similar correlation was observed only for SN410_8.

Fold change in ABA biosynthetic (*HvNCED*) and cataobolic (*HvABA8'OH*) transcript abundance, and ABA content under stress over control in flag leaf (a, c and e) and developing seeds (b, d and f) in WT (a and b), SN410_8 (c and d) and SN23_3 (e and f). Y-axis is represented in log scale to the base 2. Asterisks represent significant change in transcript or ABA abundance under stress over control, at $P < 0.01$:** and $P < 0.001$:***. DAS: days after stress

In seeds

The ABA levels in developing seeds of WT plants under stress (all time points) could not be correlated to *HvNCED1* and *HvNCED2* transcript levels. In general at all stress levels, the *HvNCEDs* and *HvABA8'OH* transcripts were either down-regulated or showed no fold change under stress compared to their levels in plants grown under control condition. Only *HvABA8'OH2* was found to be slightly up-regulated at 4 DAS (8 DAF) and could be correlated to reduction in ABA levels (Figure 9b, supplementary figure 1b). This indicates, ABA levels in developing seeds of WT plants under stress is not a result of *de-novo* synthesis, but a result of its transport from flag leaves or other source tissue.

Contrastingly, the ABA levels in seeds of SN410_8 could be correlated with expression of *HvNCED1*, *HvNCED2* and *AtNCED6* transgene. Although the low levels of ABA maintained at 12 DAS (16 DAF) could be correlated to induction of *HvABA8'OH1*, the decreased ABA levels observed at 4 and 8 DAS (8 and 12 DAF) could not be correlated to regulation of any *HvABA8'OH* genes (Figure 9d). Similar to SN410_8, the ABA levels in seeds of SN23_3 could be correlated to expression of *HvNCED1*, 2 and *AtNCED6*. The *AtNCED6* was regulated at different time points in SN23_3 and SN410_8, respectively. At 8 DAS (12DAF), seeds of SN23_3 had their lowest level of ABA which was not correlated to transcript levels of *AtNCED6* and *HvNCEDs* that were highly induced during this time point. The low levels of ABA can be explained to some extent by slight induction of *HvABA8'OH1* and 2. However, the ABA levels in SN23_3 were maintained higher at 12 DAS (16 DAF) similar to that of WT plants (Figure 9f). Nevertheless in transgenic plants, ABA accumulated in seeds is a result of its *de-novo* synthesis in addition to its import from other sources. To study the effect of altered ABA on regulation of its own catabolism, ABA catabolites were measured in WT and SN410_8 plants which showed maximum regulation of *HvABA8'OHs*.

3.4.5 Regulation of ABA catabolic products in leaves and seeds of WT and SN410_8 plants under post-anthesis drought stress:

The ABA catabolites were measured in flag leaf and seed tissue of WT and SN410_8 plants subjected to short and long term drought stress along with respective control plants. Differences in PA and DPA level between WT and SN410_8 was observed in flag leaves only under long term stress (Figure 10a and 10c). Under long term stress compared to

respective control plants, flag leaf of WT plants maintained higher PA levels (9 folds) than DPA (6 folds) levels while transgenic plants maintained higher DPA (9 folds) levels than PA (8 folds) levels in flag leaves. However, WT and transgenic plants differed in the ABA-glucose ester (ABA-GE) levels in flag leaves under both short and long term stress. Under short term stress, ABA-GE levels was 3.6 folds higher in SN410_8 while it was 2.2 folds higher in WT plants, compared to their respective control plants (Figure 10a). Under long term stress, ABA-GE levels in flag leaves increased by 13 and 7.6 folds in SN410_8 and WT plants, respectively compared to their respective control plants (Figure 10c).

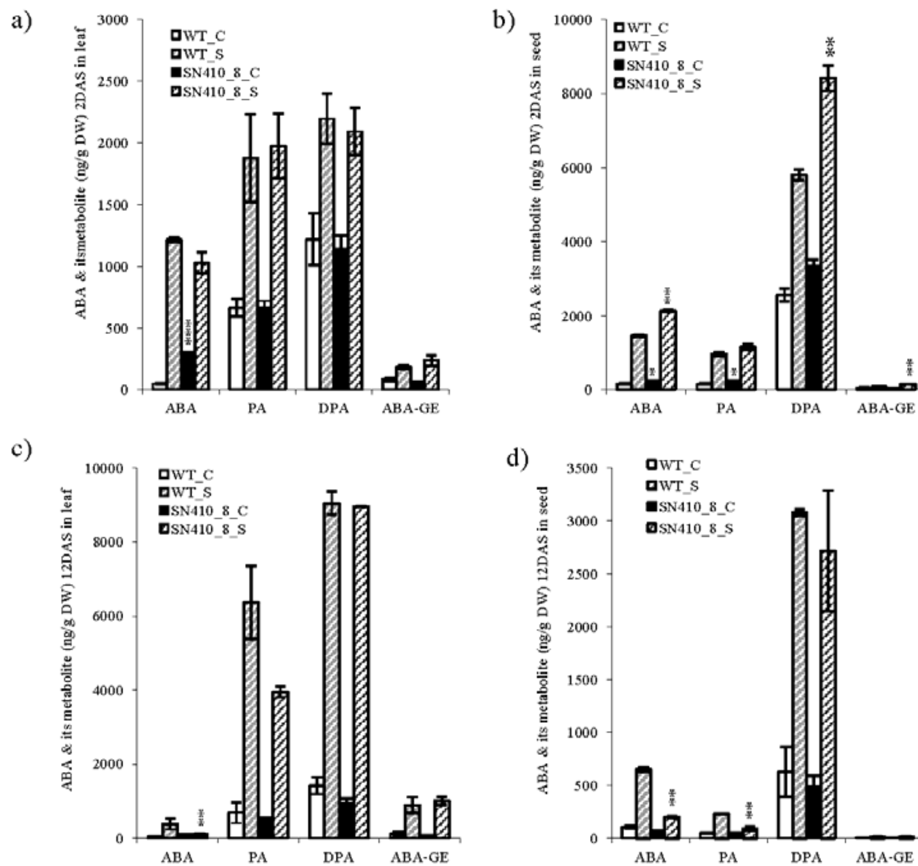


Figure 10: Accumulation of various ABA metabolites in WT and SN410 lines under short term (a and b) and long term (c and d) drought stress in flag leaf (a and c) and developing seeds (b and d). Represented are the mean ± SE (n = 4) of metabolites under control (solid bars) and stress (hatched bars). Asterisks represent significant differences in metabolite accumulation between genotypes within a given treatment group at $P < 0.05$:*,$P < 0.01$:** and $P < 0.001$:***.

In developing seeds, PA and DPA levels were similar between the WT and transgenic plants under short term stress (2 DAS: 6 DAF). Nevertheless, fold change in ABA-GE levels under short term stress compared to respective control was higher in SN410_8 plants (4.6 folds) compared to WT plants (1.7 folds) (Figure 10b). Under prolonged drought condition (12 DAS: 16 DAF), PA levels were higher only in WT plants (4.7 folds over control) and not in transgenic plants (1.8 folds over control). WT and transgenic plants at 12 DAS (16 DAF) had similar levels of DPA and ABA-GE (Figure 10d). The SN410_8 plants were also further analyzed for changes in other phytohormones that would have occurred as a result of altering ABA homeostasis in leaves and seeds.

3.4.6 Hormonal levels altered in WT and SN410_8 in leaf and seed under post-anthesis drought:

Auxin, gibberellins and cytokinin content was analyzed in leaf and seed tissue of WT and SN410_8 plants subjected to short term stress (2 DAS: 6 DAF) and long term stress (12 DAS: 16 DAF) along with their respective controls.

Auxin (IAA)

The active form of auxin, IAA, content in leaves was not significantly different between the WT and SN410_8 plants under both control and stress conditions (2 and 12 DAS). The inactive form of auxin, IAA-Asp, was detectable in SN410_8 only at 2 DAS and in WT plants only at 12 DAS, respectively in both control and stress plants. The level of IAA in seed was nearly 20-40 folds higher than that observed in leaves (Figure 11 g and 11h). Under short term stress, seeds of SN410_8 had significantly low levels of IAA compared to WT seeds but were similar under long term stress. However, the level of inactive auxin (IAA-Asp) was significantly lower in seeds of SN410_8 plants under stress (2 and 12 DAS: 6 and 16 DAF) compared to WT plants under stress; similar, reduced levels were observed in SN410_8 under control conditions only at 2 DAS: 6 DAF (Figure 11c and 11d).

Cytokinin

Three metabolites *trans*-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR) and 2 iso-pentyladenine (2iP) were measured as an estimate of active and inactive forms of cytokinin. The level of 2iP in leaves of SN410_8 control plants was significantly higher than levels found in leaves

of WT plants under control conditions (2 and 12 DAS) (Figure 11i and11j). In seeds, *t*-ZR levels at 2 DAS (6 DAF) was significantly lower in SN410_8 plants compared to WT plants, when both were grown under control conditions. However, 2iP level in seeds at 2 DAS (6 DAF) was significantly lower in SN410_8 plants compared to WT plants under stress. At 12 DAS (16 DAF), the levels of both active and inactive cytokinin were lower than levels observed at 2 DAS (6 DAF) but they were similar in both SN410_8 and WT plants, under control and stress condition (Figure 11e and 11f).

Gibberellins

The active form (GA4) and inactive form (GA34) were not detected in leaves of both WT and SN410_8 plants at 2 DAS and 12 DAS. The level in seed was higher at 12 DAS (16 DAF) compared to 2 DAS (6 DAF). Seeds of SN410_8 plants grown under control conditions had significantly lower levels of GA4 and GA34 at 2 DAS compared to seeds of WT plants grown under similar control conditions. However, the level of only GA34 was maintained lower in SN410_8 seeds compared to WT seeds under control conditions at 12 DAS: 16 DAF (Figure 11a and 11b).

3.4.7 Performance of WT and SN transgenic under stress:

Physiological performance

The leaves from WT and SN transgenic plants were detached and weighed at regular intervals till 1h to estimate the amount of water loss. Both transgenic lines, SN23_3 and SN410_8, lost significantly less water compared to WT plants in this detached leaf experiment; SN23_3 and SN410_8 did not significantly differ from each other (Figure 12a). Similarly, relative water content (RWC) parameter estimated at 4 DAS was used as a measure of stress tolerance. As expected, RWC decreased in WT and both the transgenic lines (SN23_3 and SN410_8) under stress compared to their control plants, respectively. However under stress, SN23_3 and SN410_8 lines maintained significantly high percent RWC in leaves than that of WT plants under similar stress conditions (Figure 12b).

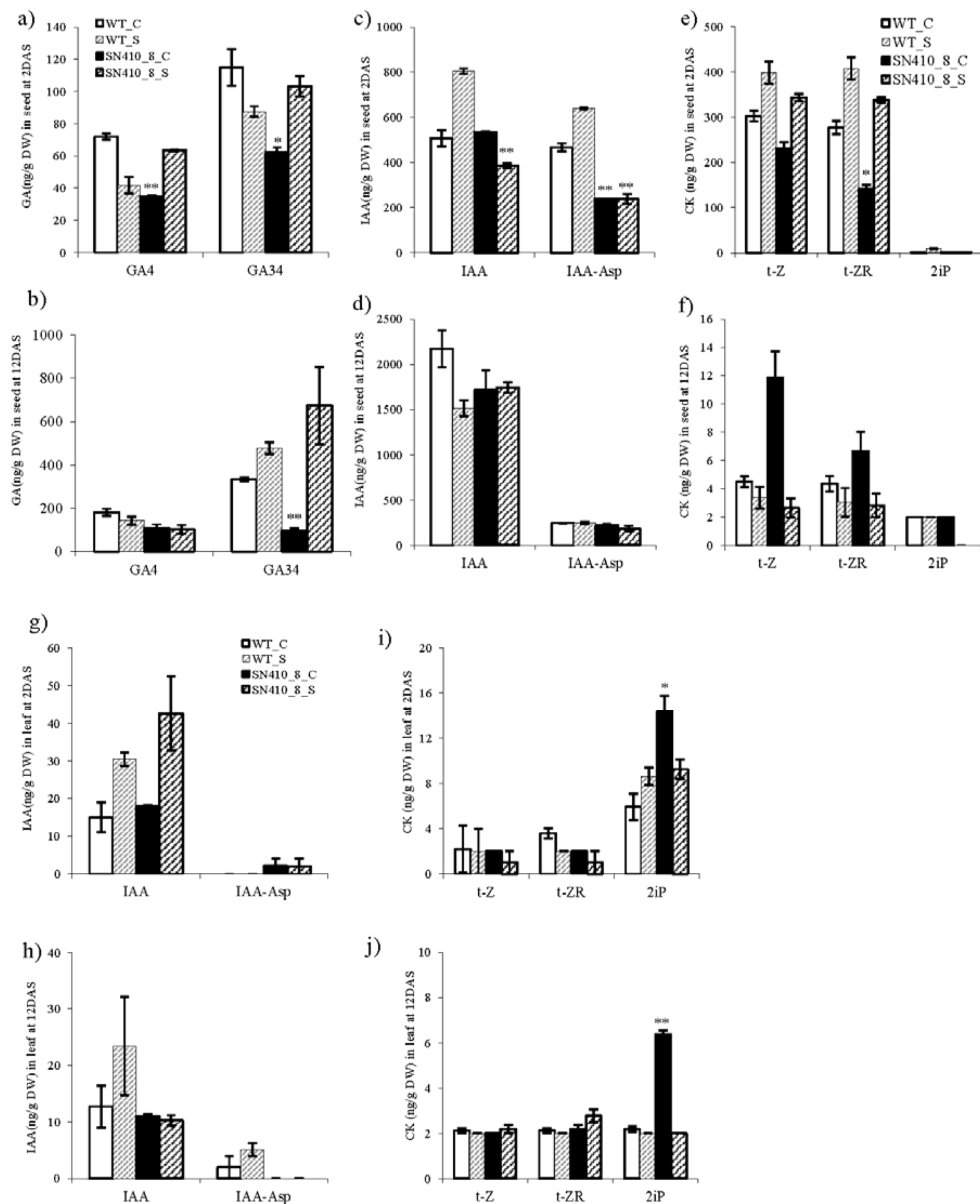


Figure 11: phytohormones other than ABA were altered in leaf and seed tissue of WT and SN410_8 transgenic plants under short and long term drought stress.

Represented are the mean ± SE (n = 4) amount of hormones gibberellins (a, b), auxins (c, d, g, h) and cytokinins (e, f, i, j) in seed tissue (a-f) and leaf tissue (g-j; no gibberellins data) under short term stress (a,c & e), under long term stress (b, d & f). Solid bars represent control and hatched bars represent stress condition. Asterisks represent significant differences in hormone levels between genotypes within a given treatment group, at P < 0.05:*,P < 0.01:** and P < 0.001:***.

The stress tolerance of SN410_8, percent RWC, could be explained due to the high levels of ABA in leaves at 2DAS. On the other hand, ABA levels were similar between SN23_3 and WT plants, but still SN23_3 could maintain high percent RWC under stress. Under stress, both transgenic lines (SN23_3 and SN410_8) maintained significantly higher stomatal conductance than WT plants. However under control condition, SN410_8 plants maintained significantly lower stomatal conductance compared to WT and SN23_3 plants (Figure 12c). In addition, stress had a drastic effect on reducing the assimilation rate of WT and transgenic lines (SN23_3 and SN410_8), compared to respective control plants.

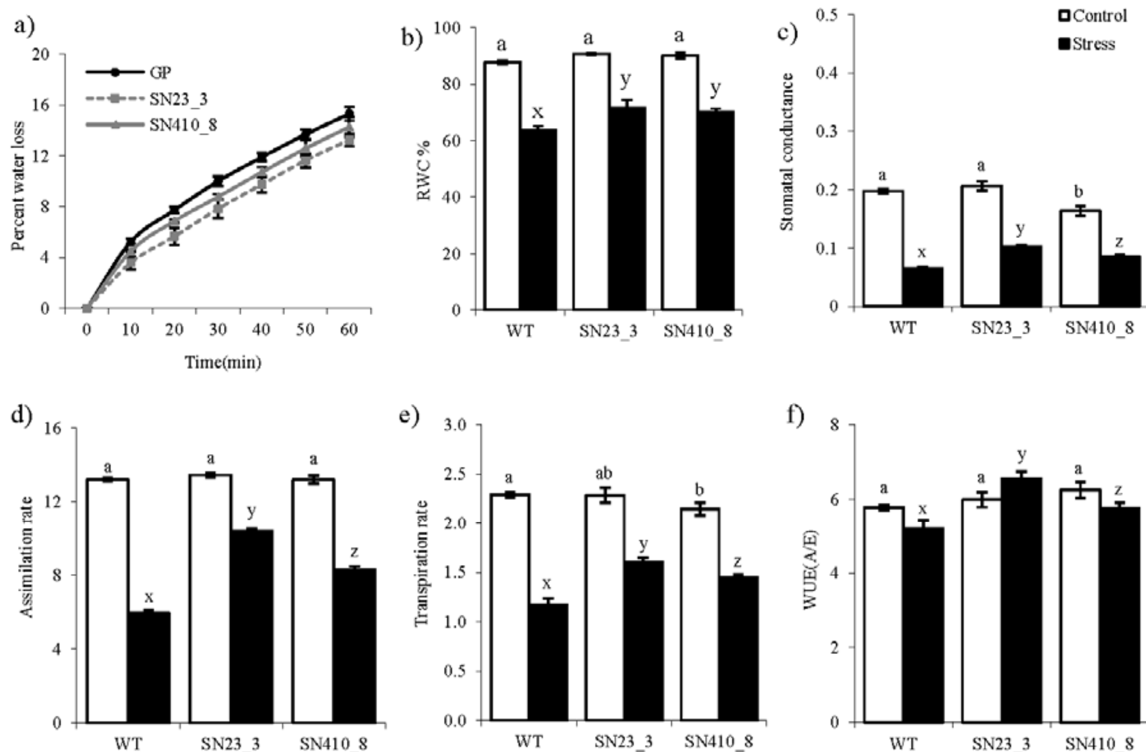


Figure 12: The physiological performance of transgenic lines, SN410_8 and SN23_3, under drought stress was significantly higher than wild type plants grown under similar stress condition.
Mean ± SE of a) rate of water loss b) RWC%, c) stomatal conductance (mol m^{-2} s^{-1}), d) assimilation rate (µmol m^{-2} s^{-1}), e) transpiration rate (mol m^{-2} s^{-1}) and f) WUE (water use efficiency). For all the parameters measured, n = 15 except for rate of water loss and RWC% n = 5; white bars: control and black bars: stress. Letters represent significant difference between genotypes under control (a, b, c) and stress (x, y, z) condition, respectively, at $P < 0.01$. Similar letters indicate no significant differences exists between the genotypes, at $P < 0.01$.

SN23_3 and SN410_8 plants had assimilation rates comparable to that of WT plants under control condition. Under stress, SN23_3 had significantly higher assimilation rate followed by SN410_8, and WT plants (Figure 12d). This correlated with the stomatal conductance under stress which was significantly higher in SN23_3 and SN410_8 compared to WT plants under stress, respectively (Figure 12c). Transpiration rate and WUE of SN23_3, SN410_8 and WT plants under stress followed similar patterns as stomatal conductance under stress (Figure 12e and 12f). However, reduced stomatal conductance of SN410_8 under control condition did not negatively affect WUE of these plants. WUE of SN410_8 plants was similar to that of WT and SN23_3 plants under control conditions. Thus, both the transgenic plants, SN23_3 and SN410_8, outperformed WT plants under stress by maintaining photosynthesis. These differences in physiological performance of the plants also had an effect on the final performance of the plants in terms of TGW and yield.

TGW and Yield related parameters:

Although SN transgenic plants maintained high photosynthesis under stress, the number of seeds put forth was comparable to that of WT plants under both control and stress conditions. Stress had no significant effect on reducing number of seeds in any of the lines (Figure 13a). However, stress resulted in significant reduction in TGW of only WT plants. Similar to assimilation rate, the TGW under stress was significantly higher than WT in SN23_3 followed by SN410_8; in addition, SN23_3 had significantly higher TGW under control conditions (Figure 13b). Nevertheless, yield estimated using 15 representative plants per replication was found to be comparable between SN lines under stress. SN transgenic plants had significantly higher yield than WT plants under stress. Stress had a significant effect in reducing yield in all lines. The increase in yield of SN23_3 under control is due to significant differences in TGW under control (Figure 13c).

The WT, SN23_3 and SN410_8 seeds differed in their size. SN410_8 had significantly lower seed area than WT and SN23_3 seeds under control condition. This reduced seed area is due to significant reduction in seed breadth. Seeds of both SN23_3 and SN410_8 maintained higher seed area than that of WT seeds under drought and this was due to significantly higher seed breadth of SN transgenic seeds. The seed breadth of SN23_3 and WT plants were significantly reduced by stress compared to their respective control seeds while seed length

was unaffected by stress (Figure 13d-f). The reduced seed size of SN410_8 under control did not negatively affect the yield. For the plants to maintain higher yield under stress, during which the grain filling period gets drastically reduced, the seeds must have higher cellular activity and they must be able to maintain higher rates of starch accumulation for maximum utilization of the filling period.

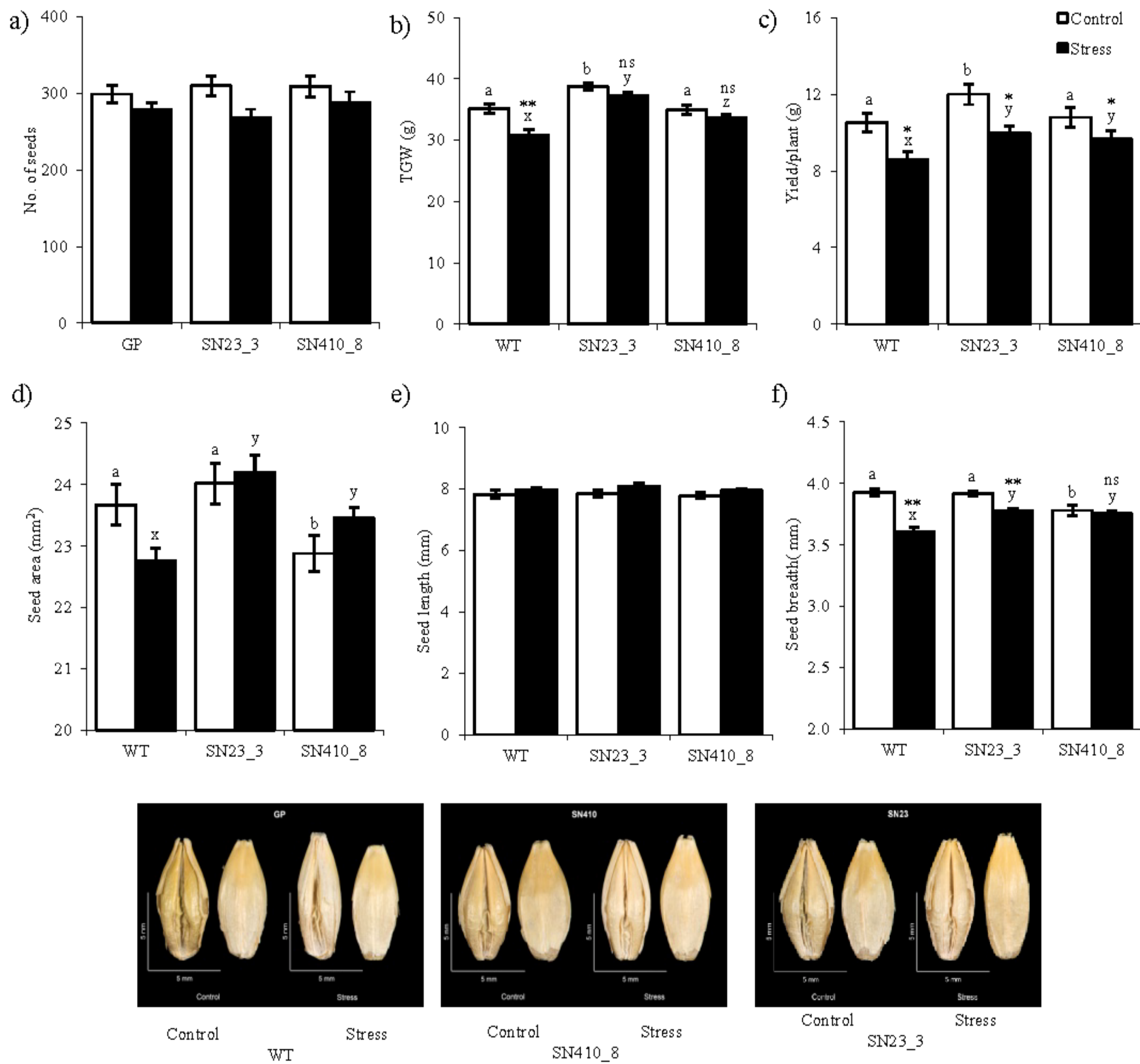


Figure 13: Transgenic lines, SN410_8 and SN23_3, performed better than WT plants by maintaining higher TGW which was an effect of increasing its seed area, mainly seed breadth, and not seed number under stress.
Mean ± SE (n = 16) of a) seed number, b) TGW, c) Yield, d) seed area, e) seed length and f) seed breadth. Lower panel represents the phenotype of WT, SN410 and SN23_3 seeds under control and stress (2 seeds each). In graph, white bars: control and black bars: stress. Letters represent significant differences between genotypes under control (a, b, c) and stress (x, y, z) condition, respectively, at $P < 0.01$. Similar letters indicate no significant differences exists between the genotypes within a given treatment, at $P < 0.01$.

3.4.8 Stress and endoreduplication

Since ABA is shown to affect cellularization during seed filling, it was tested if ABA also affects cellular activity by affecting endoreduplication. Hence, the seeds of WT plants and SN410_8 plants were analyzed for endoreduplication at later stages of seed development that coincides with long term stress (4 and 12 DAS: 8 and 16 DAF). At 4DAS (8 DAF), the developing seeds of WT plants under control had not yet initiated the process of endoreduplication while the endosperm of SN410_8 seeds under control condition had already 3 rounds of duplication (12C) and few cells had entered 4th round of duplication (24C). However, by 12 DAS (16 DAF) both the WT and SN410_8 control plants had seeds that were at similar levels of endoreduplication with the endosperm content ranging from 3C to 12C levels (Figure 14).

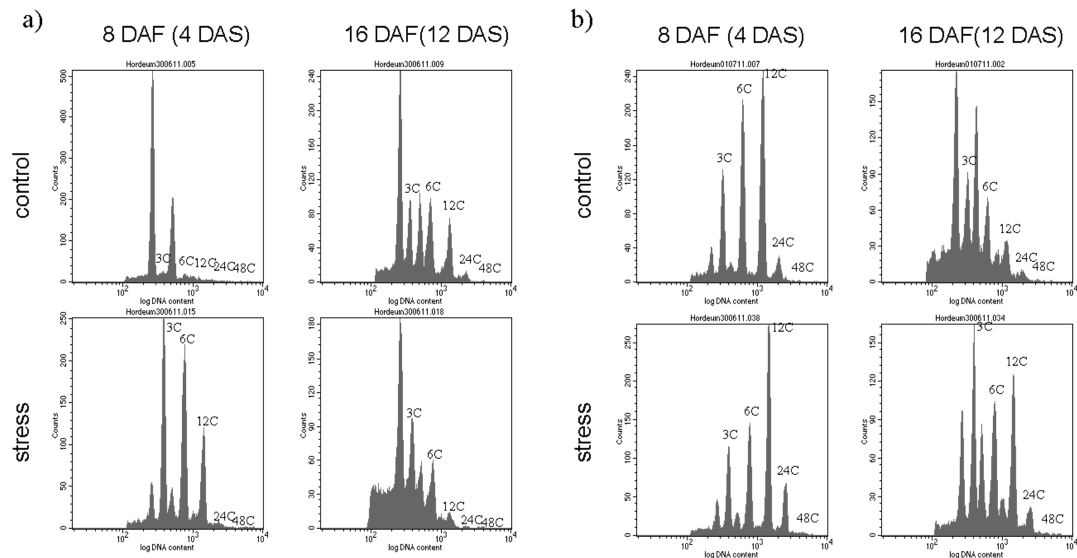


Figure 14: Endoreduplication showed opposite pattern between the WT and SN410 under stress. Logarithmic histogram of the endosperm nuclei of a) WT and b) SN410_8 at 8 and 16 DAF from control and stress plants.

Seeds of WT plants after 4 DAS (8 DAF) had maximum number of cells with normal levels of chromosome content (3C) although few cells had entered endoreduplication. In other words, the content of the cells was in the decreasing order of 3C followed by 6C and 12C, indicating that few cells had undergone endoreduplication. Nevertheless, the seeds of SN410_8 plants at 4 DAS (8 DAF) had exactly opposite pattern of cell content. The number

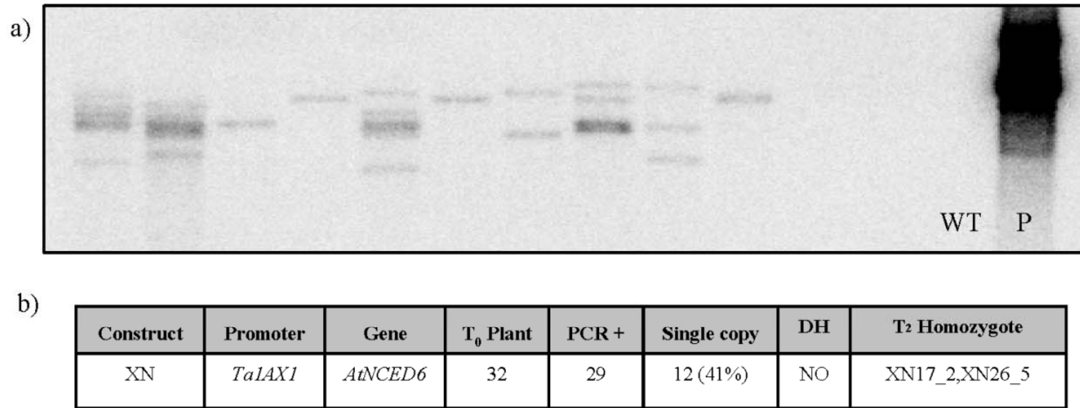
of cells with high levels of endoreduplication increased in an increasing manner. Cells with 3C<6C<12C with maximum number of cells having 12C content and few cells were also at 24C stage. At increased duration of stress, 12 DAS (16 DAF), the seeds of WT and SN410_8 plants differed similarly. However, most cells of SN410_8 seeds were having 3C, but still there were high number of cells with increased DNA content up to 24C compared to respective cells of WT seeds (Figure 14). Usually endoreduplication is closely associated with higher cellular activity and hence it indicates that seeds of SN410_8 are in highly active stage especially under stress. This could be one of the reasons for the maintenance of higher seed yield by SN410_8 plants under stress compared to WT plants under similar stress conditions.

We wanted to understand if differences observed in TGW (between WT and SN transgenic plants) is due to the difference in ABA during the critical grain filling phase of seeds. As these plants differing in their ABA content had differential cellular activity (endoreduplication) which in turn could affect starch accumulation. This could be best studied in developing seeds of SN410_8 plants that maintain ABA homeostasis at distinct time interval of grain filling period compared to ABA homeostasis in seeds of WT plants. We hypothesise that there is a critical phase of grain filling period that is sensitive to alterations in ABA homeostasis and results in reduction of TGW or yield by directly or indirectly affecting starch accumulation. To test this hypothesis transgenic plants that were targeted to alter ABA homeostasis specifically in seeds (XN plants- *Ta1AX1*::*AtNCED6*) were also characterized similar to SN plants.

3.5 Characterization of *Ta1AX1*::*AtNCED6* transgenic plants:

The endosperm specific promoter *1AX1* from wheat was used to drive the expression of *AtNCED6* in order to alter ABA levels in seeds of transgenic plants (XN). The transgenic plants were characterized by southern hybridization to identify the number of transgene copies integrated. Two representative lines, XN17 and XN26_5, both harbouring a single insertion of *AtNCED6* were used for the generation of homozygous T_2 lines (Figure 15). Unlike SN transgenic plants, XN transgenic plants did not display altered phenotype such as enhanced production of secondary spikes or early flowering except for alterations in tiller number. Interestingly, the XN plants behaved similar to SN plants by producing significantly

lower number of tillers than that of WT plants at early stages of plant development; nonetheless, by maturity the number of tillers was significantly higher in XN26_5 plants compared to WT plants (Figure 16).



Construct	Promoter	Gene	T_0 Plant	PCR +	Single copy	DH	T_2 Homozygote
XN	*Ta1AX1*	*AtNCED6*	32	29	12 (41%)	NO	XN17_2,XN26_5

Figure 15: Representative (a) southern blot of the primary transgenic plant, which was carried out to determine the copy number, and (b) summary of transgenic plants produced in barley over expressing *AtNCED6* under the control of wheat *Ta1AX1* promoter. WT: wild type, P: positive control.

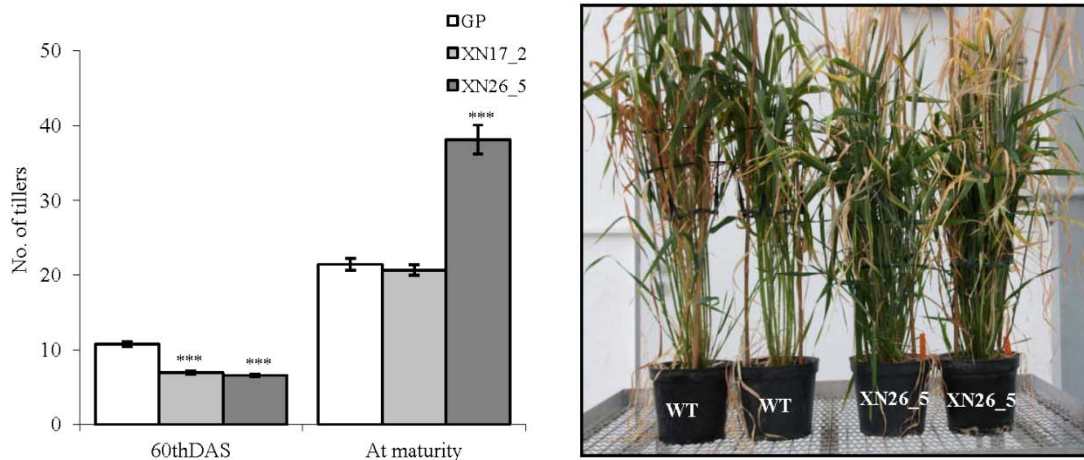


Figure 16: Mean SE number of tillers produced by XN transgenic plants differed significantly from that produced by WT plants under control condition, respectively.
a) tiller number (n = 16) and b) phenotype of WT and XN26 under control. Asterisks represent significant differences in tiller number between the WT and XN transgenic, at P < 0.001:***.

Transgene expression was analyzed in flag leaves and seeds under control and drought stress. Although both XN17 and XN26_5 line had similar pattern of *AtNCED6* transcript accumulation, the latter had high levels of expression in seed as is the expected behaviour of this promoter. In addition, very low (1000 fold low) levels the *AtNCED6* was expressed in the flag leaves (Figure 17a). Although *1AX1* promoter is known to be developmentally regulated, it was found to be significantly induced by stress in seeds (Figure 17b).

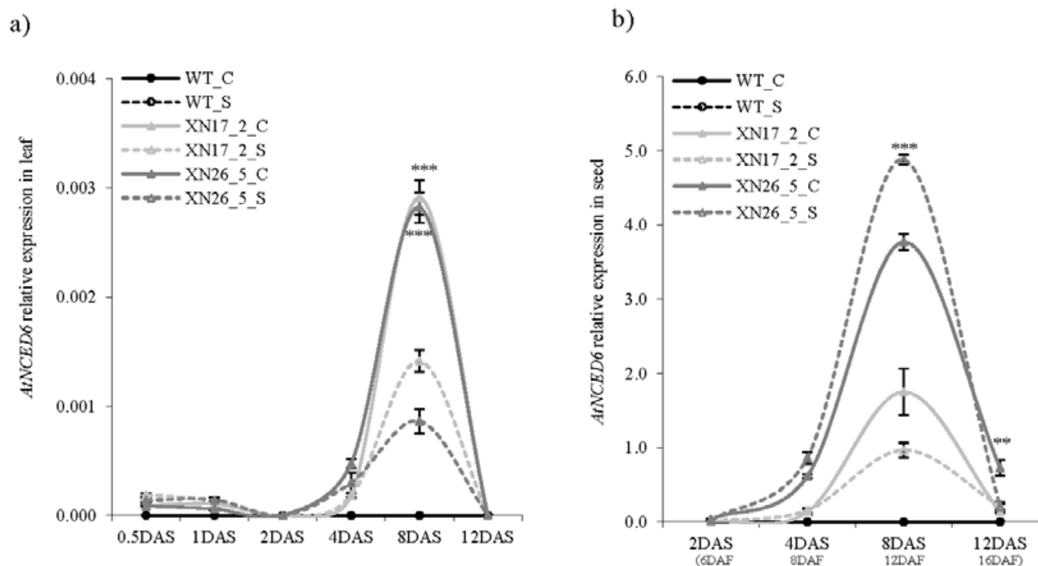


Figure 17: Expression of the transgene *AtNCED6* under the regulation of *Ta1AX1* promoter was found to be highly confined to developing seeds and was responsive to stress.
Expressed are the relative expression level of *AtNCED6* in comparison to housekeeping gene (n = 4). Solid and dashed lines represent control and drought stress, respectively. Asterisks represent significant differences in transcript abundance under stress within the genotype at $P < 0.05$:*, $P < 0.01$:** and $P < 0.001$:***.

3.5.1 ABA content in flag leaf and developing seed of XN transgenic plants:

The flag leaves of XN transgenic plants under control conditions had significantly higher ABA content at 2 DAS compared to WT plants while with increase in duration they had slightly lower ABA content than WT plants. However, under stress conditions, XN17 plants behaved similar to WT plants except at 8 DAS at which it was similar to XN26_5 plants. Leaves of XN26_5 plants had significantly higher ABA content at 2 DAS which significantly decreased compared to WT plant with increase in duration of stress. At later stages, ABA content of XN26_5 flag leaves was similar to that of WT flag leaves (Figure 18a). Under control conditions, seeds of XN transgenic lines maintained significantly lower ABA levels compared to seeds of WT plants at early stages of seed development and the trend continued

along seed development. Similarly under stress, seeds of XN transgenic plants had lower ABA content compared to seeds of WT plants during early stages of seed development. At later stages of development (12 DAS: 16 DAF), seeds of XN26_5 plants accumulated significantly higher levels of ABA compared to WT plants, under stress (Figure 18b). We used XN26_5 for further characterization and for testing the role of altered ABA homeostasis during grain filling period in regulating starch accumulation and yield.

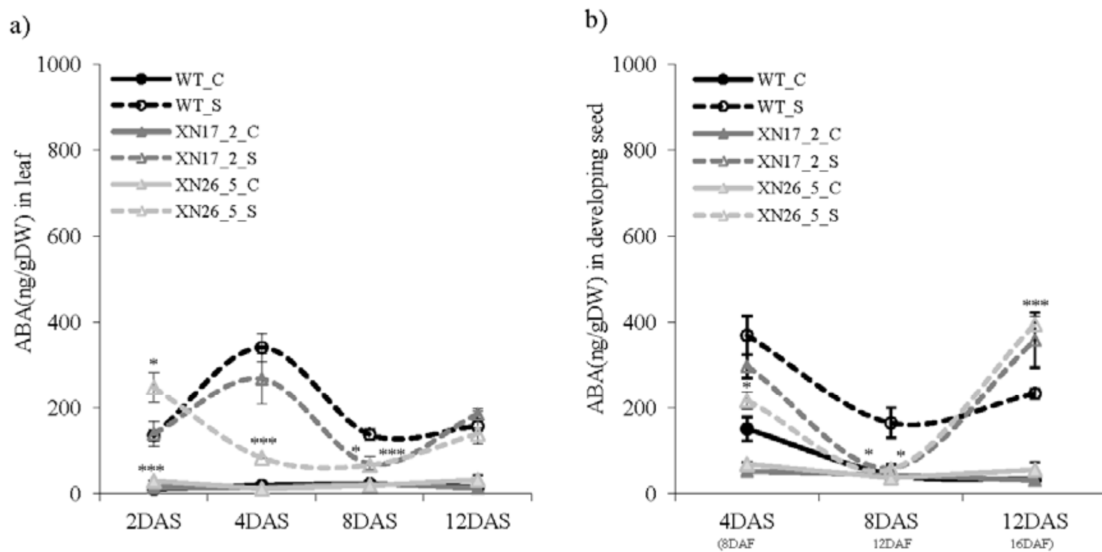


Figure 18: Under long term drought stress XN transgenic plants had (a) similar ABA levels in leaves, but (b) significantly higher ABA levels in seeds compared to wild type leaves and seeds, respectively. Represented is mean ±SE (n = 4) ABA content in wild type, XN17 and XN26 leaves and seeds of plants grown under control (solid lines) and drought stress (dashed lines). Black, dark grey and light grey represent WT, XN17 and XN26, respectively. Asterisks represent significant differences between genotypes within treatment group at $P < 0.05$:*,$P < 0.01$:** and $P < 0.001$:***. DW: dry weight, DAS: days after stress.

3.5.2 Regulation of *HvNCED* and *HvABA8′OH* family of genes in leaf and seeds of XN26_5 transgenic plant:

Compared to WT and SN410_8, the extent of change in ABA content in flag leaves of XN26_5 was lower under stress compared to its level in respective control plants. The high ABA levels in flag leaves of XN26_5 under stress can be correlated to up-regulation of *HvNCED1* and *HvNCED2* at 2 DAS and to *AtNCED6* at 4 DAS. The *HvABA8'OH* transcripts were mostly not affected by stress except for slight down-regulation at few time points (Figure 19a and Supplementary figure 2a). In seeds, the expression of *AtNCED6*

transcript was mostly similar under control and stress condition. However, the seeds of XN26_5 had high levels of ABA under stress (12 DAS: 16 DAF) that could not be explained by expression of transgene or endogenous *HvNCEDs* (Figure 19b and Supplementary figure 2b). Although the absolute levels of ABA in XN26_5 seeds under stress is higher than in seeds of WT plants at 12 DAS (16 DAF), the fold change in ABA under stress compared to their respective controls is comparable between XN26_5 and WT plants (Figure 9b and 19b).

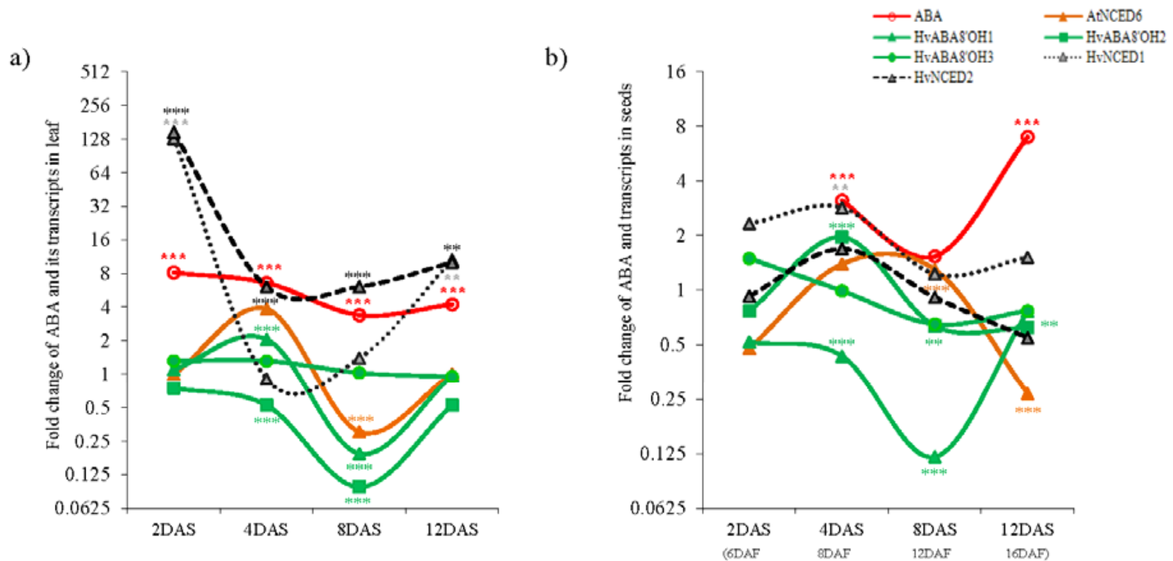


Figure 19: Change in *HvNCED* transcripts correlates with change in ABA content in (a) flag leaves and (b) seeds of XN26_5 plants.
Fold change in ABA biosynthetic (*HvNCED*) and catabolic (*HvABA8'OH*) transcript abundance, and ABA content under stress over control. Y-axis is represented in log scale to the base 2. Asterisks represent significant change in transcript or ABA abundance under stress over control, at $P < 0.01$:** and $P < 0.001$:***.

3.5.3 Physiological performance of XN26_5 transgenic plants:

Unlike SN transgenic plants, XN26_5 plants were not highly stress tolerant compared to WT plants. The percent RWC of XN26_5 leaves was not significantly different from leaves of WT plants under both control and stress. Nevertheless, XN26_5 plants were able to maintain significantly higher stomatal conductance under stress, similar to SN transgenic plants, that resulted in higher assimilation and transpiration rate than WT plants under stress condition(Figure 20a - e). However, the WUE of XN26_5 and WT plants was similar under stress condition.

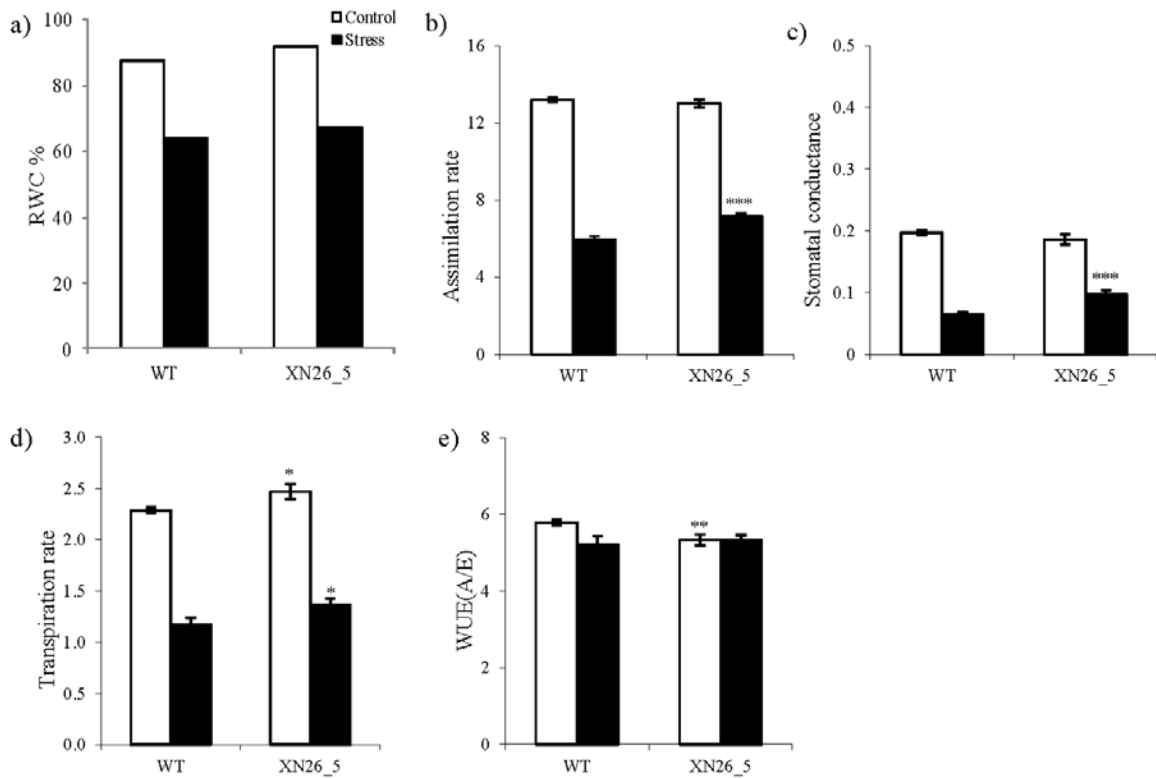


Figure 20: Physiological performance of XN26_5 transgenic plants under post-anthesis drought stress was significantly higher than that of wild type plants under similar stress condition.
Mean ± SE of a) RWC%, b) assimilation rate(µmol m^{-2} s^{-1}), c) stomatal conductance(mol m^{-2} s^{-1}), d) transpiration rate(mol m^{-2} s^{-1}) and e) WUE(water use efficiency). For all the parameters measured n = 15 except for RWC% n = 5; white and black bars represent control and drought stress, respectivley. Asterisks represent significant differences between WT and transgenic plant at a given treatment condition, at $P < 0.05$:*,$P < 0.01$:** and $P < 0.001$:***.

3.5.3 TGW and yield related parameter of XN26_5 transgenic plants:

XN26_5 plant produced significantly more number of seeds per plant compared to WT plants under control growth conditions. Contrastingly under stress, the number of seeds produced by XN26_5 plants was significantly less than that produced by WT plants (Figure 21a). The differences observed for seed number was translated to differences in yield. However, TGW of XN26_5 was not significantly different from that of WT plants under both control and stress conditions (Figure 21b and 21c). Although the length and breadth of XN26_5 seeds was significantly different from that of WT seeds under control conditions, there was no significant difference in their seed area. However, stress drastically affected the seed size of XN26_5 plants compared to its respective control (Figure 21d - f, and lower panel). The

transgenic plants (SN410_8 and XN26_5) that differed from WT and among themselves with respect to ABA homeostasis in seeds were also found to differ in their TGW and yield. These three plants (SN410_8, XN26_5 and WT plants) were analyzed for starch content and regulation of starch metabolism genes.

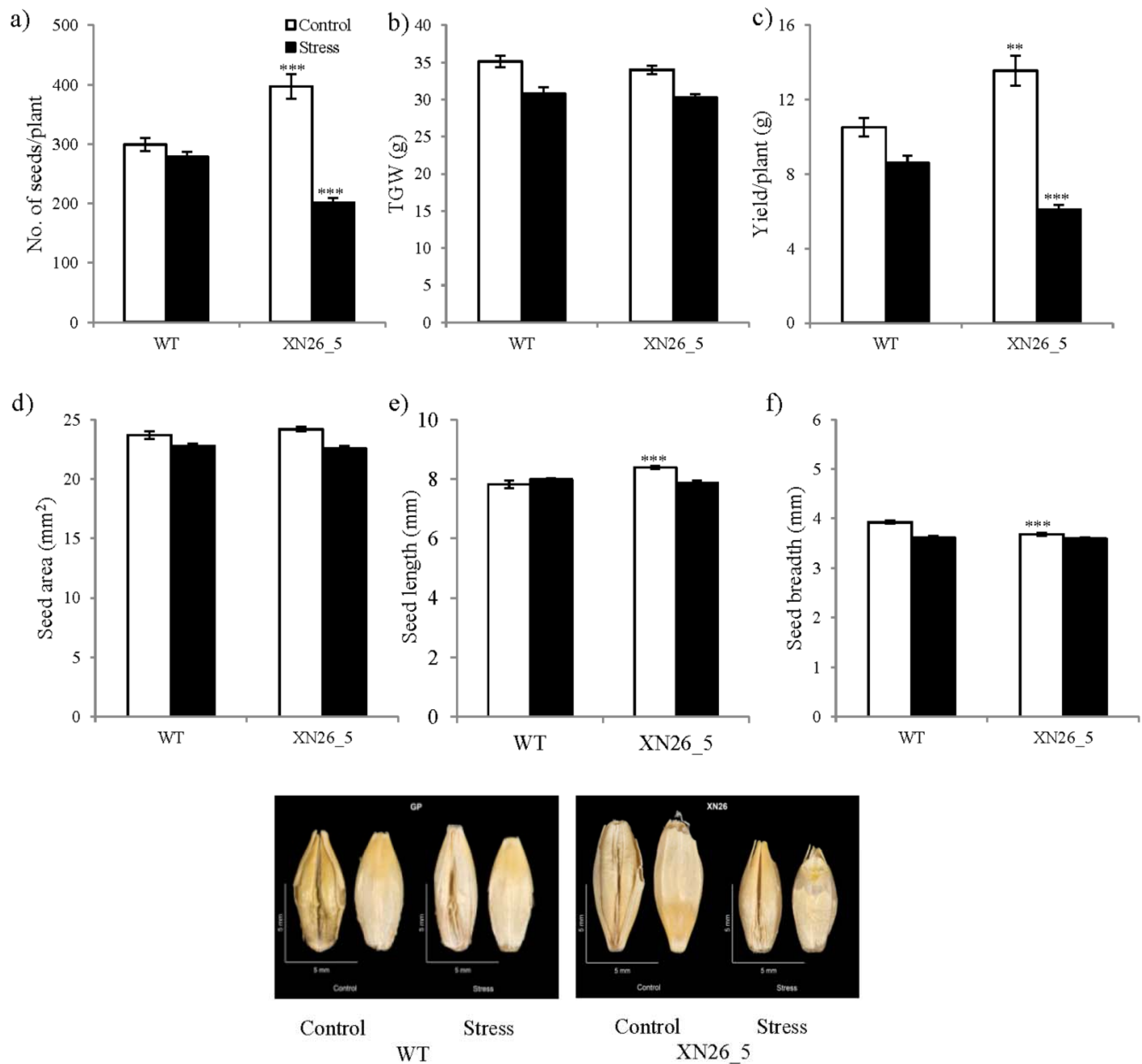


Figure 21: XN26_5 plants produced significantly less seeds and yield per plant under stress compared to WT plants under similar stress condition.
Mean ± SE (n=16) of a) seed number, b) TGW, c) Yield, d) seed area, e) seed length and f) seed breadth. Lower panel represents the phenotype of WT, and XN26 seeds under control and stress (2 seeds each) . white and black bars represent control and drought stress, respectively. Asterisks represent significant differences between WT and transgenic plant at a given treatment condition, at P<0.05:*,P<0.01:** and P<0.001:***.

3.6 Exploring the role of altered ABA homeostasis, at different time points of grain filling period, on seed starch accumulation:

Starch content which is a major contributor for seed weight was estimated in WT, SN410_8 and XN26_5 plants under control and stress conditions, respectively. The genes coding for enzymes involved in regulating starch biosynthesis and degradation was also estimated to study if ABA affects the rate of grain filling.

3.6.1 Estimation of starch content and starch accumulation rate in seeds:

Under control conditions, seeds of XN26_5 plants had significantly higher starch content at 8 DAF and significantly lower starch content at 12 DAF compared to WT plants at respective developmental stage. Starch content of SN410_8 seeds under control conditions did not differ significantly from that of either WT or XN26_5 seeds at 8 and 12 DAF. At 16 DAF, SN410 seeds had significantly lower starch compared to WT and XN26_5 seeds (Figure 22a). At maturity, starch content was comparable between the three lines under control condition (Figure 22c). However, under stress, SN410_8, XN26_5 and WT plants had similar starch content, at all the stages measured. Nevertheless, matured seeds of SN410_8, XN26_5 and WT plants under stress significantly differed in their starch content. The seeds of SN410_8 plants were found to maintain significantly higher starch content than that of WT and XN26_5 plants under stress, respectively (Figure 22c).

Though under stress the lines did not differ in their starch content, the rate of starch accumulation differed between these plants. All the plants showed a logarithmic trend of accumulation. The WT plants that showed a rate of 412 mg starch/4 days under control condition was found to have decreased its rate of accumulation to around 279 mg starch/4 days under stress. Although SN410_8 plants had slightly lower rate of starch accumulation (348 mg starch/4 days) under control, the rate of starch accumulation was only slightly decreased under stress (301 mg starch/4 days). The rate of starch accumulation of XN26_5 plants was similar to that of SN410_8 plants under control (375 mg starch/4 days) and stress (350 mg starch/4 days). The rate of starch accumulation was severely affected in WT plants under stress compared to control condition during early grain filling phase while the effect was less pronounced in transgenic lines, SN410_8 and XN26_5, respectively (Figure 22a and 22b). However, the low levels of starch in XN26_5 at maturity could be due to the effect of

high ABA levels during later stages of grain filling (12 DAS: 16 DAF) that was much higher than in SN410_8 plants during similar grain filling period; this could only be tested if the samples are collected and analyzed for rate of starch accumulation at later stages (12 DAS: 16 DAF onwards).

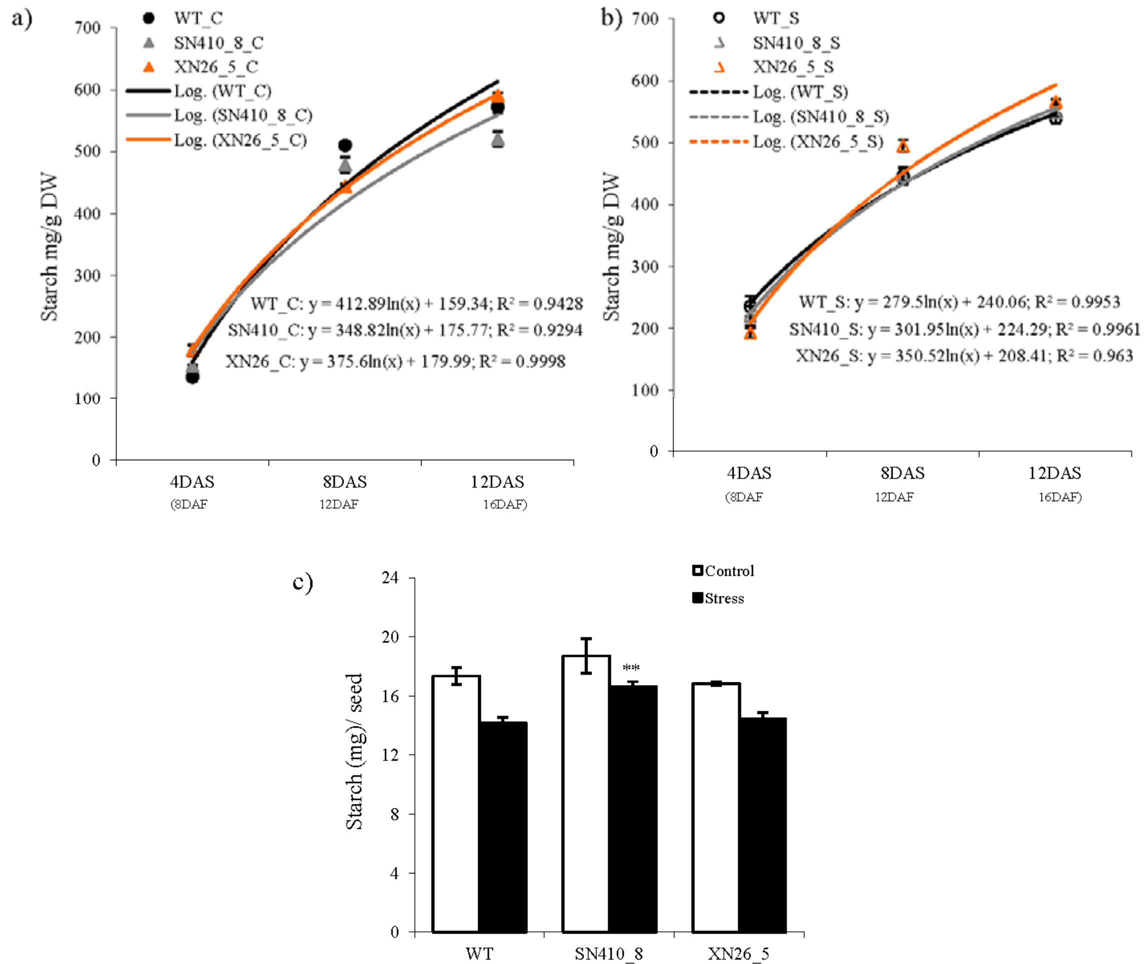


Figure 22: The mean ± SE starch accumulation rate (a and b under control and stress, respectively) and starch content per seed (c) was maintained higher in SN410_8 plants compared to XN26_5 and WT plants while these parameters were comparable between XN26_5 and WT plants, under drought stress. 4, 8 and 12 DAS corresponds to 8, 12 and 16 DAF.
N = 4; black circle: WT, grey triangle: SN410_8 and orange triangle: XN26_5, and filled: control and unfilled: stress. White and black bars represent control and stress, respectively, in the bar graph. Asterisks represent significant differences between WT and transgenic plant in a given treatment, at $P < 0.05$:*, $P < 0.01$:** and $P < 0.001$:***. DW: dry weight, DAS: days after stress, DAF: days after flowering.

In addition, the results would have been clear if the starch content was measured per seed rather than per dry or fresh weight of seed. Since major share of seed weight is made up by starch when starch is compared per weight basis we would be masking the differences per

seed. Apart from this, we also found significant negative correlation between ABA and starch under both control and stress conditions. However, this kind of correlation was only observed for WT and SN410_8 plants while there was no significant correlation between ABA and starch in XN26_5 under both control and drought stress. This could be due to the fact that increase in ABA in XN26_5 seems to start from 12 DAS: 16 DAF onwards. Until 12 DAS: 16 DAF, we observed no effect of ABA on starch in XN26_5 plants, but ABA content at later stages might affect starch accumulation as mentioned earlier (Supplementary table 2).

3.7 Expression of starch metabolism genes under control and stress in the developing seed under post anthesis drought:

3.7.1 Starch biosynthesis

The first step of starch biosynthesis in seeds is the conversion of sucrose to fructose and UDP-glucose, catalysed by sucrose synthase enzyme (*HvSUS*) in barley. Stress had significant effect in altering *HvSUS* expression (2 DAS: 6 DAF) only in SN410_8 plants compared to its respective control plants. The lines significantly differed in *HvSUS* expression under both control and drought stress. Under stress (12 DAS: 16 DAF), expression of *HvSUS* in WT plants was significantly higher than SN and XN transgenic lines (Figure 23a and b).

ADP-glu-pyrophosphorylase (heterotetramer- 2 large and 2 small subunits) catalyzes the production of ADP-glucose. The small subunit (AGP-S) and large subunit (AGP-L) in barley are encoded by 2 genes each (*HvAGP*-S1a and *HvAGP*-S1b -splice product of same gene, and *HvAGP*-S2; *HvAGP*-L1 and *HvAGP*-L2). In seeds, expression of *HvAGP*-L1 is higher than *HvAGP*-L2. Stress significantly enhanced the expression of *HvAGP*-L in all the lines compared to their respective control plants. Under control conditions, the expression of *HvAGP*-L1 was significantly higher in XN26_5 (6 DAF) compared to WT and SN410_8, and *HvAGP*-L2 was significantly higher in both SN410_8 and XN26_5 compared to WT (at 8 DAF). Under stress, only expression of *HvAGP*-L1 was significantly higher in SN410_8 and XN26_5 compared to WT plants (at 4 DAS: 8 DAF), respectively. Of the small subunit of AGP, expression of only *HvAGP*-S2 significantly reduced under stress in only SN410_8 plants (12 DAS: 16 DAF) (Figure 23a and b).

The ADP-glucose formed is either converted to amylase by granular bound starch synthase (GBSS) or to amylopectin by action of starch synthase (SS), starch branching (SBE) and starch de-branching enzyme isoamylase (ISA).

GBSS in barley is encoded by 2 genes (*HvGBSS1a* and *HvGBSS1b*), of which *HvGBSS1a* (abundantly expressed) was significantly up-regulated and *HvGBSS1b* (low expressed) was significantly down-regulated under stress in WT, SN and XN lines compared to their respective control plants. Among the three lines, expression of *HvGBSS1a* was lowest in WT plants (8 DAF) under control conditions while it was highest in XN26_5 plants (2 DAS: 6 DAF) under stress (Figure 23a and b).

Starch synthase (SS) is coded by maximum number of genes (6 genes- *HvSSI*, *HvSSIIa*, *HvSSIIb*, *HvSSIIIa*, *HvSSIIIb* and *HvSSIV*). Among these genes *HvSSI*, *HvSSIIa* and *HvSSIIIa* are highly expressed (nearly 20 fold) compared to *HvSSIIb*, *HvSSIIIb* and *HvSSIV*. Stress significantly regulated the expression of *HvSS* (at least one member) in all the three lines compared to their respective control plants. Under stress among the three lines, of the highly regulated *HvSS* genes, *HvSSI* expression was significantly higher in SN410_8 (4 DAS: 8 DAF), *HvSSIIa* expression was significantly lower in SN410_8 (4 DAS: 8 DAF), *HvSSIIIa* expression (at 12 DAS: 16 DAF) was significantly lower in XN26_5. The SBE in barley is encoded by 3 genes (*HvSBE1*, *HvSBE2a* and *HvSBE2b*), of which *HvSBE2b* is least abundant (aprrox. 20 folds lower than other two) (Figure 23a and b).

Stress significantly affected the expression of *HvSBE* genes (at least one member) in WT, SN and XN transgenic plants compared to their respective control plants. Under control condition, among the three lines, expression of *HvSBE* (except for *HvSBE2a* at 8 DAF and 16 DAF) was mostly lower in SN410_8 plants. Under stress (4 DAS: 8 DAF), expression of *HvSBE2a* and *HvSBE2b* was lowest in WT plants followed by SN410_8 and or XN26_5, respectively (Figure 23a and b). Starch de-branching enzymes under stress was significantly down- (*HvISA1* in SN410_8 and XN26_5; *HvISA3* in SN410_8) or up-regulated (*HvISA2* in XN26_5; *HvISA3* in WT and XN26_5) in all the lines compared to their respective control grown plants. Under control condition, only expression of *HvISA2* was similar between the three lines. Under stress, the lines significantly differed in the expression of *HvISA3*, highest expression in WT plants at 2 DAS: 6 DAF (Figure 23a and b).

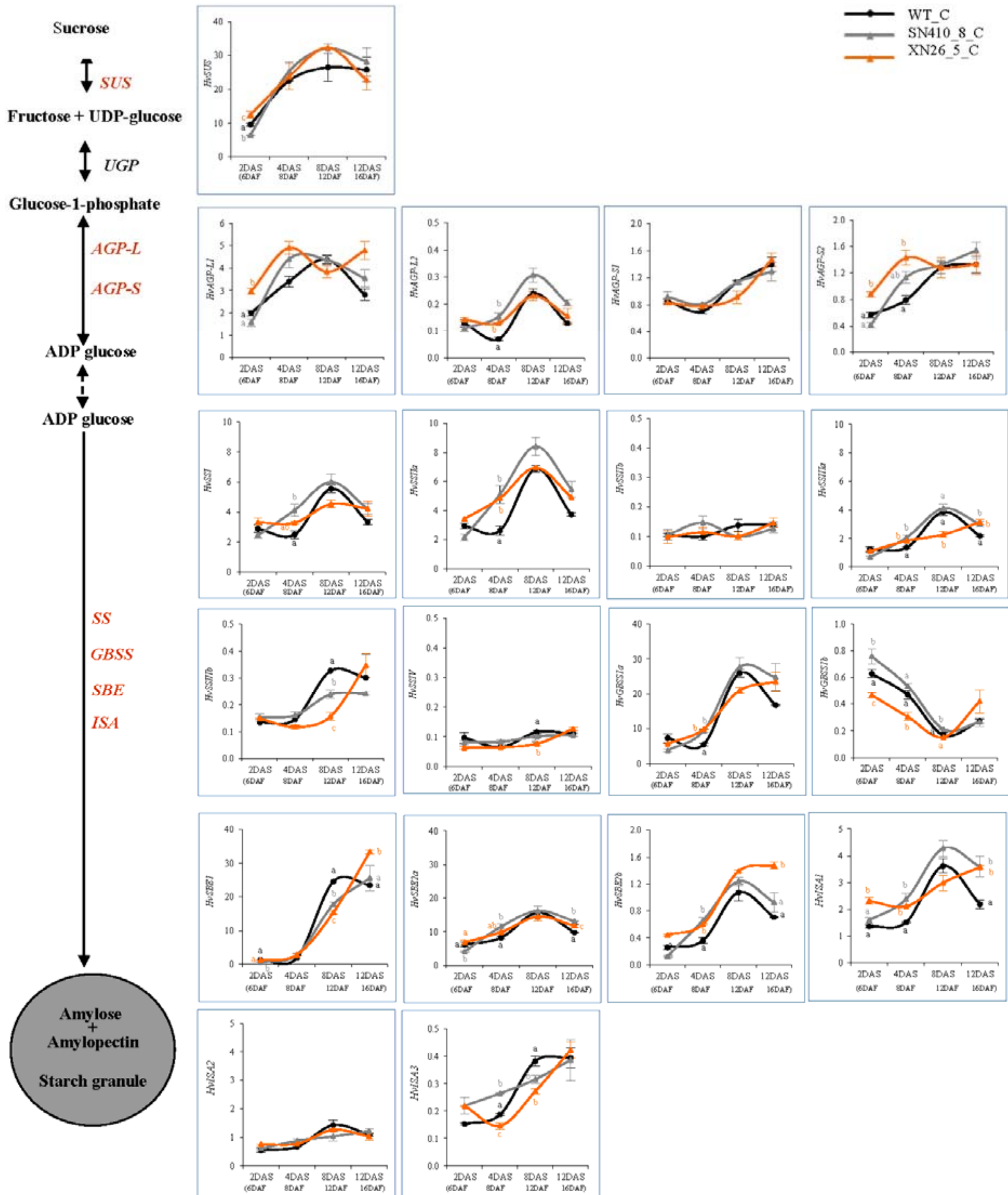


Figure 23a: Differential expression of starch biosynthesis genes across seed development (6 DAF to 16 DAF that corresponds to 2 DAS to 12 DAS) of WT, SN410_8 and XN26_5, under control condition. Expressed are the relative expression level of starch biosynthesis genes in comparison to housekeeping gene (n=4). Black, grey and orange lines indicate WT, SN410_8 and XN26_5. Letters represent significant difference between genotypes under control (a, b, c) condition, at $P < 0.01$; and, similar letters indicate no significant differences exists between the genotypes. DAS: days after stress, DAF: days after flowering.

Figure 23b: Differential expression of starch biosynthesis genes across seed development (6 DAF to 16 DAF that corresponds to 2 DAS to 12 DAS) of WT, SN410_8 and XN26_5, under stress condition. Expressed are the relative expression level of starch biosynthesis genes in comparison to housekeeping gene (n=4). Black, grey and orange lines indicate WT, SN410_8 and XN26_5. Letters represent significant difference between genotypes under stress (a, b, c) condition, at P<0.01; and, similar letters indicate no significant differences exists between the genotypes. DAF: days after flowering, DAS: days after stress.

3.7.2 Starch degradation:

Two major starch degradation encoding genes, alpha and beta amylase, were studied. In barley, alpha amylase is encoded by 4 genes (*HvAMY1-HvAMY4*) and beta amylase is encoded by 7 genes (*HvBAM1-HvBAM7*) (Figure 24a and b).

Expression of at least one member of alpha and beta amylase was significantly affected under stress in all three lines compared to its expression in respective control plants. Under control condition, the lines significantly differed in expression on *HvAMY* genes except for *HvAMY2*. Under stress, the lines significantly differed only in the expression of *HvAMY3* (Figure 24a and b). Among the beta amylase encoding genes, *HvBAM3-HvBAM7* are low expressed while *HvBAM2* (10 folds) and *HvBAM1* (100 folds) are abundantly expressed. Stress compared to control condition significantly increased or decreased the expression of *HvBAM1* and *HvBAM2* (abundantly expressed beta amylase) expression in transgenic plants and WT plants, respectively. Under control conditions, the lines significantly differed in the expression of both *HvBAM1* and *HvBAM2*. Under stress, the lines only differed in the expression of *HvBAM2*. The low expressed *HvBAMs* were mostly down-regulated in WT while they were up-regulated in SN410_8 and XN26_5 under stress compared to their control plants, respectively. The lines significant differed in the expression of low abundant *HvBAMs* under control condition. Under stress, expression of *HvBAM3* and *HvBAM5* was similar between the lines (Figure 24a and b).

Overall, the lines (WT, SN410_8 and XN26_5) differed in the rate of starch accumulation (till 12 DAS: 16 DAF), but they did not differ in their starch content at these time points. However, at maturity the lines significantly differed in their starch content. Probably, differences in starch content starts at later time points (after 16 DAF) hence we were unable to observe differences in starch content at early time points. Starch content measured on per dry weight basis and not per seed basis probably masks the small differences that probably persist. Hence, we grew the plants again under similar drought stress conditions and collected samples at 12, 16 and 20 DAS that correspond to 8, 20 and 24 DAF, respectively. Samples were analysed for starch content and transcripts coding for enzymes involved in starch biosynthesis. Only the transcripts of starch biosynthesis genes that were found to be differentially regulated were analyzed viz., *HvAGP*-L1, *HvSSIIa*, *HvSBE2a*, *HvSBE2b*,

HvAMY1, *HvAMY3*, *HvBAM1*, *HvBAM2* and *HvBAM3*. In addition, transcripts of *AtNCED6*, *Hv-NCEDs* and *-ABA8'OH* were analyzed along with ABA content to study the role of ABA in starch metabolism. The results are present in separate section as the material analysed is of plants grown in green house at different time point.

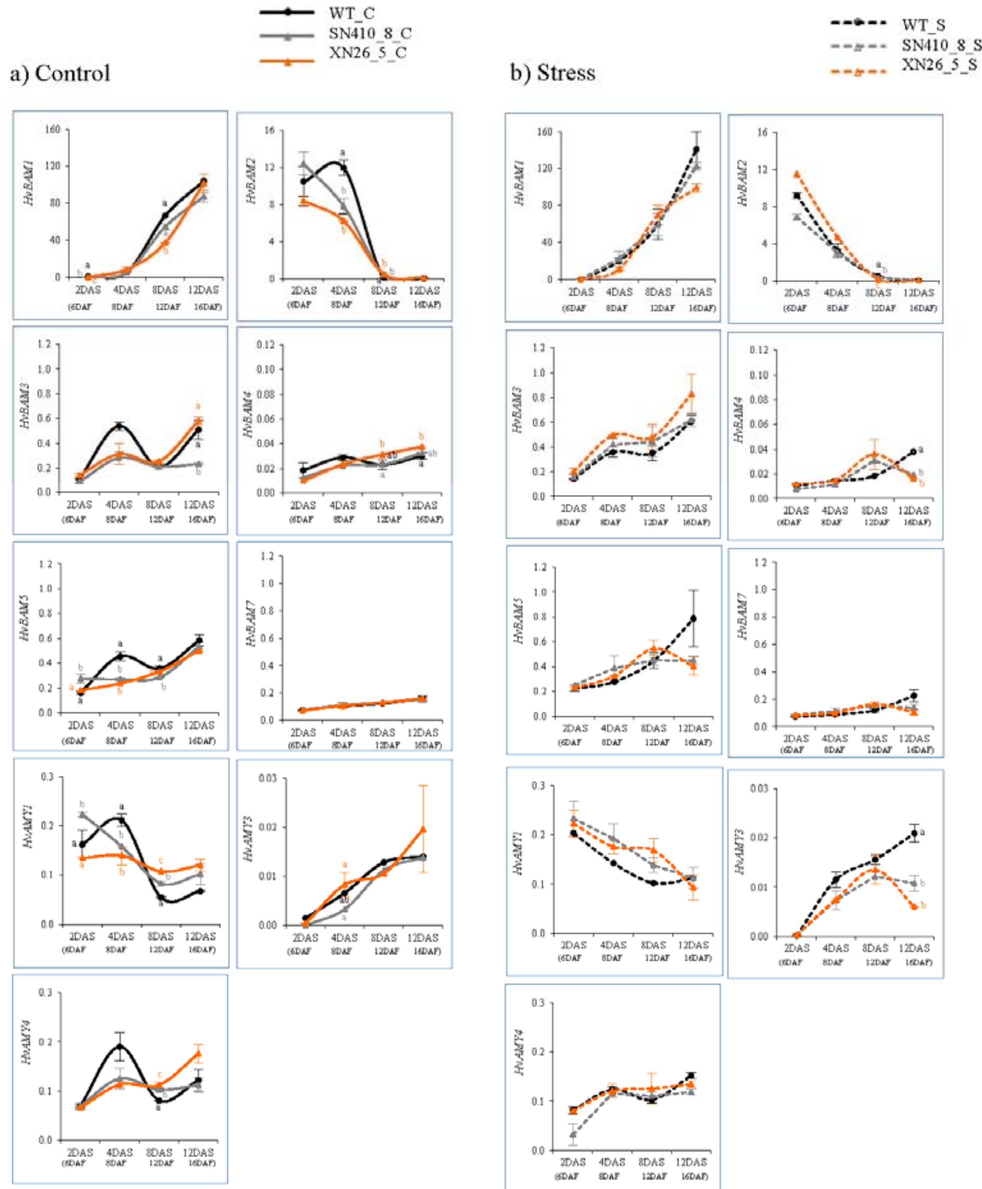


Figure 24: Differential expression of starch degradation genes across seed development (6 DAF to 16 DAF that corresponds to 2 DAS to 12 DAS) of WT, SN410_8 and XN26_5 under (a) control and (b) post-anthesis drought stress.

Expressed are the relative expression level of starch degradation genes in comparison to housekeeping gene (n = 4). Black, grey and orange lines indicate WT, SN410_8 and XN26_5; solid and dashed lines represent control and stress, respectively. Letters represent significant difference between genotypes under control (a, b, c) and stress (x, y, z) condition, at $P < 0.01$; and, similar letters indicate no significant differences exists between the genotypes. DAS: days after stress, DAF: days after flowering.

3.8 Performance of green house grown WT and transgenic plants (SN410_8 and XN26_5) under later stages of drought stress (12- 20 DAS):

3.8.1 TGW

Similar to previous season grown plants, the TGW of SN410_8 and WT was not significantly different from each other under control conditions; with exception of XN26_5 that had significantly lower TGW than that of WT and SN410_8 under similar control conditions. Under drought stress, TGW of SN410_8 was significantly higher than WT and XN26_5 plants; WT and XN26_5 had similar TGW, these results are similar to that obtained in previous season. However, there were certain differences compared to previous growth season, XN26_5 did not display any reduction in TGW under stress compared to control, but maintained low TGW than better performing transgenic line (SN410_8) under both control and stress. Also, stress reduced the TGW of WT plants under stress in both growing seasons (Figure 25a).

3.8.2 Starch and moisture content:

Under control conditions, SN410_8 and WT plants had similar starch content per seed while starch content of XN26_5 seeds was significantly lower than both WT and SN410_8 at 24 DAF. Similarly under stress, the genotypes did not significantly differ from each other for amount of starch per seed at 16 DAS (20 DAF). Nevertheless, at remaining two time points (12 and 20 DAS: 16 and 24 DAF) starch content of WT seeds was intermediate to that of transgenic lines. Starch content of SN410_8 seeds was significantly higher than that of XN26_5 seeds, under stress (Figure 25b). At all time points measured, starch content of XN26_5 seeds was similar between control and stress. Stress significantly reduced the amount of starch per seed of WT (at all time points) and SN410_8 (only at 20 DAS: 24 DAF) compared to their content in seeds of respective control plants (Figure 25b).

The difference in starch content across genotypes is also translated to differences in seed dry weight. Under control conditions, among the three lines, significantly least dry weight was observed for WT seeds at 12 DAS (16 DAF) and XN26_5 seeds at 20 DAS (24 DAF). Nevertheless under stress among the lines, SN410_8 seeds had significantly higher dry weight, at 20 DAS: 24 DAF (Figure 25b and c). Similarly under stress, the genotypes significantly differed for seed moisture content only at later stages (20 DAS: 24DAF) and

SN410_8 seeds maintained significantly higher seed moisture compared to WT and XN26_5 seeds. Moreover under control conditions, the moisture content of WT and SN410_8 seeds was similar, and higher than XN26_5 seeds, at all time points respectively (Figure 26b). For all genotypes, seed moisture content and dry weight under stress was significantly lower than that under control condition, at later stages of drought stress (Figure 25c and inset).

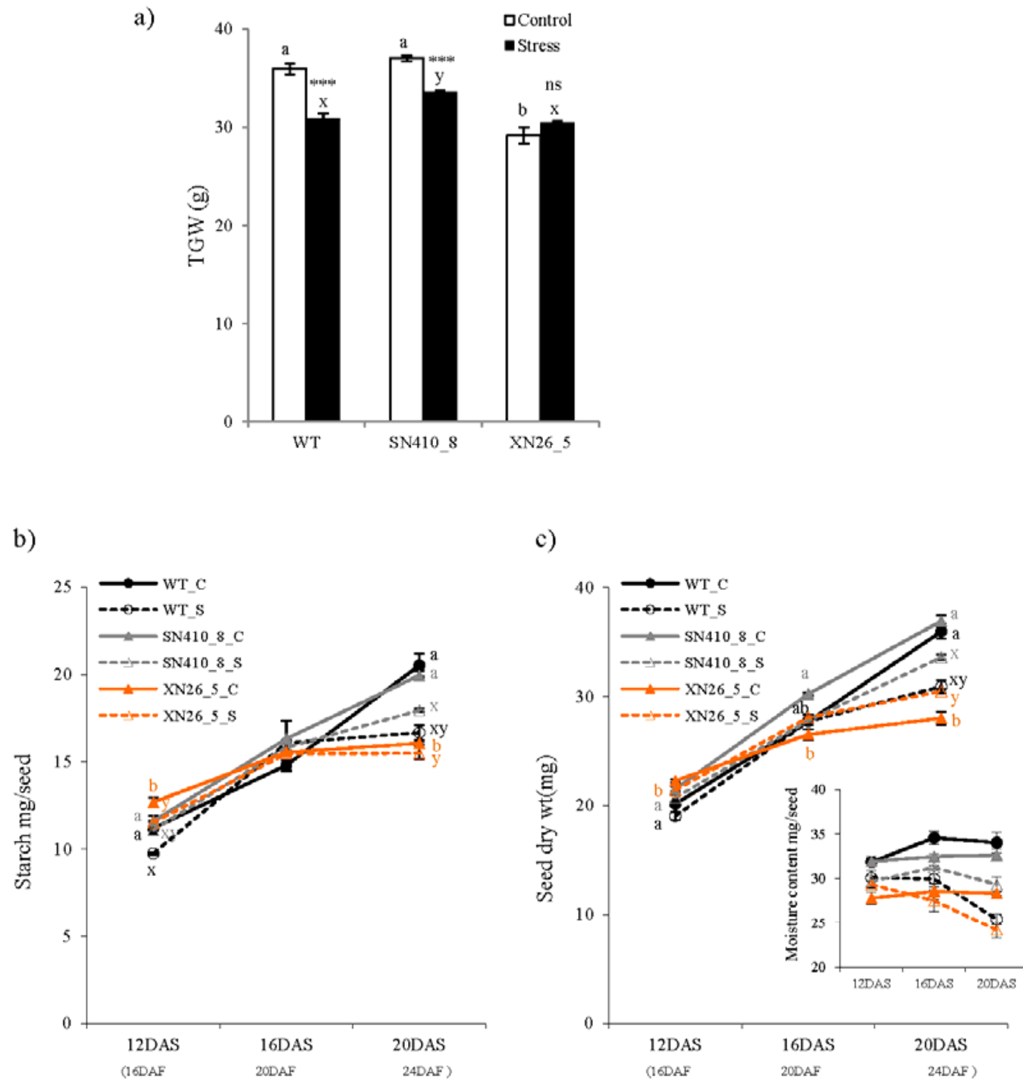


Figure 25: Stress significantly reduced the TGW in WT and SN410_8 compared to respective control plants; and under stress SN410_8 maintained significantly higher TGW than both WT and XN26_5, which was also observed for starch content and seed dry weight.
Represented are mean ± SE (n = 6) a) TGW (at maturity), b) mg starch per seed and c) seed dry weight with inset of moisture content per seed at later stages of seed development (12 DAS to 20 DAS that corresponds to 16 DAF to 24 DAF). White and black bars represent control and stress, respectively, in the bar graph. Black, grey and orange lines indicate WT, SN410_8 and XN26_5; solid and dashed lines represent control and stress, respectively. Letters represent significant differences between genotypes under control (a, b, c) and stress (x, y, z) conditions, at $P < 0.01$; and, similar letters indicate no significant differences exists between the genotypes.

3.8.3 Regulation of starch metabolism genes:

Starch metabolism genes that were found to be stress responsive in previous season study were analyzed in WT, SN410_8 and XN26_5 seeds under long term stress. In all the three lines, expression of starch metabolism genes decreased drastically from 16 DAF (12 DAS) to 24 DAF (20 DAS) except for *HvBAM3* which followed opposite trend. Indicating, regulation of starch metabolism genes almost ceased as the starch accumulation is at its end. However, under control condition with increase in seed development, decline in expression of *HvAGP*-L1 and *HvSSIIa* was not strong in XN26_5 seeds. Under control condition, expression of *HvSSIIa* (at 16 and 20 DAF: 12 and 16 DAS) and *HvSBE2a* (at 20 DAF: 16 DAS) was significantly lower in XN26_5 seeds compared to WT seeds. Contrastingly, under control condition *HvAMY1* expression was significantly higher in XN26_5 seeds (at 16 DAF: 12 DAS) than WT seeds. Decrease in major starch metabolism genes in XN26_5 seeds along with low moisture content would drastically affect starch accumulation (Figure 26).

In seeds of XN26_5, expression of *HvSSIIa* and *HvBAM3* was significantly affected (at 16 and 20 DAF: 12 and 16 DAS) under stress compared to control condition. At 20 DAS (24 DAF), except for *HvAMY3* and *HvBAM2* expression of starch metabolism genes was significantly different in XN26_5 plants under stress compared to its respective control. Under stress XN26_5 in comparison to WT, had significantly low expression of *HvSSIIa* (16 DAS: 20 DAF), *HvAMY1* (20 DAS: 24 DAF), *HvBAM1* (16 and 20 DAS: 20 and 24 DAF), and significantly high expression of *HvBAM2* (20 DAS: 24 DAF) and *HvBAM3* (12 and 20 DAS: 16 and 24 DAF) (Figure 26).

Although under control condition SN410_8 and WT did not significantly differ in their starch content, they differed in expression of starch metabolism genes. Under control condition, SN410_8 seeds had significantly lower expression of *HvSSIIa, HvSBE2b* (at 20 DAF), but significantly higher expression of *HvAMY1* (16 DAF), *HvAMY3* (20 and 24 DAF), and *HvBAM3* (all time points) compared to WT seeds. Under stress, expression of *HvAGP*-L1 (12 DAS: 16 DAF), *HvBAM2* and *HvBAM3* (12 and 20 DAS: 16 and 24 DAF) was significantly higher in SN410_8 seeds compared to WT seeds (Figure 26).

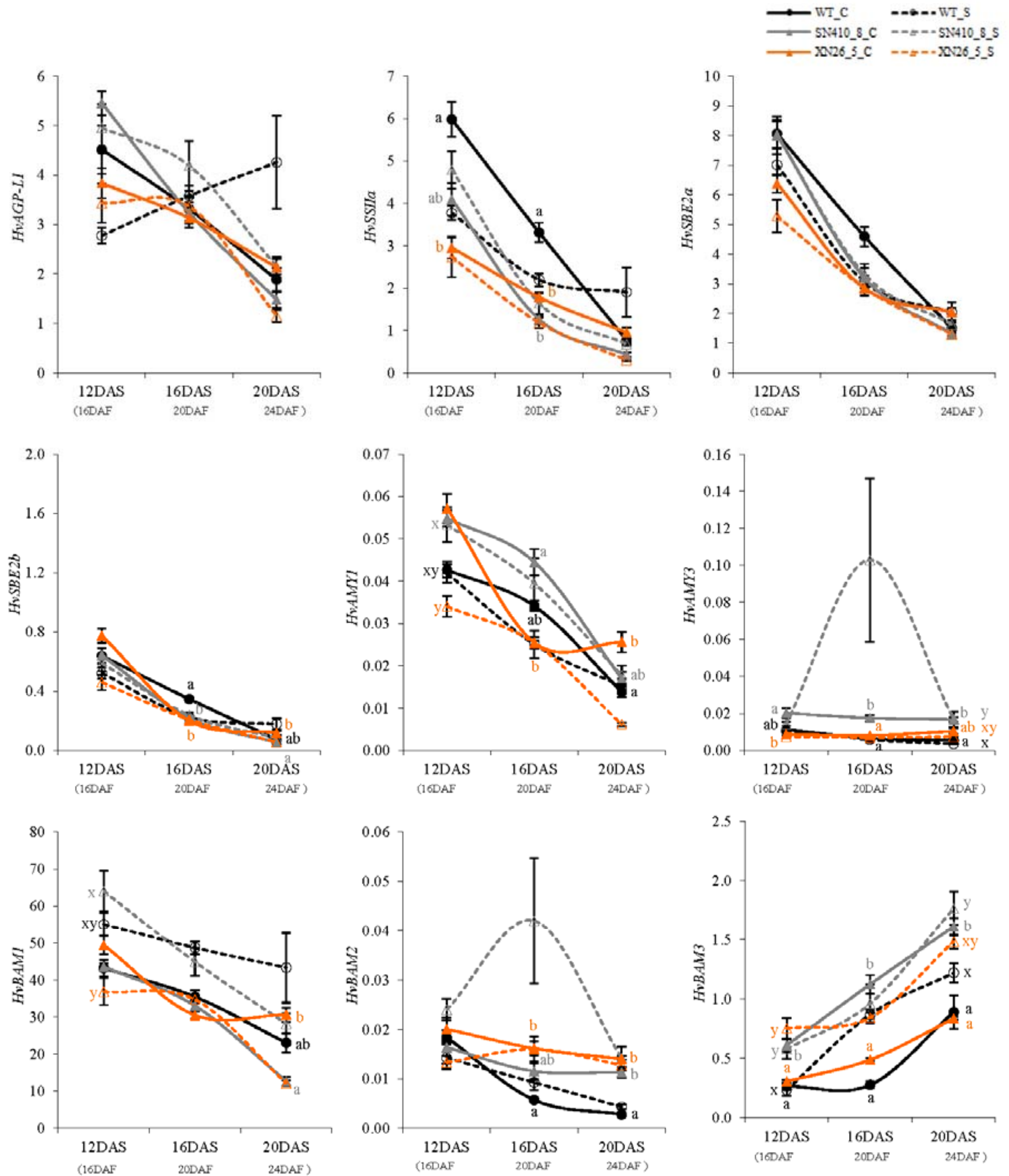


Figure 26: Differential expression of starch metabolism genes in the seeds of WT, SN410_8 and XN26_5 across later stages of seed development (12 DAS to 20 DAS that corresponds to 16 DAF to 24 DAF) under control and stress condition.

Black, grey and orange lines indicate WT, SN410_8 and XN26_5; solid and dashed lines represent control and stress, respectively. Letters represent significant difference between genotypes under control (a, b, c) and stress (x, y, z) condition, at $P < 0.01$; and, similar letters indicate no significant differences exists between the genotypes.

These differences in transcripts along with maintenance of relative high moisture content under stress would have resulted in high starch content of SN410_8 seeds. In SN410_8 seeds, expression of *HvSSIIa* (20 DAS: 24 DAF) and *HvBAM1* (12 and 20 DAS: 16 and 24 DAF) was significantly affected by stress compared to control condition (Figure 26). At these developmental stages, key genes regulating ABA (NCEDs and ABA hydroxylase) and ABA levels was analyzed in flag leaf and seed tissue of WT, SN and XN transgenic plants.

3.8.4 Regulation of *AtNCED6*, *HvNCEDs* and *HvABA8'OHs* genes and ABA content:

Expression of *HvNCED1* and *2* was highly induced in leaves of WT under stress (up to 50 folds) over control (Figure 27a). Under stress, in flag leaf tissues, the relative expression levels of *HvNCED1* and *2* was significantly higher in WT compared to SN410_8 plants (Supplementary figure 4). In leaves of WT plants under stress, expression of *HvABA8'OH1* and *2* was significantly lower under stress while expression of *HvABA8'OH3* was high compared to control plants (Figure 27a). The ABA levels significantly increased in leaves of WT plants (at 16 and 20 DAS: 20 and 24 DAF) under stress compared to control plants (Figure 27a). Under stress, WT seeds had only moderate fold change in expression of *HvNCED1* and *HvNCED2* over control (Figure 27b). The relative expression of *HvNCED1* in WT seeds was similar to that observed in XN26_5, than SN410_8 seeds (Supplementary figure 3). Among the three *HvABA8'OH's*, only *HvABA8'OH2* was highly induced under stress (16 DAS: 20 DAF) in WT seeds (Figure 27b). ABA content in WT seeds was significantly higher under stress (16 DAS: 20 DAF) compared to control. ABA content that significantly increased in leaves of WT plants under later stages of stress did not result in high ABA content in seeds during the stages we measured (till 20 DAS: 24 DAF) (Figure 27a and b).

In SN410_8 and XN26_5, the ABA levels can also be contributed by *AtNCED6* expression. In SN410_8 plants, expression of *AtNCED6* was comparable between leaf and seed tissue under both control and stress condition. In SN410_8 leaves, among the *HvNCEDs*, only *HvNCED2* was highly induced under stress (75 folds) over control; and transcripts of *HvABA8'OH's* were generally lower under stress compared to control except for *HvABA8'OH3*. There was no significant change in ABA content in leaves of SN410_8 plants at all time points measured (Figure 27c and Supplementary figure 2). Under stress, the

relative expression of *HvNCED2* was significantly lower in seeds of SN410_8 than WT and XN26_5. However, the fold change *HvNCED2* transcripts was higher in SN410_8 than other two lines (Figure 27b, d, and f; Supplementary figure 3). Expression of *HvABA8'OH3* was highly induced in SN410_8 seeds compared to that in WT and XN26_5 seeds. ABA levels were significantly higher in SN410_8 seeds during early time points (12 and 16 DAS: 16 and 20 DAF) of stress compared to control.

In XN26_5, expression of *AtNCED6* was higher in seeds than leaves under both control and drought stress (Supplementary figure 3 and 4). Expression of both *HvNCED1* and *2* in XN26_5 leaves was significantly induced to several folds (50-100 folds) under drought (16 and 20 DAS: 20 and 24 DAF) over control. Among the *HvABA8'OH's*, the highly abundant *HvABA8'OH3* followed by *HvABA8'OH1* was significantly induced in leaves of XN26_5 under stress (12 and 20 DAS) over control. ABA content was significantly higher in leaves of XN26_5 under stress (16 and 20 DAS) compared to control (Figure 28e and Supplementary figure 4). Contrasting to leaves, in XN26_5 seeds, expression of *AtNCED6* was significantly lower under stress (at 16 and 20 DAS: 20 and 24 DAF) compared to control. However, similar to leaves, expression of *HvNCED1* and *2* in XN26_5 seeds was highly significantly induced under stress (16 DAS: 20 DAF) over control. Among the three *HvABA8'OH* genes, *HvABA8'OH1* was highly induced under stress in seeds of XN26_5 which had very low expression under control condition. ABA levels were found to be significantly high in XN26_5 seeds under stress (16 and 20 DAS: 20 and 24 DAF) (Figure 27f and Supplementary figure 3).

3.9 Screening of WT and transgenic lines (SN410_8 and XN26_5) in rain shelter:

Stress imposed at 4 DAF (continued till maturity) to plants grown in rain shelter had no significant effect on altering TGW of transgenic (SN and XN) and WT plants compared to their respective control plants (Figure 28). The stress imposition was probably not as severe as observed in green house. However, TGW of XN26_5 was significantly reduced under both control and stress condition compared to WT plants. SN410_8 transgenic plant had significantly higher yield under stress compared to WT and XN26_5 transgenic line.

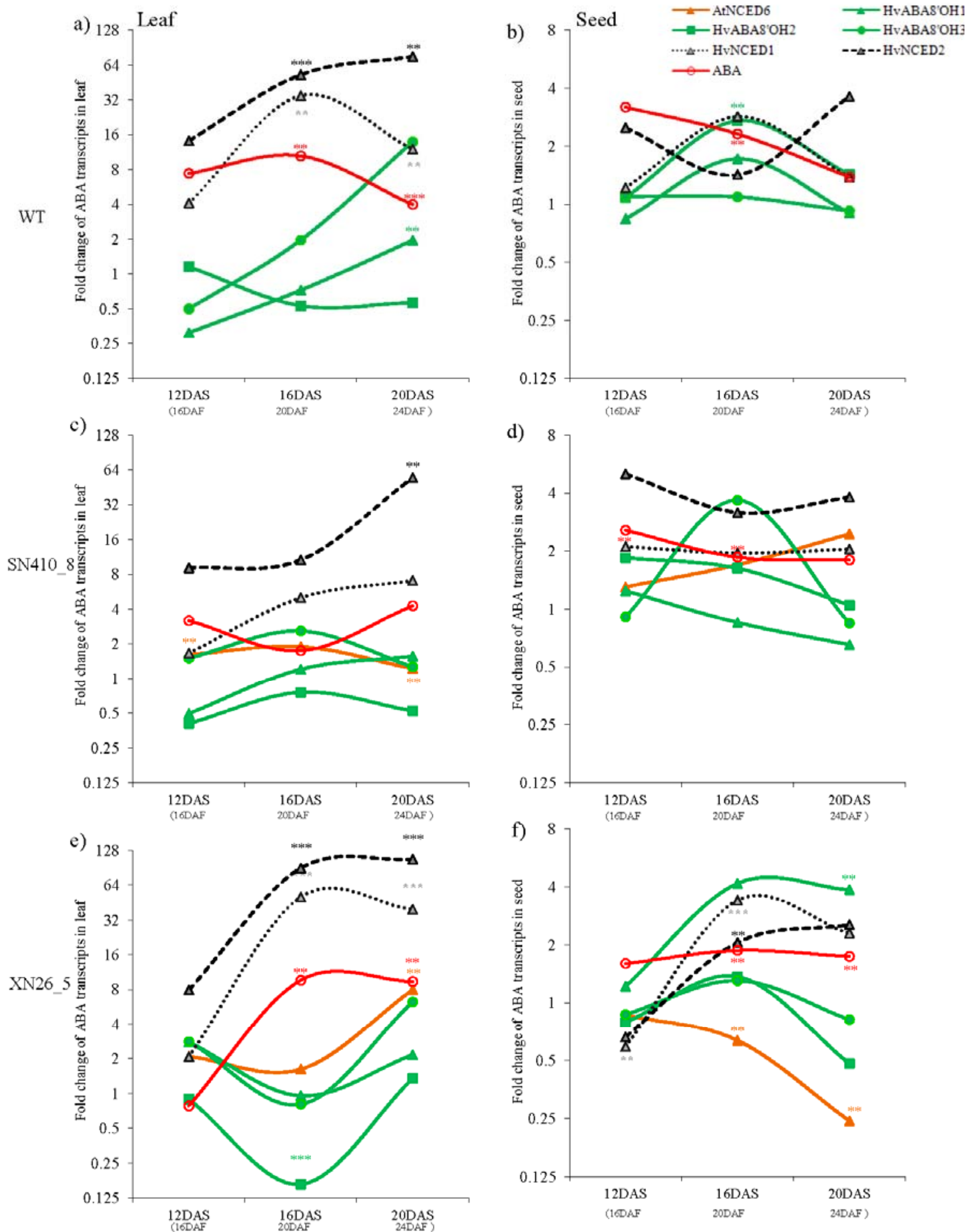


Figure 27: Fold change in ABA biosynthetic (*HvNCEDs* and *AtNCED6*) and catabolic (*HvABA8'OH*) transcripts abundance under stress over control in flag leaf (a, c and e) and developing seeds (b, d and f) in WT (a and b), SN410_8 (c and d) and XN26_5 (e and f).
Y-axis is represented in log scale to the base 2. Asterisks represent significant change in transcript abundance under stress over control, at P < 0.01:** and P < 0.001:***.

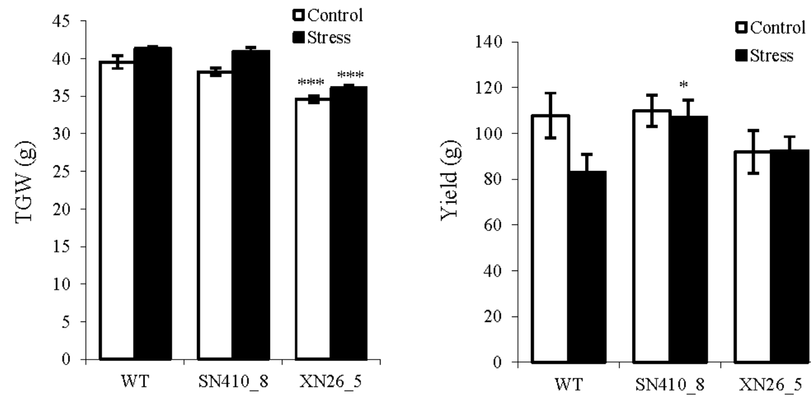


Figure 28: Under glass house in the field conditions, TGW of XN26_5 transgenic plants was significantly lower than that of WT and SN410_8 plants under both control and stress; and SN410_8 plants maintained significantly higher yield under stress compared to WT and XN26_5 plants grown under similar stress condition.

Two-hundred plants (grown in 5 rows each with 40 plants) of WT, SN410_8 and XN26_5 were grown per replication per treatment. Two replications were maintained for each treatment. Stress was imposed at 4DAF and continued till maturity. TGW and yield (measured per row) was recorded at the end of maturity. White and black bars represent control and stress, respectively. Asterisks represent significant difference between WT and transgenic plant in a given treatment, at $P < 0.05$:*, $P < 0.01$:** and $P < 0.001$:***.

4 Discussion

Transgenic crop plants altered in their ABA levels would help shed light on the role of ABA under stress. Crop plants would respond differently to changes in ABA compared to model plant (Arabidopsis) because of differences in growing conditions. In current study, ABA levels in barley plants was altered, in turn ABA homeostasis, by transforming plants with *AtNCED6* gene driven by a stress-inducible *SalT* promoter (SN) or seed specific *1AX1* promoter (XN), to study the role of ABA during post-anthesis drought stress. Physiological, biochemical and molecular analysis of untransformed and transgenic plants under control and post-anthesis drought stress (different durations) revealed the probable mechanism by which ABA affects plant performance and yield under stress. The mechanisms identified were ABA feedback, to maintain its levels, which in leaves affected physiological process and in seeds affected processes such as endoreduplication and starch biosynthesis. Differences in these mechanisms in SN410_8 compared to wild type and XN26_5 was a result for their drought tolerance.

Oxidative cleavage of 9-cis-epoxycarotenoid is considered to be the rate-limiting step and the committed step in ABA biosynthesis, catalyzed by NCED (Schwartz et al., 2003; Taylor et al., 2005). The first *NCED* gene was cloned from maize (Schwartz et al., 1997a and b; Tan et al., 1997), then on ortholog genes have been identified in other plant species and over expressed to study role of ABA in physiological processes and stress tolerance (Aswath et al., 2005; Iuchi et al., 2001; Thompson et al., 2007a; Tung et al., 2008; Qin and Zeevart 2002; Zhang et al., 2008; Zhang et al.,2009; Lefebvre et al., 2006; Hwang et al., 2010; Wan & Li 2006; Thompson et al., 2000; Zhu et al., 2007). Arabidopsis has five genes (*AtNCED2, 3, 5, 6* and *9*) that are classified as *NCED* that are involved in ABA biosynthesis (Schwartz et al., 2003; Lefebvre et al., 2006); and four genes that are classified as carotenoid cleavage dioxigenase (*CCDs*). Rice has six *NCED* genes and one *CCD* gene. Barley till date is found to have two *NCED* genes (*HvNCED1* and *2*) and three putative *CCD* members (*HvCCD3*, *4* and *5*) (Leymarie et al., 2008; Seiler et al., 2011). Most of the genetic engineering studies to increase ABA in plants have been by ectopic expression of *NCED* or *ZEP*. However, only limited studies have been carried out in crop plants (Thompson et al., 2000a, 2000b 2007a and 2007b; Wang et al., 2008; Sun et al., 2012; Melhorn et al., 2008).

4.1 Feed-back and –forward regulation by ABA regulates its homeostasis:

4.1.1 Regulation of ABA biosynthesis:

We observed differences in the regulation pattern of *AtNCED6*, *HvNCED1* and *HvNCED2* between wild type, SN410_8 and XN26_5, in leaves and seed tissue. In flag leaves, expression of *HvNCED1* and *HvNCED2* was highly induced earlier (2 DAS) in SN410_8 and XN26_5 while they were induced during later stages in wild type (4 DAS and 8 DAS) (Figure 9 and 19) when subjected to similar drought stress condition. The high expression of *HvNCEDs* corresponded to elevated ABA peaks in all three lines. One possible reason for this differential response of the plants could be due to the positive feedback by ABA (accumulated due to transgene expression) on its own biosynthesis. In recent years, it has been discovered in Arabidopsis that an initial increase in ABA can positively regulate its own biosynthesis (limited levels) by increasing the expression of key ABA biosynthesis genes like *NCED3* in Landsberg and Colombia ecotypes; however, in C24 accessions they were un-induced by ABA (Xiong et al., 2002a).

Similarly, in cowpea (Iuchi et al., 2000) and tomato (Thompson et al., 2000a, 2000b) *NCED* was not induced by external ABA application. Contrastingly, in Arabidopsis, among ABA biosynthetic genes, *AtNCED3* was found to be predominantly induced by ABA in both wild type and ABA-deficient mutants (Barrero et al., 2006). Similar report of *NCED3* induction by ABA was published by Cheng et al., 2002. In peanut also, external ABA treatment resulted in induction of *AhNCED1* (Wan and Li, 2006). In addition, induction of other ABA biosynthetic genes *AAO3*, *ABA1* and *ABA3* has also been shown to respond positively to ABA (positive feed-back regulation by ABA) (Xiong et al., 2002a; Barrero et al., 2006). However, we need to analyze expression of ABA biosynthetic genes other than NCED to confirm if ABA positively also regulates expression of all ABA biosynthesis pathway genes in barley.

Although regulation of *HvNCED1* and *2* differed between wild type, SN410_8 and XN26_5 seeds, the influence on ABA content was not similar to that observed in leaves. Only in SN410_8 seeds, ABA content could be correlated to expression of internal (*HvNCED1* and *2*) or introduced NCED gene (*AtNCED6*). One of the reasons for high ABA content in seeds of WT and XN26_5 seeds is probably due to import of ABA from maternal tissue and not

due to overexpression of NCEDs. Two peaks of ABA accumulation occur during seed development (Bentsink and Koornneef, 2008; Finkelstein et al., 2002). The first peak is mainly by contribution from maternal tissue which is confirmed by using ABA deficient mutants as female parent in reciprocal crosses (Xiong and Zhu, 2003). The other reason for this differential behaviour could be due to the combined effect of sugars and ABA on the regulation of ABA biosynthesis in endosperm and embryo. It is shown that ABA biosynthetic genes ZEP, SDR1, AAO3 and MCSU are induced by sugars to various levels and that sugar may regulate ABA biosynthesis in seeds (Cheng et al., 2002).

The probable components mediating positive feedback by ABA on its own biosynthesis was studied and one of the component was identified to be SAD1 (supersensitive to ABA and drought 1). SAD1 encodes a Sm-like small ribonucleoprotein that probably regulates turnover of transcripts by its probable involvement in mRNA splicing, export and degradation (Xiong et al., 2001). The *sad1* mutants were impaired in regulation AAO3 and MCSU genes by ABA (Xiong et al., 2001). One of the probable targets of this mechanism that regulates feedback by ABA is ABI1-like protein phosphatase 2C. Similar to *sad1* mutants, the *abi1* mutants were also partially impaired in regulation of ABA biosynthesis genes by ABA (Finkelstein et al., 2002). Since reactive oxygen species (ROS) production was impaired in *abi1* (Murata et al., 2001) and they are involved in ABA signalling (Zhao et al., 2001), it was proposed that positive feedback regulation by ABA could be mediated by a phosphorylation cascade via ROS (Xiong and Zhu, 2003). From this study, it can be hypothesised that regulation of *HvNCED* genes in barley is more likely to be ABA dose dependent, which needs to be confirmed by transient expression studies using promoters of *HvNCED1* and *HvNCED2* gene. ABA is also known for its negative feedback regulation by activating its own catabolism (Cutler and Krochko, 1999).

4.1.2 Regulation of ABA catabolism:

The differential ABA homeostasis between wild type and transgenic plants is also due to the breakdown of ABA to PA, DPA and ABA-GE. The up-regulation of *HvABA8'OHs* (all three) genes in SN410_8 seeds under stress coincided with high levels of ABA (2 DAS). Similar results have also been reported in tobacco plants overexpressing NCED; plants that over produced ABA were also found to have high PA (Qin and Zeevaart, 2002). Even much

earlier, *ABA8'OH* genes were shown to be stimulated by exogenous ABA application (Uknes and Ho, 1984). Contrastingly, no correlation between *HvABA8'OHs* transcripts and PA levels was observed in wild type seeds (12 DAS). This explains why overexpressing one ABA biosynthetic gene (*AtNCED6*) under a highly stress inducible promoter (SalT) resulted in increased ABA biosynthesis compared to wild type and XN26_5 plants. Most of the stress responsive genes are also responsive to ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). Plants that express stress responsive genes are also shown to be highly tolerant to drought stress (Kasuga et al., 1999; Sunkar et al., 2003). Overexpression of some of these ABA responsive genes is also shown to impart drought tolerance (Zhang et al., 2004).

The early induction of *AtNCED6* expression as early as 1 DAS, by highly stress responsive promoter *OsSalT*, under drought stress in leaves of SN410_8 resulted in increased ABA content. The high ABA levels at 2 DAS is by the regulation of *HvNCEDs* by ABA. This early increase of ABA in SN410_8 plants probably resulted in early induction of ABA-responsive genes that play role in maintaining cellular turgidity and reduced water loss. This probably in turn helped SN410_8 plants to maintain high RWC, assimilation, starch biosynthesis and TGW under stress.

4.2 Disturbances in ABA homeostasis affect endosperm polyploidization:

In seeds, first ABA peak is known to decrease with the onset of cellularization phase, and the seed enters the transitional reprogramming phase leading to accumulation of storage proteins and starch (Sreenivasulu et al., 2010a; Sreenivasulu et al., 2010b; Zhu et al., 2011). Also an effect on cell number, cell size and metabolic activity directly affects TGW and yield. The shrunken endosperm of *seg8* (barley mutant) is reduced in endosperm cell number, ploidy level with abnormal differentiation leading to 50 per cent reduction in starch content. This occurred due to the lack of ABA peak during pre-storage phase and the 3 folds increase in ABA that occurred during the transition phase (Sreenivasulu et al., 2010b). Cellularization in barley is completed at 4 DAF (Weschke et al., 2000), which decides the endosperm cell number. Since in this study the plants were subjected to drought stress treatments from 4 DAF onwards, endosperm cellularization and cell number differences between the plants was not studied. Significant difference in endosperm ploidy level was observed between

SN410_8 and wild type under control and stress condition, respectively. In SN410_8 seeds, the ploidy level reached up to 24C due to endoreduplication (Figure 14).

The process of endoreduplication typically occurs in large and metabolically active cells (Lee et al., 2009), and is found to be reduced by drought affecting kernel size (Artlip et al., 1995; Setter and Flanningan, 2001). Increased ploidy level in Arabidopsis leaves under stress helped in maintaining cell size and expansion (Cookson and Granier, 2006). Endoreduplication has been strongly correlated with plant cell size (Schnittger et al., 2003; Sugimoto-Shirasu and Roberts, 2003) and is considered as a means of increasing cell size and gene expression to support the rapid accumulation of storage metabolites (D'Amato, 1984; Leiva-Neto et al., 2004). It is also correlated with metabolic rate, terminal differentiation and grain filling (Kowles and Phillips, 1985; Brunori et al., 1993; Engelen-eigles et al., 2001; Setter and Flanningan, 2001). SN410_8 seeds maintained relatively higher ploidy content of the endosperm under both short and long term stress which coincides with intermediate and main storage phase. This probably resulted in higher metabolic activity of SN410_8 seeds due to which it maintained starch accumulation. This resulted in maintenance of significant higher TGW in SN410_8 plants compared to WT and XN26_5 plants, under stress. In addition, the phytohormone GA is also known to be involved in cell expansion of seeds. Mutant plants defective in active GA production were retarded in growth (Swain et al., 1995; Borisjuk et al., 2004). SN410_8 seeds that had higher seed size compared to wild type under stress were found to have relatively higher levels of active GA (GA4) under short term stress (2DAS). Hence, it can be proposed that SN410_8 plants maintained higher TGW under stress by maintaining higher ploidy level (in endosperm) and seed size compared to respective wild type under stress which resulted in higher sink strength.

4.3 De-regulation of ABA homeostasis in seeds affects starch metabolism:

The metabolic activity of seeds that is measured in terms of starch accumulation was found to be significantly different between SN410_8 and wild type or XN26_5. Since the starch content initially was measured on dry weight basis, the differences between the wild type and transgenic was not so high. However, wild type, SN410_8 and XN26_5 differed for their starch content when measured on per seed basis. Seeds of SN410_8 plants accumulated significantly higher starch per seed than XN26_5 and the levels were comparable to that of

wild type plants under drought stress (Figure 25). Similarly, the lines also significantly differed for their seed size and TGW.

The starch content in seeds was found to be negatively and significantly weakly correlated with ABA. Of all the starch biosynthetic genes analyzed, most of them (*HvAGP_S1b*, *HvAMY3*, *HvBAM1*, *HvBAM7*, *HvCWINV2*, *HvCWINV3*, *HVSBE1*, *HvSSIIIb* and *HvSSIV*) were found to be positively significantly correlated ($P < 0.001$) with starch content while *HvAMY1* and *HvBAM2* were, negatively significantly correlated ($P < 0.001$). Nevertheless, most of these genes showed negative correlation (many significantly correlated) although not as strong as starch (Supplementary table 4). Overall, most of the starch biosynthesis genes in wild type and XN26_5 were found to be highly induced during later stages of seed developmental and stress treatment (4 DAS onwards). On the other hand, in SN410_8 the starch biosynthetic genes were induced much during early stages of seed development and stress treatment (2 DAS). The net rate of starch synthesis in SN410_8 seeds was also maintained during main storage phase. Higher SUS activity during early stages of grain filling is correlated to higher grain filling rate and starch accumulation (Mohapatra et al., 2009; Tang et al., 2009). Thus, under stress during which grain filling period is limiting, SN410_8 plants could efficiently use the photosynthesis assimilates while they would be wasted in wild type and XN26_5 plants due to their poor starch synthesizing capacity during early stages. This is also true for rate of starch accumulation under stress, which is higher in SN410_8 compared to wild type and XN26_5 plants. Similar mechanism is reported to cause differences between the superior and inferior spikelets in rice (Zhu et al., 2011). Under stress, seed filling period drastically reduces. During this condition, plants that are able to maintain high rate of starch synthesis during early to main storage phase of seed development would be less affected by stress for reduction in their TGW. This trait was observed in SN410_8 seeds and they were also found to maintain significantly higher TGW compared to WT plants, under stress respectively.

Based on transcript analysis it looks like the key genes that play probable role in creating this difference between the wild type and SN410_8 plants are *HvSUS*, *HvAGP*, *HvISA*. Among them, *HvSUS* and *HvAGP* were up-regulated while *HvISA* was down-regualted in SN410_8 seeds under stress compared control condition. These genes in wild type and XN26_5 plants

under stress were oppositely regulated (compared to SN410_8 under similar stress conditions). However, we have no data for the activity levels of these enzymes. The low activities of starch synthesis enzymes (*SUS*, *AGPase*, *SS* and *SBE*) are usually associated with low grain weight (Jiang et al., 2003; Kato et al., 2007; Panda et al., 2009; Tang et al., 2009). Not only at protein level, but also the differential expression of some of these genes at transcript level was correlated to inferior and superior grain filling (Ishimaru et al., 2005). It is also speculated by Zhu et al., (2011) that SUS may be the primary target of ABA in seeds where in high ABA levels suppresses SUS expression leading to inferior spikelets.

However, ABA is shown to regulate the activity of SUS (decrease and or increase), and regulate the expression of other starch biosynthesis genes. High ABA levels are also known to reduce the amount of sucrose transported to seeds; in this case, high ABA levels were found to stimulate the expression of starch biosynthesis gene (Ahmadi and Baker, 1999; Bhatia and Singh, 2002; Tang et al., 2009; Akihiro et al., 2005, 2006). Hence, during long term stress we could find induction of these genes in wild type and XN26_5 that had relatively high levels of ABA. Sucrose content has to be measured in seeds of both SN410_8 and wild type seeds to see if this holds true and could be one of the reasons for differential drought tolerance behaviour. In addition, ABA is also known to stimulate better mobilization of carbohydrates form vegetative tissue to developing grain and accelerate grain filling; and influence sugar metabolism (Gibson, 2004; Rook et al., 2006; Akihiro et al., 2005, 2006). Nevertheless under post-anthesis drought, in our study we found the grain filling period between 8/10 DAF to 16 DAF is the critical phase that is sensitive to high ABA levels resulting in reduced yield.

In summary, the transgenic study helps understand how yield and TGW could be regulated or maintained under stress by fine-tuning ABA homeostasis. The SN410_8 plants were found to have high ABA levels during early period of stress (2DAS) which then onwards was maintained to basal levels, in both leaves and seeds. Early ABA increase in SN410_8 leaves under stress probably lead to induction of ABA-regulated genes which helped it in maintaining high RWC, assimilation and WUE under stress. Similarly, early ABA increase (6 DAF) in seeds of SN410_8 plants under stress, resulted in high sink strength due to high metabolic activity of endosperm cells (by process of endoreduplication). Hence, in SN410_8

plants under stress, it resulted in early initiation of starch accumulation that made the best use of all resources; under stress the grain filling period drastically reduces. It can be concluded that high levels of ABA during 8 DAF – 16 DAF, a phase most sensitive to alterations in basal ABA homeostasis levels, affects TGW especially when grain filling period is limiting.

4.4 Conclusion: how altered ABA homeostasis affects starch metabolism, TGW and yield:

Transgenic plants over expressing *AtNCED6* under stress and developmentally induced promoter resulting in high ABA levels in leaves (under stress) and seeds (under control and or stress) were produced. These plants along with WT plants differed in their ABA levels. Early peaking of ABA (2 DAS) in leaves of SN410_8 plants under stress probably helped in triggering genes that play a role in cellular protection. This was evident by the maintenance of high percent leaf RWC content by SN410_8 plants under stress (4 DAS) compared to WT and XN26_5 plants that had high ABA relatively late. This also helped SN410_8 plants to maintain higher photosynthetic rate than WT and XN26_5 plants, under stress. In addition, under stress, ABA levels reached basal levels (8/10 DAF- 16/20 DAF) after an initial increase of ABA (6 DAF) in seeds of SN410_8 while in WT and XN26_5 seeds the ABA levels did not reach basal levels. The impact of this was seen in terms of low rate of starch accumulation in WT and XN26_5 under stress.

Based on our study with transgenic plants (altered for ABA), we propose how changes in ABA levels in leaf and seeds affects the performance of plants (superior and inferior performers) under post-anthesis drought stress (imposed 4 DAF). In addition, we also propose that alteration in ABA levels during critical grain filling period between 8/10 DAF – 16/20 DAF in barley affects starch metabolism, in turn TGW and yield.

Under stress, if plants are able to immediately increase ABA and reach back to basal levels in leaves, trigger ABA responsive genes involved in cell protection and help maintain RWC and high photosynthetic activity (Figure 29c: green dash line). Hence, these plants would not be limited in supply of photosynthesis products to developing seeds. In addition, plants that are able to reach basal levels of ABA after the first peak of ABA (4 – 6 DAF) in seeds would maintain high rate of starch accumulation (triggering and activating starch biosynthesis genes) (Figure 29a: green dash line). These plants would make the best use of available

resources under stress during which the seed filling period and moisture content decreases (Figure 29b). The above mentioned reprogramming was observed in SN410_8 plants and they performed superior under drought stress compared to other lines.

In addition under stress, plants that are not so stress responsive have late increase in their ABA (in leaves) which results in high water loss and decreased photosynthesis when water is limiting (Figure 29c: black dash line). Similarly in seeds of plants under stress, if the ABA levels do not reach back to basal levels after first peak, it greatly affects the rate of starch accumulation. In these lines although the starch metabolism genes are triggered the ABA levels need to come back to basal levels for the coded enzyme to be active (Figure 29c: orange and black dash line). Hence, these plants would have low starch accumulation rate when the seed filling period and moisture content decreases. This would result in reduced TGW and yield under stress. The above mentioned reprogramming was observed in WT and XN26_5 plants that performed inferior under stress.

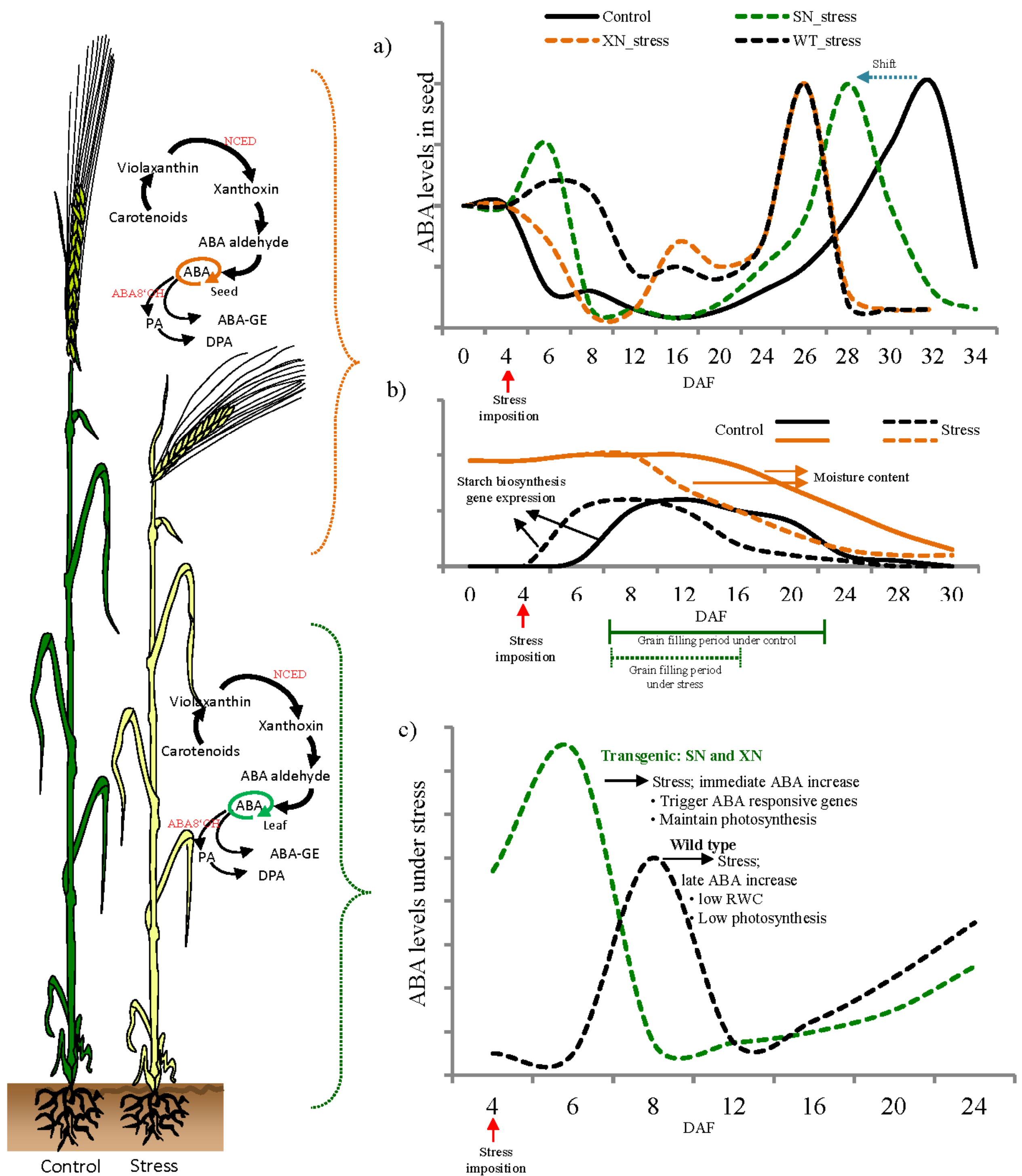


Figure 29: Schematic representation of probable changes in ABA homeostasis in leaf and seeds and its impact on starch metabolism under drought stress, which decreases the grain filling period. Represented is a) ABA levels in plants under control and under stress in better (SN410_8)and poor (WT and XN26_5) performing plants; b) moisture and starch gene expression under stress relating to ABA levels in a); and, c) ABA levels in leaf under stress in better and poor performing plants.

5 Summary

Drought is one of the major abiotic stresses that largely impair plant growth and development thereby limiting plant performance resulting in reduced yield. However, plants perceive and respond to abiotic stress with adaptive processes mainly controlled by sesquiterpene phytohormone abscisic acid (ABA). ABA acts as a messenger in regulating plant water status and many essential plant developmental processes. Exogenous treatment of ABA revealed its importance in maintaining water relations in plants by regulating stomatal opening affecting photosynthesis. Similarly, in this study with barley, when the spikes were exogenously treated with the ABA biosynthesis inhibitor fluridone, there was also significant reduction in TGW and yield. Under drought stress there was significant reduction in yield (6 %) compared to control conditions. However, exogenous ABA treatment compensated for the reduction in TGW under stress, indicating the importance of ABA in regulating yield. In addition, ABA levels profoundly increased under drought stress in both flag leaves and developing seeds, and high ABA levels negatively correlated with starch content especially at 12 and 16 DAF under stress. Moreover, slight decrease in later stages indicates the possibility that ABA accelerates the rate of starch accumulation and reduces the duration of seed filling.

Based on this and the expression analysis of ABA and starch metabolism genes in elite line of barley, LP110, under control and post-anthesis drought stress we hypothesised that deviation in ABA levels from basal levels have negative impact on starch accumulation that in turn affects TGW and yield. To test this hypothesis that alterations in ABA homeostasis during grain filling period (under control and post-anthesis drought) regulates TGW and yield, transgenic plants altered in their ABA were produced. These transgenic plants would also be useful to study the impact of altered ABA homeostasis on starch metabolism during grain filling period. Transgenic plants expressing *AtNCED6* under *OsSalT* (SN410_8) and *Ta1AX1* (XN26_5) promoter that are stress and developmentally inducible, respectively, were produced to regulate or alter ABA levels in leaves and seeds under stress and control conditions, respectively.

The *SalT* promoter was found to be highly induced in both leaf and seed tissue under early stages of drought while the *1AX1* promoter was more specific to seed tissue. SN410_8 plants

were more drought tolerant as they were able to maintain significantly higher yield (TGW) than WT plants under stress, under both green house and rain shelter conditions. On the other hand, performance of XN26_5 plants under stress and sometimes under control conditions was either lower or on par with that of WT plants under stress. These three lines (WT, SN410_8 and XN26_5) significantly differed in their ABA accumulation pattern. SN410_8 plants accumulated maximum ABA (17 folds over control) in leaves within 2 days of drought stress that reached to basal levels very fast (12 DAS). This helped the plants to maintain significantly high leaf RWC, photosynthesis and WUE compared to WT and XN26_5 plants that had maximum ABA (17 and 20 folds over control, respectively) levels 4 DAS and ABA levels in these plants did not reach basal levels (still high at 12 DAS).

Similarly in seeds of stressed plants, ABA levels in SN410_8 plants was high only at 2 DAS (6 DAF) that reduced to basal levels immediately (8 DAS: 12 DAF) while ABA levels in WT and XN26_5 plants were maintained higher even at 8 DAS (12 DAF) and later stages. SN410_8 plants under stress compared to control were able to maintain comparatively higher seed moisture content and seed dry weight that was also reflected in terms of starch accumulation. The seeds of SN410_8 plants also maintained higher rate of starch accumulation under stress compared to WT and XN26_5. The high rate of starch accumulation could be explained by the increased expression of key starch metabolism genes between 4 DAS to 12 DAS (8 DAF to 16 DAF). Since under drought stress grain filling period reduces, SN410_8 plants performed better by maintaining moisture content and completing starch accumulation in this narrow window by maintaining basal levels of ABA which if high have negative effect on starch metabolism.

Moreover, the poor performance of WT and XN26_5 plants under stress resulted because of lower rate of starch accumulation. Although regulation of starch metabolism genes in these lines is similar to SN410_8 plants during early grain filling phase, the input of sucrose form leaves would have been limiting in these plants. Since these plants had significantly lower photosynthetic rate and low percent leaf RWC. In addition, some of the key starch biosynthesis genes were found to be up-regulated in WT and XN26_5 at intermediate stages of seed development under stress, by which time the moisture content of these seeds was significantly lower. In addition, WT and XN26_5 under stress maintained high ABA levels

during later stages of seed development that is known to effect starch metabolism by triggering starch degradation. Since ABA homeostasis did not reach basal levels during main grain filling period in WT and XN26_5 under stress, it could not make best use of the shortened grain filling period in accumulating starch. It becomes very clear from this study that in barley it is very crucial to maintain basal levels of ABA during the critical period of 8 DAF to 16 or 20 DAF (peak grain filling period). Plants that are able to maintain basal levels of ABA during this critical window of grain filling under drought stress would be promising in maintaining yield under stress provided similar ABA homeostasis is also achieved in leaf tissues.

If regulating ABA homeostasis levels to basal levels is so crucial, it would be expected that plants must have evolved such mechanism during evolution to combat stress. However, under stress we find that the untransformed plants (Golden promise) are not capable of doing so and hence result in low yields. It is likely that plants would have definitely evolved with such mechanism but their priority would have been to perpetuate and not yield. We find that under severe drought stress (20 % Field capacity) the stress plants are still able to produce enough seeds. In human and agronomic interest, we want plants that are able to maintain TGW and yield under stress and apart from just perpetuating. This study is an attempt to understand the role and mechanism by which ABA regulates TGW and yield under stress. An understanding of drought tolerance mechanisms would help in future to develop plants that are able to produce or maintain yield apart from just surviving under stress.

6 Zusammenfassung

Trockenheit ist einer der Hauptstressfaktoren, der Pflanzenwachstum und Entwicklung stark beeinträchtigt und dadurch die Leistung der Pflanze limitiert, was wiederum zu Ertragsverlusten führt. Pflanzen nehmen jedoch den abiotischen Stress wahr und reagieren darauf mit adaptiven Prozessen, die hauptsächlich durch das Sesquiterpen-Phytohormon Abscisinsäure (ABA) kontrolliert werden. ABA agiert dabei grundsätzlich als Messenger der Regulation des Wasserstatus der Pflanze und vieler essentieller pflanzlicher Entwicklungsprozesse. Die exogene Behandlung mit ABA deckte seine Wichtigkeit in der Aufrechterhaltung der Wasserrelationen in Pflanzen auf. ABA reguliert das Schließen und Öffnen der Stomata was wiederum die Photosynthese beeinflusst. Gleichermaßen wurde in dieser Arbeit mit Gerste eine signifikante Reduktion des Tausendkorngewichtes (thousand grain weight, TGW) und des Ertrages beobachtet, wenn die Ähren exogen mit dem ABA Biosyntheseinhibitor Fluridon behandelt wurden. Dennoch wurde ebenso unter Trockenstress eine signifikante Reduktion des Ertrages (6 %) verglichen mit Kontrollbedingungen, gemessen. Eine exogene Behandlung mit ABA kompensierte das unter Stress reduzierte TGW, was auf die Wichtigkeit von ABA bei der Ertragsregulierung hindeutet. Zusätzlich wurde herausgefunden, dass die ABA-Gehalte unter Trockenstress in Fahnenblättern und sich entwickelnden Samen stark ansteigen, und diese erhöhten ABA-Gehalte korrelieren mit einem erhöhten Stärkegehalt besonders 12 und 16 Tage nach der Blüte (days after flowering, DAF) unter Stressbedingungen. Desweiteren deutet ein leichtes Absinken des Stärkegehaltes in späteren Stadien auf die Möglichkeit hin, dass ABA die Stärkeakkumulationsrate beschleunigt und dadurch die Dauer der Samenfüllung verkürzt.

Aufgrund dessen und der Expressionsanalyse von Genen des ABA- und Stärkestoffwechsels in einer Gersten-Elitelinie (LP110) unter Kontrollbedingungen und Trockenstress nach der Blüte kann die Hypothese aufgestellt werden, dass die Abweichung der ABA-Homöostase von ihren Basalgehalten einen negativen Einfluss auf die Stärkeakkumulation hat, was wiederum den Ertrag und das TGW beeinflusst. Um diese Hypothese der Rolle der ABA-Homöostase während der Kornfüllungsphase unter Trockenstress und ihren Einfluss auf den Ertrag zu überprüfen, wurden transgene Pflanzen erzeugt, die in ihrer ABA-Homöostase verändert sind. Diese transgenen Pflanzen wären ebenfalls nützlich, um die Auswirkung einer geänderten ABA-Homöostase auf den Stärkestoffwechsel während der Kornfüllungsphase zu untersuchen. Transgene Pflanzen, die *AtNCED6* unter Kontrolle des *OsSalT* (SN410_8) und *Ta1AX1* (XN26_5) Promotors (stress- bzw. entwicklungsspezifisch induziert) exprimieren, wurden

erstellt, um die ABA-Homöostase in Blättern und Samen unter Trockenstress- bzw. Kontrollbedingungen zu regulieren oder zu verändern.

Der *SalT* Promotor wird stark in Blättern und Samengewebe unter Trockenstress induziert während der *1AX1* Promotor eher samenspezifisch ist. SN410_8 Pflanzen waren trockentoleranter, da sie sowohl unter Gewächshaus- als auch unter Feldbedingungen in der Lage waren, einen höheren Ertrag (TGW) als die Wildtyppflanzen unter Stress zu halten. Auf der anderen Seite war die Leistung der XN26_5 Pflanzen unter Stress und zum Teil auch unter Kontrollbedingungen entweder niedriger als die der Wildtyppflanzen oder mit dieser vergleichbar. Diese drei Linien (WT, SN410_8 und XN26_5) zeigten deutliche Unterschiede in ihrem Muster der ABA-Akkumulation. SN410_8 Pflanzen akkumulierten das meiste ABA in Blättern innerhalb zwei Tagen nach Stressbeginn (17 fach erhöht im Vergleich zu Kontrollbedingungen) und danach sanken die Werte wieder auf Grundniveau etwa 12 Tage nach Stressbeginn (12 DAS). Dies half den Pflanzen einen signifikant höheren prozentualen Wassergehalt in den Blättern, sowie auch eine deutlich höhere Photosyntheserate und WUE verglichen mit dem WT und den XN26_5 Pflanzen aufrecht zu erhalten. WT und XN26_5 hatten den maximalen ABA-Gehalt (17 bzw. 20 fach erhöht im Vergleich zu Kontrolle) erst vier Tage nach Stressbeginn und auch 12 DAS waren die Werte immer noch höher als in SN410_8.

Gleichermaßen waren die ABA-Gehalte auch in Samen von gestressten SN410_8 Pflanzen nur 2 Tage nach Stressbeginn erhöht und sanken dann bis 8 DAS wieder auf ihr Grundniveau wohingegen die ABA-Gehalte in Samen von WT und XN26_5 Pflanzen auch noch 8 DAS und später erhöht waren. SN410_8 Pflanzen waren in der Lage unter Stress eine höhere Samenfeuchtigkeit sowie ein höheres Samentrockengewicht zu halten, was sich auch hinsichtlich der Stärkeakkumulation widerspiegelte. Die Samen von SN410_8 Pflanzen wiesen auch eine höhere Stärkeakkumulationsrate unter Stress auf, verglichen mit WT und XN26_5. Diese hohe Stärkeakkumulationsrate kann durch eine erhöhte Expression von Schlüsselgenen des Stärkemetabolismus während 4 und 12 DAS (entspricht 8 – 16 DAF) erklärt werden. Trockenstress führt normalerweise zu einer Reduktion der Dauer der Kornfüllungsphase. Die SN410_8 Pflanzen umgehen dies, indem ihre Samen die Feuchtigkeit halten und sie die Stärkespeicherung innerhalb dieses Fensters weitestgehend abschließen, was wiederum durch das Halten der ABA-Homöostase begründet ist. Andererseits hätten erhöhte ABA-Gehalte einen negativen Einfluss auf den Stärkemetabolismus.

Desweiteren ergab sich die schlechtere Leistung der WT und XN26_5 Pflanzen aufgrund einer geringeren Stärkeakkumulationsrate. Obwohl es während der frühen Kornfüllungsphase keine nennenswerten Unterschiede in der Regulation der Gene des Stärkestoffwechsels im Vergleich mit SN410_8 gab, könnte der Eintransport von Saccharose aus den Blättern limitierend sein, da diese Pflanzen eine deutlich geringere Photosyntheserate und geringeren Prozentsatz an RWC aufwiesen. Zusätzlich waren einige Schlüsselgene der Stärkesynthese im WT und XN26_5 in späteren Stadien der Samenentwicklung unter Stress hochreguliert. Zu dieser Zeit war der Samenfeuchtegehalt deutlich geringer. Die WT und XN26_5 Pflanzen wiesen auch in späteren Stadien der Samenentwicklung höhere ABA-Gehalte auf, was sich auf den Stärkestoffwechsel auswirkt. Da in WT und XN26_5 Pflanzen keine ABA-Homöostase während der Samenentwicklung unter Stressbedingungen erreicht wurde, konnten diese Pflanzen die verkürzte Samenfüllungsphase nicht hinreichend nutzen, um Stärke zu speichern. Aus dieser Arbeit wird sehr deutlich, dass es in Gerste sehr wichtig ist, eine ABA-Homöostase zu halten, die ähnlich dem ABA-Grundgehalt ist, besonders während der kritischen Phase von 8 DAF bis 16 oder 20 DAF, welche den Höhepunkt der Speicherphase darstellt. Pflanzen die während dieser kritischen Phase der Kornfüllung unter Stress in der Lage sind, ABA auf Grundniveau zu halten, sind vielversprechend, um auch den Ertrag stabil zu halten. Gleichermaßen muss dafür ABA auch in den Blättern auf Grundniveau gehalten werden.

Wenn die Regulierung der ABA-Homöostase so wichtig ist, würde man annehmen, dass in der Evolution Pflanzen mit solchen Mechanismen entstanden sind, um Stress zu bekämpfen. Jedoch wurde in dieser Arbeit herausgefunden, dass Wildtyppflanzen dazu nicht in der Lage sind und dadurch ein Ertragsverlust resultiert. Es ist wahrscheinlich, dass solche Pflanzen evolutionär entstanden, ihre Priorität jedoch das Überleben und nicht ein hoher Ertrag gewesen wäre. In dieser Arbeit wurde festgestellt, dass die Pflanzen unter starkem Trockenstress (20 % FC) immer noch in der Lage sind, genug Samen zu produzieren. Jedoch ist es im menschlichen und agronomischen Interesse, auch Pflanzen zu haben, die das TGW und einen hohen Ertrag auch unter Trockenstress halten können, abgesehen davon ihre Art zu erhalten. Diese Arbeit ist ein Versuch, die Rolle und Mechanismen zu verstehen, inwiefern Aba das TGW und den Ertrag unter Stress reguliert. Die Kenntnis von Trockentoleranzmechanismen würde in der Zukunft dazu beitragen, Pflanzen zu entwickeln, die in der Lage sind, gute Erträge zu liefern anstatt unter Stressbedingungen nur das Überleben zu sichern.

7 References

Addicott, F., Lyon, J., Ohkuma, K., Thiessen, W., Carns, H., Smith, O., Cornforth, J., Milborrow, B., Ryback, G. and Wareing, P. (1968) Abscisic acid: a new name for abscisin II (dormin). *Science*, **159**, 1493.

Addicott, F.T. (1983) *Abscisic acid*: Praeger New York.

Addicott, F.T. and Lyon, J.L. (1969) Physiology of abscisic acid and related substances. *Annual review of plant physiology*, **20**, 139-164.

Ahmadi, A. and Baker, D. (1999) Effects of abscisic acid (ABA) on grain filling processes in wheat. *Plant Growth Regulation*, **28**, 187-197.

Ahmadi, A. and Baker, D. (2001) The effect of water stress on grain filling processes in wheat. *The Journal of Agricultural Science*, **136**, 257-269.

Akihiro, T., Mizuno, K. and Fujimura, T. (2005) Gene expression of ADP-glucose pyrophosphorylase and starch contents in rice cultured cells are cooperatively regulated by sucrose and ABA. *Plant and cell physiology*, **46**, 937.

Akihiro, T., Umezawa, T., Ueki, C., Lobna, B.M., Mizuno, K., Ohta, M. and Fujimura, T. (2006) Genome wide cDNA-AFLP analysis of genes rapidly induced by combined sucrose and ABA treatment in rice cultured cells. *FEBS letters*, **580**, 5947-5952.

Araus, J., Slafer, G., Reynolds, M. and Royo, C. (2002) Plant breeding and drought in C3 cereals: What should we breed for? *Annals of botany*, **89**, 925-940.

Artlip, T., Madison, J. and Setter, T. (1995) Water deficit in developing endosperm of maize: cell division and nuclear DMA endoreduplication. *Plant, cell & environment*, **18**, 1034-1040.

Aswath, C.R., Kim, S.H., Mo, S.Y. and Kim, D.H. (2005) Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-cis-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. *Plant Growth Regulation*, **47**, 129-139.

Audran, C., Liotenberg, S., Gonneau, M., North, H., Frey, A., Tap-Waksman, K., Vartanian, N. and Marion-Poll, A. (2001) Localisation and expression of zeaxanthin epoxidase mRNA in Arabidopsis in response to drought stress and during seed development. *Functional Plant Biology*, **28**, 1161-1173.

Balsevich, J.J., Cutler, A.J., Lamb, N., Friesen, L.J., Kurz, E.U., Perras, M.R. and Abrams, S.R. (1994) Response of cultured maize cells to (+)-abscisic acid,(-)-abscisic acid, and their metabolites. *Plant physiology*, **106**, 135-142.

Barnabás, B., Jäger, K. and FehER, A. (2008) The effect of drought and heat stress on reproductive processes in cereals. *Plant, cell & environment*, **31**, 11-38.

Barow, M. and Meister, A. (2003) Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. *Plant, cell & environment*, **26**, 571-584.

Barrero, J., RodrÍGuez, P.L., Quesada, V.Í.C., Piqueras, P., Ponce, M.Í.A.R. and Micol, J.É.L. (2006) Both abscisic acid (ABA)-dependent and ABA-independent pathways govern the induction of NCED3, AAO3 and ABA1 in response to salt stress. *Plant, cell & environment*, **29**, 2000-2008.

Bentsink, L. and Koornneef, M. (2008) Seed dormancy and germination. *The Arabidopsis Book*, **6**, 1-18.

Bhatia, S. and Singh, R. (2002) Phytohormone-mediated transformation of sugars to starch in relation to the activities of amylases, sucrose-metabolising enzymes in sorghum grain. *Plant Growth Regulation*, **36**, 97-104.

Bianco-Colomas, J., Barthe, P., Orlandini, M. and Le Page-Degivry, M.T. (1991) Carrier-mediated uptake of abscisic acid by suspension-cultured Amaranthus tricolor cells. *Plant physiology*, **95**, 990.

Bittner, F., Oreb, M. and Mendel, R.R. (2001) ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in Arabidopsis thaliana. *Journal of Biological Chemistry*, **276**, 40381.

Blum, A. (2005) Drought resistance, water-use efficiency, and yield potential—are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research*, **56**, 1159-1168.

Borel, C., Audran, C., Frey, A., Marion-Poll, A., Tardieu, F. and Simonneau, T. (2001a) N. plumbaginifolia zeaxanthin epoxidase transgenic lines have unaltered baseline ABA accumulations in roots and xylem sap, but contrasting sensitivities of ABA accumulation to water deficit. *Journal of experimental botany*, **52**, 427.

Borel, C., Audran, C., Frey, A., Marion-Poll, A. and Tardieu, F. (2001b) Root physiology. *N. plumbaginifolia* zeaxanthin epoxidase transgenic lines have unaltered baseline ABA accumulations in roots and xylem sap, but contrasting sensitivities of ABA accumulation to water deficit. *Journal of Experimental Botany, 52, Special Issue 1*, **427**, 8.

Borisjuk, L., Rolletschek, H., Radchuk, R., Weschke, W., Wobus, U. and Weber, H. (2004) Seed development and differentiation: a role for metabolic regulation. *Plant biology*, **6**, 375-386.

Boudsocq, M., Barbier-Brygoo, H. and Laurière, C. (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *Journal of Biological Chemistry*, **279**, 41758.

Boudsocq, M., Droillard, M.J., Barbier-Brygoo, H. and Lauriere, C. (2007) Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant molecular biology*, **63**, 491-503.

Boyer, J.S. (1982) Plant productivity and environment. *Science*, **218**, 443.

Bray, E.A. (1997) Plant responses to water deficit. *Trends in plant science*, **2**, 48-54.

Bray, E.A. (2007) Molecular and physiological responses to water-deficit stress. *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops*, 121-140.

Brunori, A., Forino, L., Frediani, M. and Ruberti, F. (1993) Cell number and polyploidy in the starchy endosperm of Triticum aestivum in relation to seed weight. *Journal of Genetics and Breeding*, **47**, 217-217.

Burbidge, A., Grieve, T.M., Jackson, A., Thompson, A., McCarty, D.R. and Taylor, I.B. (1999) Characterization of the ABA-deficient tomato mutantnotabilisand its relationship with maizeVp14. *The Plant Journal*, **17**, 427-431.

Cheng, W.H., Endo, A., Zhou, L., Penney, J., Chen, H.C., Arroyo, A., Leon, P., Nambara, E., Asami, T. and Seo, M. (2002) A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. *The Plant Cell Online*, **14**, 2723-2743.

Chernys, J.T. and Zeevaart, J.A.D. (2000) Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant physiology*, **124**, 343-354.

Chiwocha, S.D.S., Abrams, S.R., Ambrose, S.J., Cutler, A.J., Loewen, M., Ross, A.R.S. and Kermode, A.R. (2003) A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (Lactuca sativa L.) seeds. *The Plant Journal*, **35**, 405-417.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proceedings of the National Academy of Sciences*, **81**, 1991.

Cookson, S.J. and Granier, C. (2006) A dynamic analysis of the shade-induced plasticity in Arabidopsis thaliana rosette leaf development reveals new components of the shade-adaptative response. *Annals of botany*, **97**, 443.

Cornforth, J., Milborrow, B. and Ryback, G. (1965) Chemistry and Physiology ofDormins' In Sycamore: Identity of SycamoreDormin'with Abscisin II. *Nature*, **205**, 1269-1270.

Coronado, M.J., Hensel, G., Broeders, S., Otto, I. and Kumlehn, J. (2005) Immature pollen-derived doubled haploid formation in barley cv. Golden Promise as a tool for transgene recombination. *Acta Physiologiae Plantarum*, **27**, 591-599.

Cutler, A.J. and Krochko, J.E. (1999) Formation and breakdown of ABA. *Trends in plant science*, **4**, 472-478.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. *Annual Review of Plant Biology*, **61**, 651-679.

D'amato, F. (1984) Role of polyploidy in reproductive organs and tissues. *Embryology of the angiosperms*, 519-566.

Delseny, M., Bies-Etheve, N., Carles, C., Hull, G., Vicient, C., Raynal, M., Grellet, F. and Aspart, L. (2001) Late embryogenesis abundant (LEA) protein gene regulation during Arabidopsis seed maturation. *Journal of plant physiology*, **158**, 419-427.

Ding, Z., Li, S., An, X., Liu, X., Qin, H. and Wang, D. (2009) Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in Arabidopsis thaliana. *Journal of Genetics and Genomics*, **36**, 17-29.

Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H., Seo, M., Toyomasu, T., Mitsuhashi, W. and Shinozaki, K. (2008) Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant physiology*, **147**, 1984-1993.

Engelen-Eigles, G.J. and RJ Phillips, R. (2001) DNA endoreduplication in maize endosperm cells is reduced by high temperature during the mitotic phase. *Crop Science*, **41**, 1114.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. and Basra, S. (2009) Plant drought stress: effects, mechanisms and management. *Sustainable Agriculture*, 153-188.

Finkelstein, R.R., Gampala, S.S.L. and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. *The Plant Cell Online*, **14**, S15-S45.

Finkelstein, R.R. and Gibson, S.I. (2002) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? *Current opinion in plant biology*, **5**, 26-32.

Finkelstein, R.R. and Rock, C.D. (2002) Abscisic acid biosynthesis and response. *The Arabidopsis Book, CR Somerville and EM Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab*, **58**.

Frey, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. (1999) Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. *Plant molecular biology*, **39**, 1267-1274.

Frey, A., Boutin, J.P., Sotta, B., Mercier, R. and Marion-Poll, A. (2006) Regulation of carotenoid and ABA accumulation during the development and germination of Nicotiana plumbaginifolia seeds. *Planta*, **224**, 622-632.

Frey, A., Godin, B., Bonnet, M., Sotta, B. and Marion-Poll, A. (2004) Maternal synthesis of abscisic acid controls seed development and yield in Nicotiana plumbaginifolia. *Planta*, **218**, 958-964.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y., Cutler, S.R., Sheen, J., Rodriguez, P.L. and Zhu, J.K. (2009) In vitro reconstitution of an abscisic acid signalling pathway. *Nature*, **462**, 660-664.

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. *The Plant Cell Online*, **17**, 3470-3488.

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K. and Ishiyama, K. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant and cell physiology*, **50**, 2123.

Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 1988.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P. and Firoozabady, E. (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science*, **220**, 1049-1051.

Gibson, S.I. (2004) Sugar and phytohormone response pathways: navigating a signalling network. *Journal of experimental botany*, **55**, 253-264.

Gillard, D.F. and Walton, D.C. (1976) Abscisic Acid Metabolism by a Cell-free Preparation from Echinocystis lobata Liquid Endoserum. *Plant physiology*, **58**, 790-795.

Goda, H., Sasaki, E., Akiyama, K., Maruyama-Nakashita, A., Nakabayashi, K., Li, W., Ogawa, M., Yamauchi, Y., Preston, J. and Aoki, K. (2008) The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access. *The Plant Journal*, **55**, 526-542.

Goetz, H., Christine, K., Sylwia, O., Jan, R. and Jochen, K. (2009) Agrobacterium-mediated gene transfer to cereal crop plants: current protocols for barley, wheat, triticale, and maize. *International Journal of Plant Genomics*, **2009**.

González-Guzmán, M., Apostolova, N., Bellés, J.M., Barrero, J.M., Piqueras, P., Ponce, M.R., Micol, J.L., Serrano, R. and Rodríguez, P.L. (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *The Plant Cell Online*, **14**, 1833-1846.

Gubler, F., Hughes, T., Waterhouse, P. and Jacobsen, J. (2008) Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. *Plant physiology*, **147**, 886-896.

Gusta, L., Ewan, B., Reaney, M. and Abrams, S. (1992) The effect of abscisic acid and abscisic acid metabolites on the germination of cress seed. *Canadian journal of botany*, **70**, 1550-1555.

Hao, Q., Yin, P., Yan, C., Yuan, X., Li, W., Zhang, Z., Liu, L., Wang, J. and Yan, N. (2010) Functional mechanism of the abscisic acid agonist pyrabactin. *Journal of Biological Chemistry*, **285**, 28946-28952.

Harris, D., Tripathi, R. and Joshi, A. (2002) On-farm seed priming to improve crop establishment and yield in dry direct-seeded rice. *Direct seeding: research strategies and opportunities. Manila (Philippines): International Rice Research Institute. p*, 231-240.

Hawker, J. and Jenner, C. (1993) High temperature affects the activity of enzymes in the committed pathway of starch synthesis in developing wheat endosperm. *Functional Plant Biology*, **20**, 197-209.

Hensel, G. and Kumlehn, J. (2004) Genetic transformation of barley (Hordeum vulgare L.) by co-culture of immature embryos with Agrobacteria. *Transgenic Crops of the World: Essential Protocols. Kluwer Academic Publishers, Dordrecht, The Netherlands*, 35-45.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant molecular biology*, **42**, 819-832.

Heyne, E. and Brunson, A.M. (1940) Genetic studies of heat and drought tolerance in maize. *Journal of the American Society of Agronomy*, **32**, 803-814.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research*, **27**, 297-300.

Hopkins, W. and Huner, N. (1999) Introduction to plant Physiology. John Willey and Sons. *Inc. New York*.

Hubbard, K.E., Nishimura, N., Hitomi, K., Getzoff, E.D. and Schroeder, J.I. (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes & development*, **24**, 1695.

Hurkman, W.J., McCue, K.F., Altenbach, S.B., Korn, A., Tanaka, C.K., Kothari, K.M., Johnson, E.L., Bechtel, D.B., Wilson, J.D. and Anderson, O.D. (2003) Effect of temperature on expression of genes encoding enzymes for starch biosynthesis in developing wheat endosperm. *Plant Science*, **164**, 873-881.

Hwang, S.G., Chen, H.C., Huang, W.Y., Chu, Y.C., Shii, C.T. and Cheng, W.H. (2010) Ectopic expression of rice OsNCED3 in Arabidopsis increases ABA level and alters leaf morphology. *Plant Science*, **178**, 12-22.

Imber, D. and Tal, M. (1970) Phenotypic reversion of flacca, a wilty mutant of tomato, by abscisic acid. *Science*, **169**, 592.

Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Biology*, **47**, 377-403.

Ishimaru, T., Hirose, T., Matsuda, T., Goto, A., Takahashi, K., Sasaki, H., Terao, T., Ishii, R., Ohsugi, R. and Yamagishi, T. (2005) Expression patterns of genes encoding carbohydrate-metabolizing enzymes and their relationship to grain filling in rice (Oryza sativa L.): comparison of caryopses located at different positions in a panicle. *Plant and cell physiology*, **46**, 620.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *The Plant Journal*, **27**, 325-333.

Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2000) A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant physiology*, **123**, 553-562.

Jacobsen, J.V., Pearce, D.W., Poole, A.T., Pharis, R.P. and Mander, L.N. (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiologia Plantarum*, **115**, 428-441.

Jeanneau, M., Gerentes, D., Foueillassar, X., Zivy, M., Vidal, J., Toppan, A. and Perez, P. (2002) Improvement of drought tolerance in maize: towards the functional validation of the Zm-Asr1 gene and increase of water use efficiency by over-expressing C4-PEPC. *Biochimie*, **84**, 1127-1135.

Ji, X., Dong, B., Shiran, B., Talbot, M.J., Edlington, J.E., Hughes, T., White, R.G., Gubler, F. and Dolferus, R. (2011) Control of Abscisic Acid Catabolism and Abscisic Acid Homeostasis Is Important for Reproductive Stage Stress Tolerance in Cereals. *Plant physiology*, **156**, 647.

Jiang, F. and Hartung, W. (2008) Long-distance signalling of abscisic acid (ABA): the factors regulating the intensity of the ABA signal. *Journal of experimental botany*, **59**, 37.

Jiang, H., Dian, W. and Wu, P. (2003) Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme. *Phytochemistry*, **63**, 53-59.

Jones, R.J. and Mansfield, T. (1970) Suppression of stomatal opening in leaves treated with abscisic acid. *Journal of experimental botany*, **21**, 714.

Kang, J., Hwang, J.U., Lee, M., Kim, Y.Y., Assmann, S.M., Martinoia, E. and Lee, Y. (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proceedings of the National Academy of Sciences*, **107**, 2355.

Kanno, Y., Hanada, A., Chiba, Y., Ichikawa, T., Nakazawa, M., Matsui, M., Koshiba, T., Kamiya, Y. and Seo, M. (2012) Identification of an abscisic acid transporter by functional screening using the receptor complex as a sensor. *Proceedings of the National Academy of Sciences*, **109**, 9653-9658.

Karssen, C., Brinkhorst-Van der Swan, D., Breekland, A. and Koornneef, M. (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of Arabidopsis thaliana (L.) Heynh. *Planta*, **157**, 158-165.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology*, **17**, 287-291.

Kato, T., Shinmura, D. and Taniguchi, A. (2007) Activities of enzymes for sucrose-starch conversion in developing endosperm of rice and their association with grain filling in extra-heavy panicle types. *Plant production science*, **10**, 442-450.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J. and Harter, K. (2007) The AtGenExpress global

stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *The Plant Journal*, **50**, 347-363.

Knight, C.D., Sehgal, A., Atwal, K., Wallace, J.C., Cove, D.J., Coates, D., Quatrano, R.S., Bahadur, S., Stockley, P.G. and Cuming, A.C. (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. *The Plant Cell Online*, **7**, 499-506.

Koiwai, H., Nakaminami, K., Seo, M., Mitsuhashi, W., Toyomasu, T. and Koshiba, T. (2004) Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in Arabidopsis. *Plant physiology*, **134**, 1697-1707.

Kong, L., Abrams, S.R., Owen, S.J., Graham, H. and Von Aderkas, P. (2008) Phytohormones and their metabolites during long shoot development in Douglas-fir following cone induction by gibberellin injection. *Tree Physiology*, **28**, 1357-1364.

Koornneef, M., Jorna, M., Brinkhorst-Van der Swan, D. and Karssen, C. (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of Arabidopsis thaliana (L.) Heynh. *TAG Theoretical and Applied Genetics*, **61**, 385-393.

Koornneef, M., Léon-Kloosterziel, K.M., Schwartz, S.H. and Zeevaart, J.A.D. (1998) The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in Arabidopsis. *Plant Physiology and Biochemistry*, **36**, 83-89.

Kowles, R.V. and Phillips, R.L. (1985) DNA amplification patterns in maize endosperm nuclei during kernel development. *Proceedings of the National Academy of Sciences*, **82**, 7010.

Kozloski, G.V., Rocha, J.B.T., Ribeiro Filho, H. and Perottoni, J. (1999) Comparison of acid and amyloglucosidase hydrolysis for estimation of non-structural polysaccharides in feed samples. *Journal of the Science of Food and Agriculture*, **79**, 1112-1116.

Kuromori, T., Miyaji, T., Yabuuchi, H., Shimizu, H., Sugimoto, E., Kamiya, A., Moriyama, Y. and Shinozaki, K. (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. *Proceedings of the National Academy of Sciences*, **107**, 2361.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y. and Nambara, E. (2004) The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. *The EMBO journal*, **23**, 1647-1656.

Lee, H.O., Davidson, J.M. and Duronio, R.J. (2009) Endoreplication: polyploidy with purpose. *Genes & development*, **23**, 2461.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J. and Hwang, I. (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell*, **126**, 1109-1120.

Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M., Nambara, E. and Marion-Poll, A. (2006) Functional analysis of Arabidopsis *NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *The Plant Journal*, **45**, 309-319.

Leiva-Neto, J.T., Grafi, G., Sabelli, P.A., Dante, R.A., Woo, Y., Maddock, S., Gordon-Kamm, W.J. and Larkins, B.A. (2004) A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm. *The Plant Cell Online*, **16**, 1854-1869.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P. and Rombauts, S. (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research*, **30**, 325-327.

Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. *Annual Review of Plant Biology*, **49**, 199-222.

Levitt, J. (1980) Responses of plants to environmental stresses.

Leymarie, J., Robayo-Romero, M.E., Gendreau, E., Benech-Arnold, R.L. and Corbineau, F. (2008) Involvement of ABA in induction of secondary dormancy in barley (Hordeum vulgare L.) seeds. *Plant and cell physiology*, **49**, 1830.

Lin, P.C., Hwang, S.G., Endo, A., Okamoto, M., Koshiba, T. and Cheng, W.H. (2007) Ectopic expression of ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1 in Arabidopsis promotes seed dormancy and stress tolerance. *Plant physiology*, **143**, 745-758.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. and Grill, E. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, **324**, 1064.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A. and Marion-Poll, A. (1996) Molecular identification of zeaxanthin epoxidase of Nicotiana plumbaginifolia, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of Arabidopsis thaliana. *The EMBO journal*, **15**, 2331.

Martínez-Andújar, C., Ordiz, M.I., Huang, Z., Nonogaki, M., Beachy, R.N. and Nonogaki, H. (2011) Induction of 9-cis-epoxycarotenoid dioxygenase in Arabidopsis

thaliana seeds enhances seed dormancy. *Proceedings of the National Academy of Sciences*, **108**, 17225-17229.

McCarty, D.R. (1995) Genetic control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Biology*, **46**, 71-93.

Melhorn, V., Matsumi, K., Koiwai, H., Ikegami, K., Okamoto, M., Nambara, E., Bittner, F. and Koshiba, T. (2008) Transient expression of AtNCED3 and AAO3 genes in guard cells causes stomatal closure in Vicia faba. *Journal of plant research*, **121**, 125-131.

Milborrow, B. (1967) The identification of (+)-abscisin II [(+)-dormin] in plants and measurement of its concentrations. *Planta*, **76**, 93-113.

Millar, A.A., Jacobsen, J.V., Ross, J.J., Helliwell, C.A., Poole, A.T., Scofield, G., Reid, J.B. and Gubler, F. (2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8′-hydroxylase. *The Plant Journal*, **45**, 942-954.

Mizuno, T. and Yamashino, T. (2008) Comparative transcriptome of diurnally oscillating genes and hormone-responsive genes in Arabidopsis thaliana: insight into circadian clock-controlled daily responses to common ambient stresses in plants. *Plant and cell physiology*, **49**, 481.

Mohapatra, P.K., Sarkar, R. and Kuanar, S. (2009) Starch synthesizing enzymes and sink strength of grains of contrasting rice cultivars. *Plant Science*, **176**, 256-263.

Murata, Y., Pei, Z.M., Mori, I.C. and Schroeder, J. (2001) Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cytosolic NAD (P) H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *The Plant Cell Online*, **13**, 2513-2523.

Nam, N., Subbarao, G., Johansen, C. and Chauhan, Y. (1998) Importance of canopy attributes in determining dry matter accumulation of pigeonpea under contrasting moisture regimes.

Nambara, E. and Marion-Poll, A. (2003) ABA action and interactions in seeds. *Trends in plant science*, **8**, 213-217.

Nambara, E. and Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Annu. Rev. Plant Biol.*, **56**, 165-185.

Nemhauser, J.L., Hong, F. and Chory, J. (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell*, **126**, 467-475.

Ohkuma, K., Lyon, J., Addicott, F. and Smith, O. (1963) Abscisin II, an abscission-accelerating substance from young cotton fruit. *Science*, **142**, 1592.

Olsen, O., Potter, R. and Kalla, R. (1992) Histo-differentiation and molecular biology of developing cereal endosperm. *Seed Sci. Res*, **2**, 117-131.

Panda, B.B., Kariali, E., Panigrahi, R. and Mohapatra, P.K. (2009) High ethylene production slackens seed filling in compact panicled rice cultivar. *Plant Growth Regulation*, **58**, 141-151.

Pandey, S., Nelson, D.C. and Assmann, S.M. (2009) Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. *Cell*, **136**, 136-148.

Park, H.Y., Seok, H.Y., Park, B.K., Kim, S.H., Goh, C.H., Lee, B., Lee, C.H. and Moon, Y.H. (2008) Overexpression of Arabidopsis ZEP enhances tolerance to osmotic stress. *Biochemical and biophysical research communications*, **375**, 80-85.

Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A. and Chow, T.F. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science*, **324**, 1068.

Patrick, J. (1997) Phloem unloading: sieve element unloading and post-sieve element transport. *Annual Review of Plant Biology*, **48**, 191-222.

Patrick, J.W. and Offler, C.E. (2001) Compartmentation of transport and transfer events in developing seeds. *Journal of experimental botany*, **52**, 551-564.

Peng, Y.B., Lu, Y.F. and Zhang, D.P. (2003) Abscisic acid activates ATPase in developing apple fruit especially in fruit phloem cells. *Plant, cell & environment*, **26**, 1329-1342.

Perras, M.R., Abrams, S.R. and Balsevich, J.J. (1994) Characterization of an abscisic acid carrier in suspension-cultured barley cells. *Journal of experimental botany*, **45**, 1565.

Peterson, F.C., Burgie, E.S., Park, S.Y., Jensen, D.R., Weiner, J.J., Bingman, C.A., Chang, C.E.A., Cutler, S.R., Phillips Jr, G.N. and Volkman, B.F. (2010) Structural basis for selective activation of ABA receptors. *Nature structural & molecular biology*, **17**, 1109-1113.

Pilot, G., Lacombe, B., Gaymard, F., Chérel, I., Boucherez, J., Thibaud, J.B. and Sentenac, H. (2001) Guard Cell Inward K^+ Channel Activity inArabidopsis Involves Expression of the Twin Channel Subunits KAT1 and KAT2. *Journal of Biological Chemistry*, **276**, 3215.

Price, A.H., Cairns, J.E., Horton, P., Jones, H.G. and Griffiths, H. (2002) Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL

approach: progress and new opportunities to integrate stomatal and mesophyll responses. *Journal of experimental botany*, **53**, 989-1004.

Qin, X. and Zeevaart, J.A.D. (2002) Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in Nicotiana plumbaginifolia increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant physiology*, **128**, 544-551.

Raghavendra, A.S., Gonugunta, V.K., Christmann, A. and Grill, E. (2010) ABA perception and signalling. *Trends in plant science*, **15**, 395-401.

Raz, V., Bergervoet, J. and Koornneef, M. (2001) Sequential steps for developmental arrest in Arabidopsis seeds. *Development*, **128**, 243-252.

Ritchie, S., Swanson, S.J. and Gilroy, S. (2000) Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: new insights from cell and molecular biology. *Seed Science Research*, **10**, 193-212.

Rock, C. and Quatrano, R. (1995) The role of hormones during seed development. *Plant Hormones: Physiology, Biochemistry and Molecular Biology, PJ Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers)*, 671-697.

Rook, F., Hadingham, S.A., Li, Y. and Bevan, M.W. (2006) Sugar and ABA response pathways and the control of gene expression. *Plant, cell & environment*, **29**, 426-434.

Rucker, K., Kvien, C., Holbrook, C. and Hook, J. (1995) Identification of Peanut Genotypes with Improved Drought Avoidance Traits 1. *Peanut science*, **22**, 14-18.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., Sakata, K. and Mizutani, M. (2004) Arabidopsis CYP707As encode (+)-abscisic acid 8'-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. *Plant physiology*, **134**, 1439-1449.

Sambrook, J. and Russell, D.W. (2001) *Molecular cloning: a laboratory manual*: CSHL press.

Sauter, A., Davies, W.J. and Hartung, W. (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *Journal of experimental botany*, **52**, 1991-1997.

Sauter, A., Dietz, K.J. and Hartung, W. (2002) A possible stress physiological role of abscisic acid conjugates in root to shoot signalling. *Plant, cell & environment*, **25**, 223-228.

Sauter, A. and Hartung, W. (2002) The contribution of internode and mesocotyl tissues to root to shoot signalling of abscisic acid. *Journal of experimental botany*, **53**, 297.

Schmittgen, T.D. and Livak, K.J. (2008) Analyzing real-time PCR data by the comparative CT method. *Nature protocols*, **3**, 1101-1108.

Schnittger, A., Weinl, C., Bouyer, D., Schöbinger, U. and Hülskamp, M. (2003) Misexpression of the cyclin-dependent kinase inhibitor ICK1/KRP1 in single-celled Arabidopsis trichomes reduces endoreduplication and cell size and induces cell death. *The Plant Cell Online*, **15**, 303-315.

Schwartz, S.H., Leon-Kloosterziel, K.M., Koornneef, M. and Zeevaart, J.A.D. (1997a) Biochemical characterization of the aba2 and aba3 mutants in Arabidopsis thaliana. *Plant physiology*, **114**, 161.

Schwartz, S.H., Qin, X. and Zeevaart, J.A.D. (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant physiology*, **131**, 1591-1601.

Schulte, D., Close, T.J., Graner, A., Langridge, P., Matsumoto, T., Muehlbauer, G., Sato, K., Schulman, A.H., Waugh, R. and Wise, R.P. (2009) The international barley sequencing consortium—at the threshold of efficient access to the barley genome. Plant physiology, **149,** 142-147.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A.D. and McCarty, D.R. (1997b) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science*, **276**, 1872.

Schweighofer, A., Hirt, H. and Meskiene, I. (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends in plant science*, **9**, 236-243.

Seiler, C., Harshavardhan, V.T., Rajesh, K., Reddy, P.S., Strickert, M., Rolletschek, H., Scholz, U., Wobus, U. and Sreenivasulu, N. (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. *Journal of Experimental Botany*, **62**, 2615-2632.

Seki, M., Ishida, J., Narusaka, M., Fujita, M., Nanjo, T., Umezawa, T., Kamiya, A., Nakajima, M., Enju, A. and Sakurai, T. (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. *Functional & integrative genomics*, **2**, 282-291.

Seo, M., Aoki, H., Koiwai, H., Kamiya, Y., Nambara, E. and Koshiba, T. (2004) Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. *Plant and cell physiology*, **45**, 1694-1703.

Seo, M., Hanada, A., Kuwahara, A., Endo, A., Okamoto, M., Yamauchi, Y., North, H., Marion-Poll, A., Sun, T. and Koshiba, T. (2006) Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid

metabolism and abscisic acid regulation of gibberellin metabolism. *The Plant Journal*, **48**, 354-366.

Seo, M. and Koshiba, T. (2002) Complex regulation of ABA biosynthesis in plants. *Trends in plant science*, **7**, 41-48.

Seo, M. and Koshiba, T. (2011) Transport of ABA from the site of biosynthesis to the site of action. *Journal of plant research*, 1-7.

Setter, T.L. and Flannigan, B.A. (2001) Water deficit inhibits cell division and expression of transcripts involved in cell proliferation and endoreduplication in maize endosperm. *Journal of experimental botany*, **52**, 1401.

Sharp, R.E., Poroyko, V., Hejlek, L.G., Spollen, W.G., Springer, G.K., Bohnert, H.J. and Nguyen, H.T. (2004) Root growth maintenance during water deficits: physiology to functional genomics. *Journal of experimental botany*, **55**, 2343.

Shen, Y.Y., Wang, X.F., Wu, F.Q., Du, S.Y., Cao, Z., Shang, Y., Wang, X.L., Peng, C.C., Yu, X.C. and Zhu, S.Y. (2006) The Mg-chelatase H subunit is an abscisic acid receptor. *Nature*, **443**, 823-826.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response. Plant physiology, **115**, 327**.**

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current opinion in plant biology*, **3**, 217-223.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Current opinion in plant biology*, **6**, 410-417.

Smith, A.M., Quinton-Tulloch, J. and Denyer, K. (1990) Characteristics of plastids responsible for starch synthesis in developing pea embryos. Planta, **180,** 517-523.

Simons, G. and Georgatsos, J. (1988) Lactose-hydrolyzing [beta]-glycosidases of barley meal. *Biochimica et Biophysica Acta (BBA)-General Subjects*, **967**, 17-24.

Sreenivasulu, N., Borisjuk, L., Junker, B.H., Mock, H.P., Rolletschek, H., Seiffert, U., Weschke, W. and Wobus, U. (2010a) Barley Grain Development:: Toward an Integrative View. *International Review of Cell and Molecular Biology*, **281**, 49-89.

Sreenivasulu, N., Radchuk, V., Alawady, A., Borisjuk, L., Weier, D., Staroske, N., Fuchs, J., Miersch, O., Strickert, M. and Usadel, B. (2010b) De-regulation of abscisic acid contents causes abnormal endosperm development in the barley mutant seg8. *The Plant Journal*, **64**, 589-603.

Sreenivasulu, N., Radchuk, V., Strickert, M., Miersch, O., Weschke, W. and Wobus, U. (2006) Gene expression patterns reveal tissue specific signaling networks controlling programmed cell death and ABA regulated maturation in developing barley seeds. *The Plant Journal*, **47**, 310-327.

Sreenivasulu, N., Sopory, S. and Kavi Kishor, P. (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. *Gene*, **388**, 1-13.

Sreenivasulu, N., Usadel, B., Winter, A., Radchuk, V., Scholz, U., Stein, N., Weschke, W., Strickert, M., Close, T.J. and Stitt, M. (2008) Barley grain maturation and germination: metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. *Plant physiology*, **146**, 1738-1758.

Sugimoto-Shirasu, K. and Roberts, K. (2003) "Big it up": endoreduplication and cell-size control in plants. *Current opinion in plant biology*, **6**, 544-553.

Sunkar, R., Bartels, D. and Kirch, H.H. (2003) Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. The Plant Journal, **35**, 452-464.

Sun, L., Sun, Y., Zhang, M., Wang, L., Ren, J., Cui, M., Wang, Y., Ji, K., Li, P. and Li, Q. (2012) Suppression of 9-cis-Epoxycarotenoid Dioxygenase, Which Encodes a Key Enzyme in Abscisic Acid Biosynthesis, Alters Fruit Texture in Transgenic Tomato. *Plant physiology*, **158**, 283-298.

Swain, S., Ross, J., Reid, J. and Kamiya, Y. (1995) Gibberellins and pea seed development. *Planta*, **195**, 426-433.

Szostkiewicz, I., Richter, K., Kepka, M., Demmel, S., Ma, Y., Korte, A., Assaad, F.F., Christmann, A. and Grill, E. (2010) Closely related receptor complexes differ in their ABA selectivity and sensitivity. *The Plant Journal*, **61**, 25-35.

Taiz, L. and Zeiger, E. (2006) Plant Physiology, Forth Edition. Sinaur Associates. *Inc. Sunderland, MA*.

Tal, M., Imber, D. and Itai, C. (1970) Abnormal Stomatal Behavior and Hormonal Imbalance in flacca, a Wilty Mutant of Tomato: I. Root Effect and Kinetin-like Activity. *Plant physiology*, **46**, 367.

Tan, B.C., Joseph, L.M., Deng, W.T., Liu, L., Li, Q.B., Cline, K. and McCarty, D.R. (2003) Molecular characterization of the Arabidopsis 9'cis epoxycarotenoid dioxygenase gene family. *The Plant Journal*, **35**, 44-56.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D. and McCarty, D.R. (1997) Genetic control of abscisic acid biosynthesis in maize. *Proceedings of the National Academy of Sciences*, **94**, 12235.

Tang, T., Xie, H., Wang, Y., Lü, B. and Liang, J. (2009) The effect of sucrose and abscisic acid interaction on sucrose synthase and its relationship to grain filling of rice (Oryza sativa L.). *Journal of experimental botany*, **60**, 2641.

Taylor, I.B., Sonneveld, T., Bugg, T.D.H. and Thompson, A.J. (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. *Journal of Plant Growth Regulation*, **24**, 253-273.

Thomas, T., Wareing, P. and Robinson, P. (1965) Chemistry And Physiology of Dormins' In Sycamore: Action of the Sycamore Dormin as a Gibberellin Antagonist. *Nature*, **205**, 1270-1272.

Thompson, A.J., Andrews, J., Mulholland, B.J., McKee, J.M.T., Hilton, H.W., Horridge, J.S., Farquhar, G.D., Smeeton, R.C., Smillie, I.R.A. and Black, C.R. (2007a) Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant physiology*, **143**, 1905-1917.

Thompson, A.J., Jackson, A.C., Parker, R.A., Morpeth, D.R., Burbidge, A. and Taylor, I.B. (2000a) Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9'cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. *Plant molecular biology*, **42**, 833-845.

Thompson, A.J., Jackson, A.C., Symonds, R.C., Mulholland, B.J., Dadswell, A.R., Blake, P.S., Burbidge, A. and Taylor, I.B. (2000b) Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *The Plant Journal*, **23**, 363-374.

Thompson, A.J., Mulholland, B.J., Jackson, A.C., McKee, J.M.T., Hilton, H.W., Symonds, R.C., Sonneveld, T., Burbidge, A., Stevenson, P. and Taylor, I.B. (2007b) Regulation and manipulation of ABA biosynthesis in roots. *Plant, cell & environment*, **30**, 67-78.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S. and Brettell, R. (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *The Plant Journal*, **11**, 1369-1376.

Ton, J., Flors, V. and Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends in plant science*, **14**, 310-317.

Tottman, D. (1987) The decimal code for the growth stages of cereals, with illustrations. *Annals of Applied Biology*, **110**, 441-454.

Tung, S.A., Smeeton, R., White, C.A., Black, C.R., Taylor, I.B., Hilton, H.W. and Thompson, A.J. (2008) Over-expression of LeNCED1 in tomato (*Solanum lycopersicum* L.) with the rbcS3C promoter allows recovery of lines that accumulate very high levels of abscisic acid and exhibit severe phenotypes. *Plant, cell & environment*, **31**, 968-981.

Uknes, S.J. and Ho, T.H.D. (1984) Mode of action of abscisic acid in barley aleurone layers: abscisic acid induces its own conversion to phaseic acid. *Plant physiology*, **75**, 1126.

Umezawa, T., Okamoto, M., Kushiro, T., Nambara, E., Oono, Y., Seki, M., Kobayashi, M., Koshiba, T., Kamiya, Y. and Shinozaki, K. (2006) CYP707A3, a major ABA 8′-hydroxylase involved in dehydration and rehydration response in *Arabidopsis thaliana*. *The Plant Journal*, **46**, 171-182.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T. and Shinozaki, K. (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proceedings of the National Academy of Sciences*, **106**, 17588-17593.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences*, **97**, 11632.

Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N., Suzuki, H., Saito, K., Shibata, D. and Kobayashi, M. (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *The Plant Journal*, **57**, 1065-1078.

Vahisalu, T., Kollist, H., Wang, Y.F., Nishimura, N., Chan, W.Y., Valerio, G., Lamminmäki, A., Brosché, M., Moldau, H. and Desikan, R. (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature*, **452**, 487-491.

Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J. and Zhu, J.K. (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal*, **45**, 523-539.

Voisin, A.S., Reidy, B., Parent, B., Rolland, G.Ë.L., Redondo, E., Gerentes, D., Tardieu, F.Ç.O. and Muller, B. (2006) Are ABA, ethylene or their interaction involved in the response of leaf growth to soil water deficit? An analysis using naturally occurring variation or genetic transformation of ABA production in maize. *Plant, cell & environment*, **29**, 1829-1840.

Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P.L., Laurière, C. and Merlot, S. (2009) Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. *The Plant Cell Online*, **21**, 3170-3184.

Walton, D.C. and Li, Y. (1995) Abscisic acid biosynthesis and metabolism. *Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed*, **2**, 140-157.

Wan, X.R. and Li, L. (2006) Regulation of ABA level and water-stress tolerance of Arabidopsis by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. *Biochemical and biophysical research communications*, **347**, 1030-1038.

Wang, N., Fang, W., Han, H., Sui, N., Li, B. and Meng, Q.W. (2008) Overexpression of zeaxanthin epoxidase gene enhances the sensitivity of tomato PSII photoinhibition to high light and chilling stress. *Physiologia Plantarum*, **132**, 384-396.

Wareing, P., Eagles, C. and Robinson, P. (1964) Natural inhibitors as dormancy agents. *Regulateurs naturels de la croissance vegetale. Paris: Centre Nationale de la Recherche Scientifique*, 1-9.

Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., dit Frey, N.F. and Leung, J. (2008) An update on abscisic acid signaling in plants and more…. *Molecular plant*, **1**, 198-217.

Weber, H., Borisjuk, L. and Wobus, U. (1997) Sugar import and metabolism during seed development. *Trends in plant science*, **2**, 169-174.

Weber, H., Buchner, P., Borisjuk, L. and Wobus, U. (1996) Sucrose metabolism during cotyledon development of Vicia faba L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. *The Plant Journal*, **9**, 841-850.

Weber, H., Heim, U., Borisjuk, L. and Wobus, U. (1995) Cell-type specific, coordinate expression of two ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development of Vicia faba L. *Planta*, **195**, 352-361.

Weigel, D. and Glazebrook, J. (2002) *Arabidopsis: a laboratory manual*: Cold Spring Harbor Laboratory Pr.

Weiner, J.J., Peterson, F.C., Volkman, B.F. and Cutler, S.R. (2010) Structural and functional insights into core ABA signaling. *Current opinion in plant biology*, **13**, 495-502.

Weschke, W., Panitz, R., Sauer, N., Wang, Q., Neubohn, B., Weber, H. and Wobus, U. (2000) Sucrose transport into barley seeds: molecular characterization of two

transporters and implications for seed development and starch accumulation. *The Plant Journal*, **21**, 455-467.

White, C.N. and Rivin, C.J. (2000) Gibberellins and seed development in maize. II. Gibberellin synthesis inhibition enhances abscisic acid signaling in cultured embryos. *Plant physiology*, **122**, 1089-1098.

Wilkinson, S. (1999) pH as a stress signal. *Plant Growth Regulation*, **29**, 87-99.

Wilkinson, S. and Davies, W.J. (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant, cell & environment*, **25**, 195-210.

Wilkinson, S. and Hartung, W. (2009) Food production: reducing water consumption by manipulating long-distance chemical signalling in plants. *Journal of experimental botany*, **60**, 1885.

Windsor, M.L., Milborrow, B. and McFarlane, I.J. (1992) The uptake of (+)-S-and (−)-R-abscisic acid by suspension culture cells of Hopbush (Dodonaea viscosa). *Plant physiology*, **100**, 54.

Xiong, L., Gong, Z., Rock, C.D., Subramanian, S., Guo, Y., Xu, W., Galbraith, D. and Zhu, J.K. (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in Arabidopsis. *Developmental Cell*, **1**, 771-781.

Xiong, L., Lee, H., Ishitani, M. and Zhu, J.K. (2002a) Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis. *Journal of Biological Chemistry*, **277**, 8588.

Xiong, L., Schumaker, K.S. and Zhu, J.K. (2002b) Cell signaling during cold, drought, and salt stress. *The Plant Cell Online*, **14**, S165-S183.

Xiong, L. and Zhu, J.K. (2003) Regulation of abscisic acid biosynthesis. *Plant physiology*, **133**, 29-36.

Xu, Z.J., Nakajima, M., Suzuki, Y. and Yamaguchi, I. (2002) Cloning and characterization of the abscisic acid-specific glucosyltransferase gene from adzuki bean seedlings. *Plant physiology*, **129**, 1285-1295.

Yadav, R.S., Hash, C., Bidinger, F., Devos, K. and Howarth, C.J. (2004) Genomic regions associated with grain yield and aspects of post-flowering drought tolerance in pearl millet across stress environments and tester background. *Euphytica*, **136**, 265-277.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.*, **57**, 781-803.

Yang, J. and Zhang, J. (2006) Grain filling of cereals under soil drying. *New phytologist*, **169**, 223-236.

Yang, J., Zhang, J., Liu, K., Wang, Z. and Liu, L. (2006a) Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. *New phytologist*, **171**, 293-303.

Yang, J., Zhang, J., Wang, Z., Liu, K. and Wang, P. (2006b) Post-anthesis development of inferior and superior spikelets in rice in relation to abscisic acid and ethylene. *Journal of experimental botany*, **57**, 149.

Yang, J., Zhang, J., Wang, Z., Xu, G. and Zhu, Q. (2004) Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. *Plant physiology*, **135**, 1621-1629.

Yang, S.H. and Zeevaart, J.A.D. (2006) Expression of ABA 8′-hydroxylases in relation to leaf water relations and seed development in bean. *The Plant Journal*, **47**, 675-686.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *The Plant Journal*, **61**, 672-685.

Yue, Y., Zhang, M., Zhang, J., Duan, L. and Li, Z. (2011) Arabidopsis LOS5/ABA3 overexpression in transgenic tobacco (*Nicotiana tabacum* cv. Xanthi-nc) results in enhanced drought tolerance. *Plant Science*.

Zhang, Y., Tan, J., Guo, Z., Lu, S., He, S., Shu, W. and Zhou, B. (2009) Increased abscisic acid levels in transgenic tobacco over-expressing 9 cis-epoxycarotenoid dioxygenase influence H_2O_2 and NO production and antioxidant defences. *Plant, cell & environment*, **32**, 509-519.

Zhang, J.Z., Creelman, R.A. and Zhu, J.K. (2004) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant physiology, **135,** 615-621.

Zhang, Y., Yang, J., Lu, S., Cai, J. and Guo, Z. (2008) Overexpressing SgNCED1 in tobacco increases ABA level, antioxidant enzyme activities, and stress tolerance. *Journal of Plant Growth Regulation*, **27**, 151-158.

Zhao, Z., Chen, G. and Zhang, C. (2001) Interaction between reactive oxygen species and nitric oxide in drought-induced abscisic acid synthesis in root tips of wheat seedlings. *Functional Plant Biology*, **28**, 1055-1061.

Zhou, R., Cutler, A.J., Ambrose, S.J., Galka, M.M., Nelson, K.M., Squires, T.M., Loewen, M.K., Jadhav, A.S., Ross, A.R.S. and Taylor, D.C. (2004) A new abscisic acid catabolic pathway. *Plant physiology*, **134**, 361.

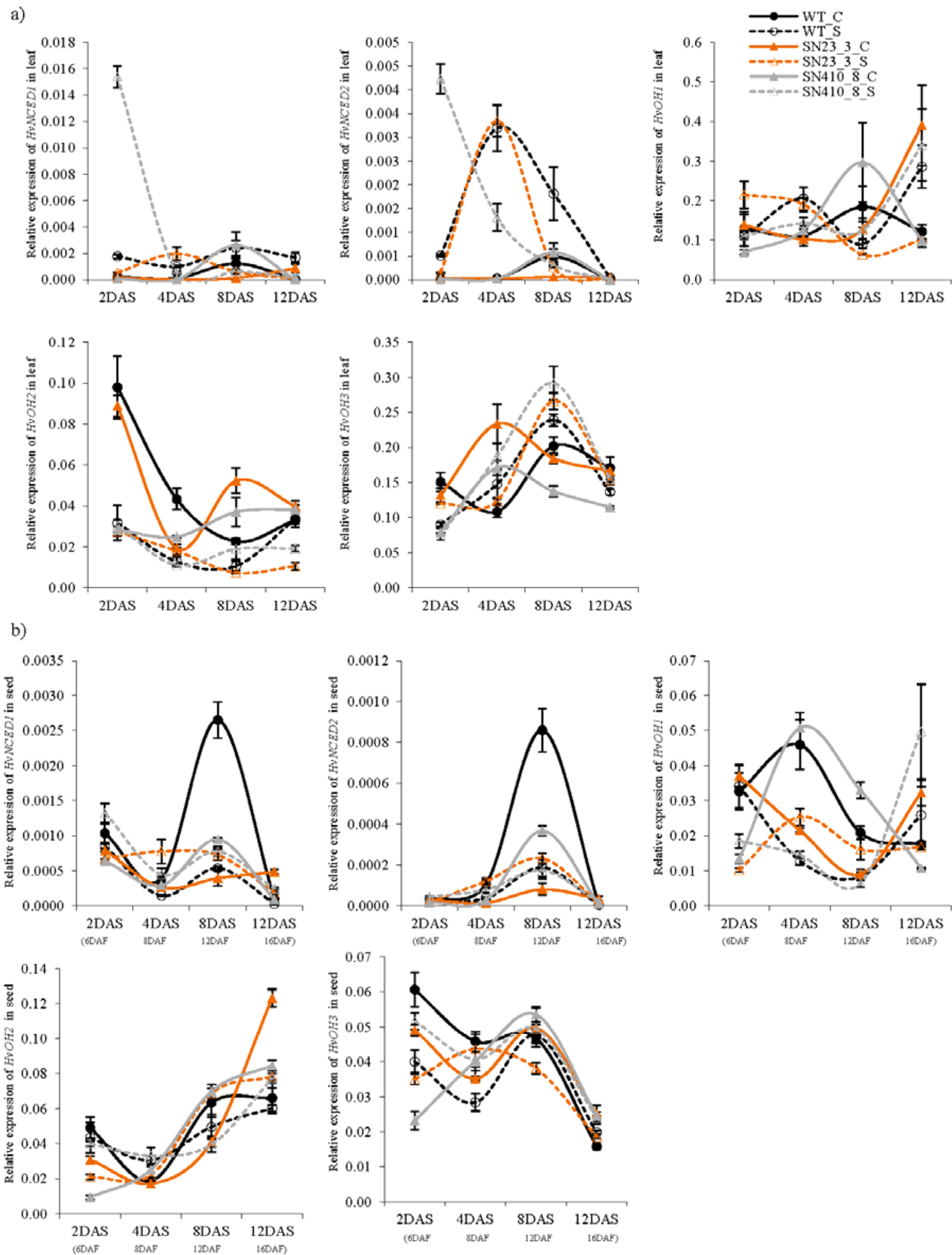
Zhu, C., Kauder, F., Romer, S. and Sandmann, G. (2007) Cloning of two individual cDNAs encoding 9-cis-epoxycarotenoid dioxygenase from *Gentiana lutea*, their tissue-specific expression and physiological effect in transgenic tobacco. *Journal of plant physiology*, **164**, 195-204.

Zhu, G., Ye, N., Yang, J., Peng, X. and Zhang, J. (2011) Regulation of expression of starch synthesis genes by ethylene and ABA in relation to the development of rice inferior and superior spikelets. *Journal of experimental botany*, **62**, 3907-3916.

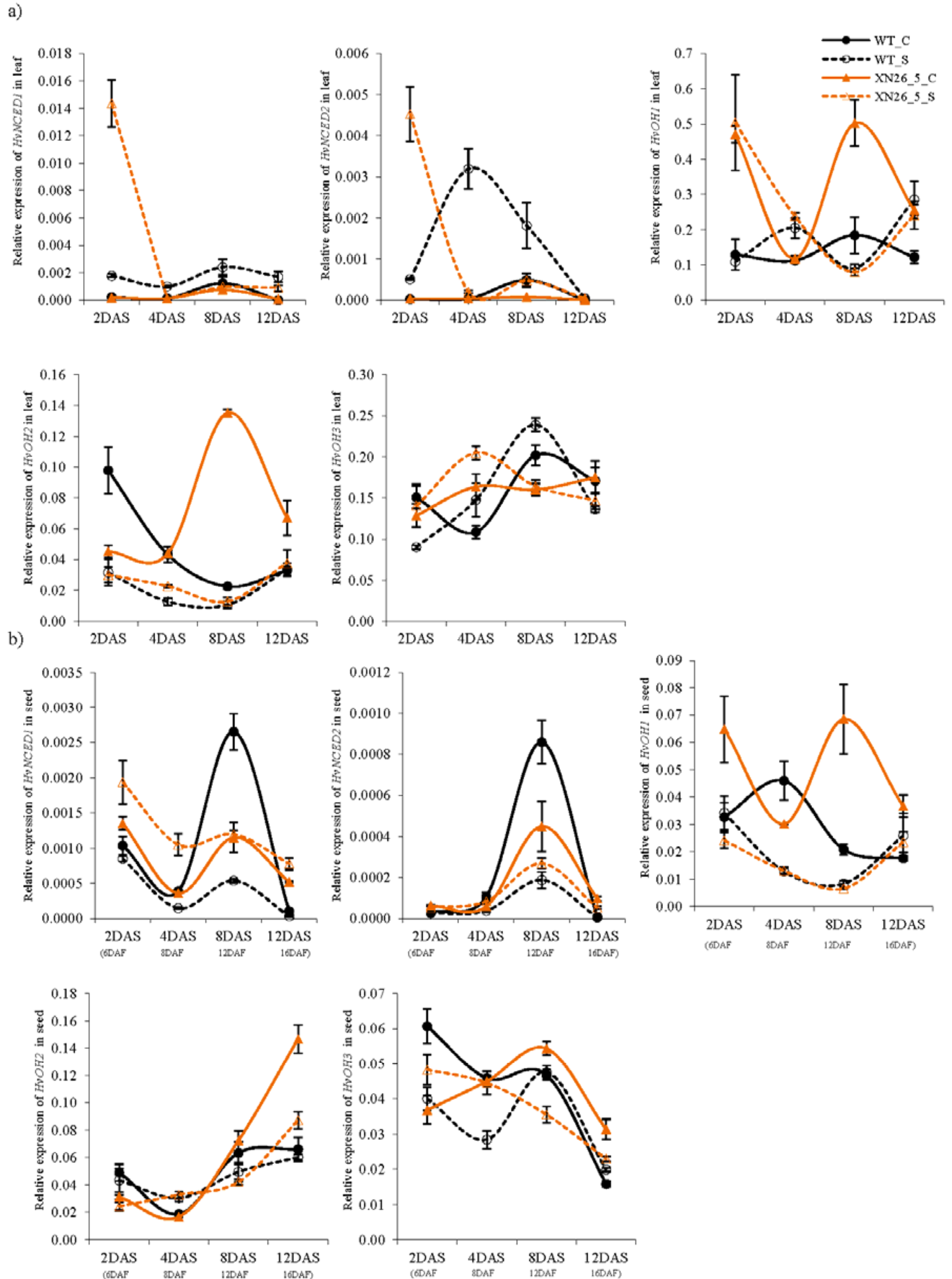
Zinselmeier, C., Jeong, B.R. and Boyer, J.S. (1999) Starch and the control of kernel number in maize at low water potentials. *Plant physiology*, **121**, 25-36.

Zou, J., Abrams, G.D., Barton, D.L., Taylor, D.C., Pomeroy, M.K. and Abrams, S.R. (1995) Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of *Brassica napus* L. cv Reston (biological responses in the presence of 8 [prime]-hydroxyabscisic acid). *Plant physiology*, **108**, 563-571.

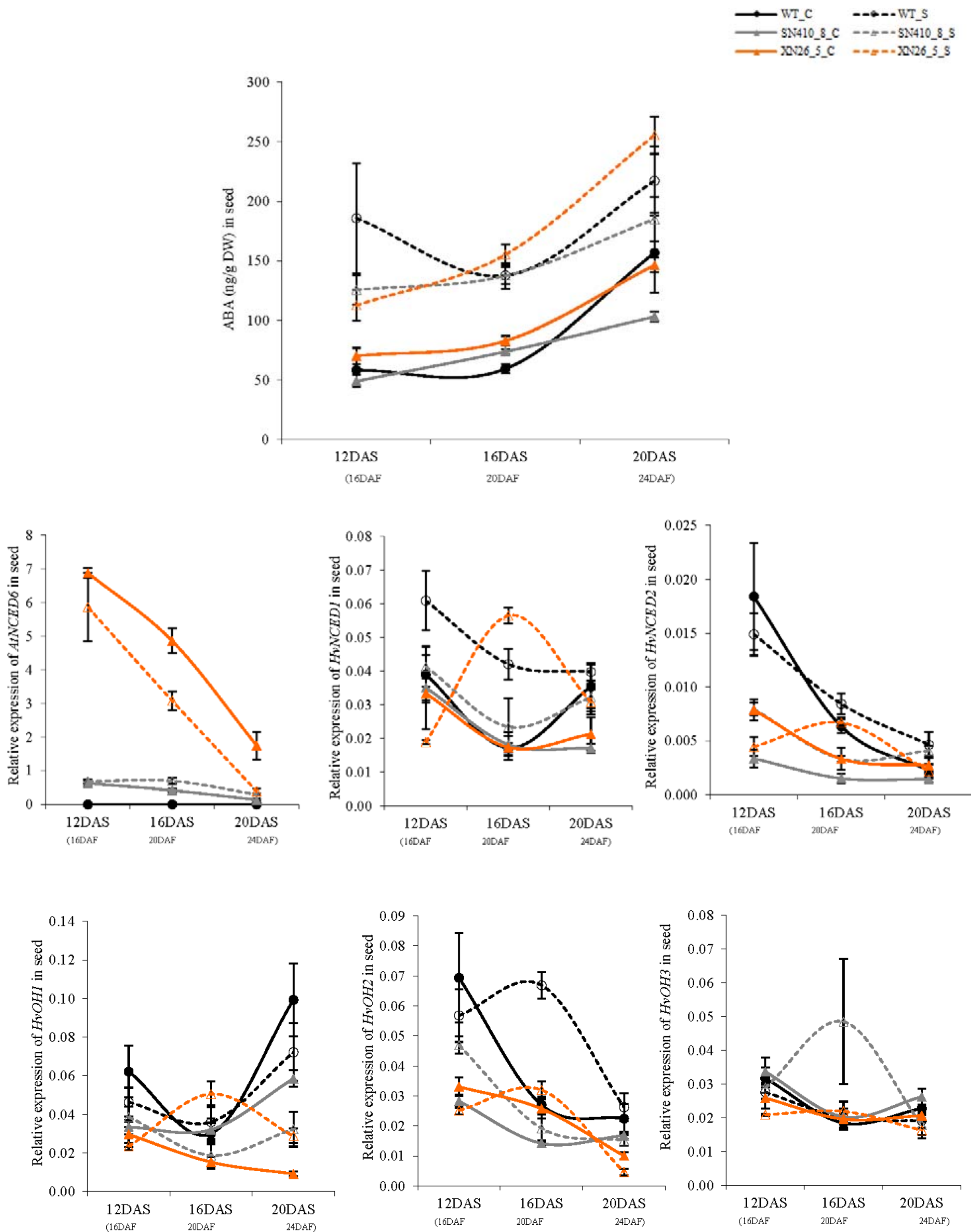
8 Supplementary figures and tables:



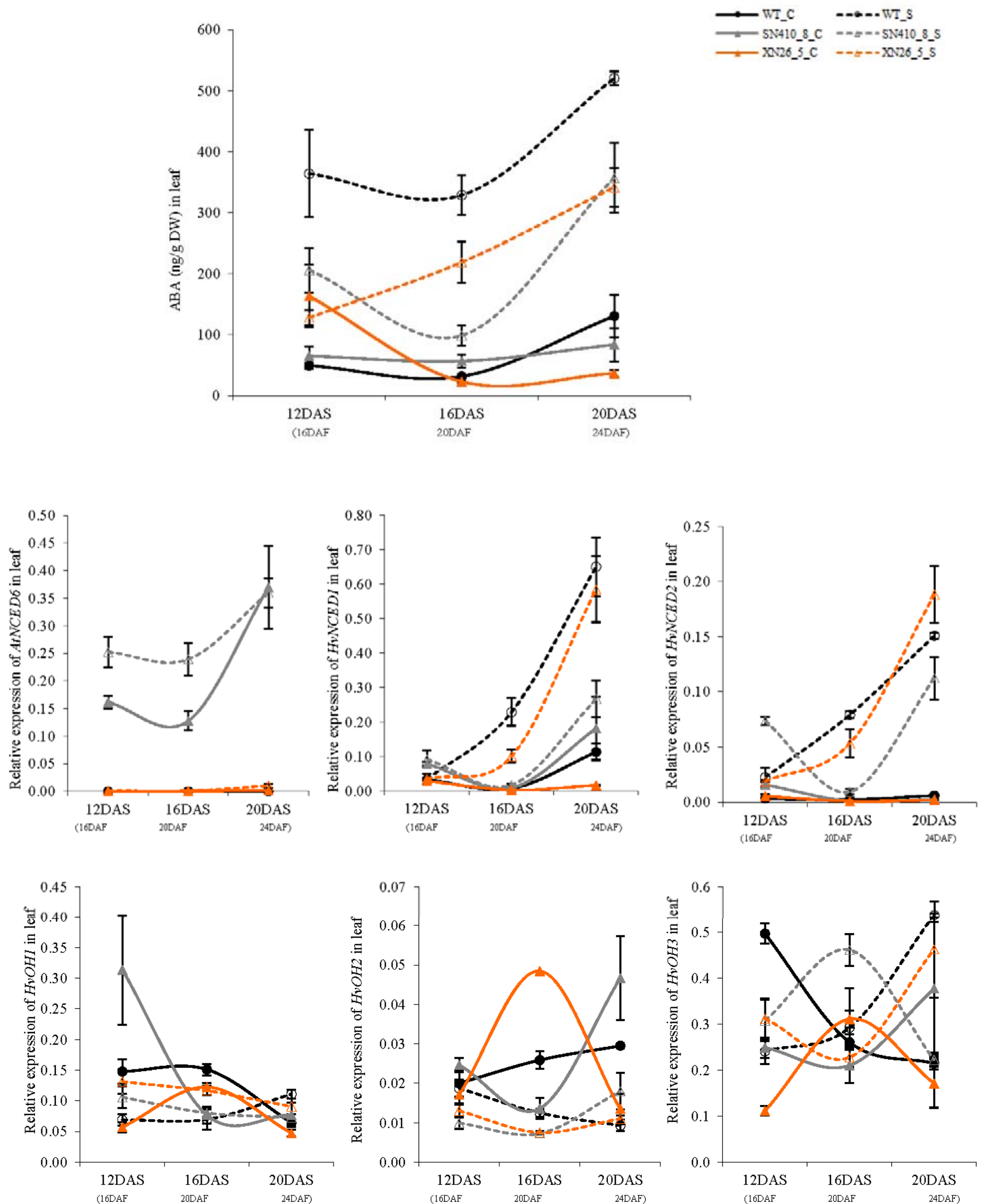
Supplementary figure 1: The relative expression of the endogenous *HvNCEDs* and *HvABA8'OH s* gene in comparison to house keeping genes in the a)flag leaf and b)seed under control and stress condition. Mean ±SE (n = 4) ,Solid lines represent control condition and dashed lines represent drought stress conditions, respectively. WT: black lines; SN410_8: light grey lines; and SN23_3: orange lines.



Supplementary figure 2: The relative expression of the endogenous HvNCEDS and *HvABA8'OH s* gene in comparison to house keeping genes in the a)flag leaf and b)seed under control and stress condition. Mean ± SE (n = 4) ,Solid lines represent control condition and dashed lines represent drought stress conditions, respectively. WT: black lines; and XN26_5: orange lines.



Supplementary figure 3: ABA content and the relative expression of the transgene, endogenous *HvNCED*s and *HvABA8'OH s* gene in comparison to house keeping genes in the seed under control and stress condition. Mean ± SE (n = 6) ,Solid lines represent control condition and dashed lines represent drought stress conditions, respectively. WT: black lines; SN410_8: light grey lines; and XN26_5: orange lines.



Supplementary figure 4 : ABA content and the relative expression of the transgene, endogenous *HvNCEDs* and *HvABA8'OH s* gene in comparison to house keeping genes in the flag leaf under control and stress condition. Mean ± SE (n = 6) ,Solid lines represent control condition and dashed lines represent drought stress conditions, respectively. WT: black lines; SN410_8: light grey lines; and XN26_5: orange lines.

Supplementary table 1: List of primers used for qRT-PCR in this study

Name	Sequence(5' to 3')	Source/Tm (°C)
R2-HZ44D03-F	GATGACTGCAACGCTCACAC	Seiler et al., 2011
R2-HZ44D03-R	CTCAAAGGAAATAATCAGGCGTC	Seiler et al., 2011
5'HZ42K12-F	AAGGAAGCCGCTGAGATGAACA	Seiler et al., 2011
5'HZ42K12-R	AGCACCCACGCGTACTTGAATG	Seiler et al., 2011
3'HZ42K12-F	TTACCCTCCTCTTGGTCGTTTTG	Seiler et al., 2011
3'HZ42K12-R	TCTTCTTGATGGCAGCCTTGG	Seiler et al., 2011
AtNCED6-F	GACAAAGGTTATGTAATGGGG	57
AtNCED6-R	ACTTGTTCCTTCAACTGATTC	55
HvABA8'OH1-F	GTACAACTCGATGCCGGTG	59
HvABA8'OH1-R	CGGGCTGAGATGATGTGG	58
HvABA8'OH2-F	ACTCCTTCCCCAACAGCTTC	60
HvABA8'OH2-R	GCTCCCTACGCTCGTGCAC	64
HvABA8'OH3-F	TAGTACGTAGTGAGTAATTCCGGA	62
HvABA8'OH3-R	TGGGAGATTCTTTCAAATTTACATC	59
HvNCED1-F	CCAGCACTAATCGATTCC	Millar et al., 2006
HvNCED1-R	GAGAGTGGTGATGAGTAA	Millar et al., 2006
HvNCED2-F	CATGGAAAGAGGAAGTTG	Millar et al., 2006
HvNCED2-R	GAAGCAAGTGTGAGCTAAC	Millar et al., 2006
HvAGP-S1b-F	GCCGCCTCCCCTTCCAAGAT	61
HvAGP-S1b-R	CAGAGGAGGCGGAGGGAGTCG	62
HvAGP-S2-F	TCCCATGCAGCAAGTTCACCAA	60
HvAGP-S2-R	TTCTTTCCGCCGACGACACTTC	60
HvAGP-L1-F	AAAGTGGCAGCGAGCAAACAAA	59
HvAGP-L1-R	GACAGAGGCAGCGGGAAAACC	60
HvAGP-L2-F	GCCGGCGCAAGAAGCACA	60
HvAGP-L2-R	ATCGCGCAACCAATGAATCC	57
HvSSI-F	CCGCCGAGCAGTACGAGCAGA	62
HvSSI-R	GGCCGCGGAAGGATGAGGAA	63
HvSSIIa-F	CTACCGGGACCACAAGGAGAGC	58
HvSSIIa-R	TGGACCGGGTAGCAGCGTTCA	62
HvSSIIb-F	CTGACGCTGACGCCTCTCCTG	60
HvSSIIb-R	TCGCGTGATCCTTGGTTACTCC	58
HvSSIIIa-F2	ATCCTCTTGCCTCCTCCATCTG	56
HvSSIIIa-R2	CGTCACTGCGGTTCTTATCTCG	56
HvSSIIIb-F	TGGCGGGGAGAGGAACAAATGA	62
HvSSIIIb-R	GTCGCGAAACCTGGCAAACACA	62

Supplementary table 1 : continued….

Name	Sequence(5' to 3')	Source/Tm (°C)
HvSSIV-F	GCTCTCGACGCCCATTTCAGGT	61
HvSSIV-R	CCCATTTTTCGCCCCCGTTCT	63
HvGBSS1a-F	TGCCGGTGGACAGGAAGGT	57
HvGBSS1a-R	CCCGGTGCCAAGGAGAATGA	59
HvGBSS1b-F	GGTTCCACATGGGTTCGTTCAA	58
HvGBSS1b-R	ACCGGCGTTCTGTACTGTTTCA	56
HvSBE1-F	TCCGATGCTTTGATGTTTGGTG	57
HvSBE1-R	GGGCGGTTGTTGAAGTTTGTTT	56
HvSBE2a-F	ACAACAGGCCACGCTCTTTCTC	57
HvSBE2a-R	TTGCCCGTCGCTTCGCTCAC	63
HvSBE2b-F	GACGCTGGACTCTTTGGTGGAT	57
HvSBE2b-R	ACGAATGGGGCCTGTTGTCA	57
HvISA1-F	CACAAAAGGGGGCAACAACAAT	57
HvISA1-R	GGCCAAGACCCTCGCACTCC	60
HvISA2-F	AGGGGACCGGCATTTTCTTTCA	60
HvISA2-R	GGACTGCACTGTTCTCGGATGG	58
HvISA3-F	GGCATTCACGATACACGACCAC	57
HvISA3-R	CCACACGGTTCCAGCTTTTATG	56
HvAMY1-F	CGATCGAGCATGAATTTCCTGA	57
HvAMY1-R	TCCGCGTGAATACCAGAAGTGA	57
HvAMY2-F	TAGTCCGAGCTCGTGTTGTCCA	57
HvAMY2-R	CGAAACCATGGGTAGCGAAAAT	57
HvAMY3-F	TTAGTCCGAGCTCACGCTGTTC	56
HvAMY3-R	CAATGCATGTCCCTCATCCTCA	57
HvAMY4-F	CGCCAGATTACCGCAGCATACC	60
HvAMY4-R	GCAGGAGAAGCGCGGCATTAG	61
HvBAM1-F	ATGGGTGGGCAGGCTGAAGG	61
HvBAM1-R	GAGCTCCCCACCCATGCCACTA	62
HvBAM2-F	GCGCTCCTTGCCCCTGTGG	62
HvBAM2-R	CTTGGCGGCTTATTTCCTGTGC	60
HvBAM3-F	GGAGTAGTTGCCGCGTCTGAAG	58
HvBAM3-R	CGCCGAAACTGAAACCGAAACC	61
HvBAM4-F	CGGGAGGAGCGGATGGTG	58
HvBAM4-R	GCAGCGCCGAGGAGGACTA	58

Supplementary table 1 : continued….

Name	Sequence(5' to 3')	Source/Tm (°C)
HvBAM5-F	GACGCCGCCAAGGAGCAGAG	62
HvBAM5-R	CCCGGCGCACGTTACCACAT	62
HvBAM6-F	CGCCGCTCTAGGATGTAAACCA	58
HvBAM6-R	CAAAGGCCTGGGACAACAAGAA	58
HvBAM7-F	GTTGGTGGGGGATTGTAGAAGC	56
HvBAM7-R	ACCAGATCCACACTCCCCAGAC	56
HvCWINV1-F	ACGGCGTGGGAGATGAAGAAGC	61
HvCWINV1-R	CCGCATGTCGATCCACCCTAAA	60
HvCWINV2-F	GGTCGTGGGCCAGTGAATCC	59
HvCWINV2-R	TGCCATCTCCGTCCAGCCATAC	61
HvCWINV3-F	ACCCTCAGCACGCCGAAAACAA	62
HvCWINV3-R	CGAGGCCGGTCCCGTTATTGA	62
HvVacINV-F	TGGCCGGTGGAGGAAGTGGAG	63
HvVacINV-R	GGTGGCGCGATGAAGGTTGAGT	62
HvSucSyn-F	GGCGCATTCATCGAGCAGGAG	61
HvSucSyn-R	ACCCCACGACAGACGGACCAA	61

Supplementary table 2 :List of primers used for transgenic study

Name	Sequence(5' to 3')	Tm(°C)	Application
pOsSalT-F	GAATTCGCCCTTCAACAACC	60	Promoter amplification
pOsSalT-R	GAATTCGCCCTTTGTAGATT	60	
AtNCED6-F	CCACCATGCAACACTCTCTTCGT	65	Full length gene isolation
AtNCED6-R	GATCAGAAAACTTGTTCCTTCAAC	60	
AtNCED6_HindIII-F	ACAAGCTTCCAATGCAACACTCTC *	64	Subcloning in to pNos-AB-M
AtNCED6_HindIII-R	ACAAGCTTTCAGAAAACTTGTTCC *	65	
AtNCED6_PstI-F	ACCTGCAGCCAATGCAACACTCTC *	67	Subcloning in to pAXi-pNOS-ABM
AtNCED6_BanHI-R	ACGGATCCTCAGAAAACTTGTTCC *	64	
pOsSalT_1320-F	TGTGAAAGTACAAAAGCACTCG	58	Confirmation of transgenic plants
pOsSalT_1465-F	AACAAAACTGAGTGGGAATAC	58	
AtNCED6_385-R	AGACAAGAAGGAATCTGACCAA	58	
p1AX1_2083-F	CTAGGATTAAGCCGATTACGTG	60	
p1AX1_1968-F	AATGGCTAACGGACACATATTC	58	

* Restriction sites are written in red

Supplementary table 3: Relative mRNA expression levels of ABA biosynthesis, catabolism and deconjugation related genes in LP110 under control and stress conditions analyzed by qRT-PCR. A: expression in seeds. B: expression in flag leaves. n.d.: not detected. Serine/threonine protein phosphatase PP2A-4 catalytic subunit (reference EST: HZ44D03) was used as reference gene and for delta Ct calculations.

A: Expression in seeds

Gene	12DAF control	12DAF stress	16DAF control	16DAF stress	20DAF control	20DAF stress	25DAF control	25DAF stress
HvZEP1	0.1467	0.1372	0.3179	0.1821	0.1425	0.2453	0.1737	0.2131
HvZEP2	0.0135	0.0062	0.0258	0.0292	0.0184	0.0473	0.0125	0.0151
HvZEP3	0.0003	0.0002	0.0001	0.0001	0.0004	0.0007	0.0019	0.0011
HvZEP4	0.0024	0.0017	0.0008	0.0010	0.0020	0.0019	0.0056	0.0048
HvZEP5	0.0003	0.0011	0.0013	0.0016	0.0032	0.0026	0.0058	0.0131
HvNCED1	0.0024	0.0005	0.0016	0.0178	0.0069	0.0032	0.0028	0.0035
HvNCED2	0.0003	0.0022	0.0004	0.0013	0.0001	0.0099	0.0043	0.0062
HvCCD3	0.0057	0.0139	0.0169	0.0150	0.0079	0.0131	0.0038	0.0114
HvCCD4	0.0699	0.0811	0.0596	0.0552	0.0567	0.0697	0.1712	0.1038
HvCCD5	0.0600	0.0832	0.1783	0.2419	0.2293	0.2425	0.3776	0.3272
HvSDR1	0.1312	0.1373	0.1234	0.2559	0.3389	0.4081	0.2219	0.1178
HvSDR2	0.2179	0.2115	0.1976	0.4028	0.4797	0.5531	0.3366	0.1698
HvSDR3	0.3669	0.5244	0.6299	0.7092	0.6034	0.5939	0.5246	0.3584
HvSDR4	0.4911	0.5086	0.2399	0.2101	0.1008	0.2736	0.0750	0.0704
HvSDR5	0.0346	0.0106	0.0113	0.0187	0.0258	0.0258	0.0027	0.0053
HvSDR6	0.1082	0.0976	0.1150	0.1565	0.1602	0.1499	0.0651	0.0655
HvSDR7	0.1353	0.1832	0.1898	0.2017	0.1795	0.2375	0.1396	0.1015
HvMCSU1	0.1941	0.1450	0.2524	0.2333	0.2981	0.3334	0.3044	0.2733
HvMCSU2	0.4161	0.3830	0.5436	0.5365	0.5960	0.7675	0.8412	0.7069
HvMCSU3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HvAO1	0.0238	0.1667	5.5512	6.9087	15.4193	20.9077	22.1224	17.8885
HvAO2	0.0059	0.0079	0.0637	0.0092	0.0080	0.0091	0.0653	0.1530
HvAO3	0.1141	0.0154	0.0161	0.0228	0.0291	0.0320	0.0199	0.0358
HvAO4	0.2191	0.2215	0.4794	0.3953	0.6803	0.7643	0.9736	1.4236
HvAO5a	0.0954	0.1297	0.3039	0.2452	0.3914	0.5777	0.6240	0.9601
HvAO5b	0.0301	0.0178	0.1874	0.2464	0.5622	0.6722	0.8703	1.2127
HvAO6	0.0056	0.0013	0.0040	0.0034	0.0049	0.0085	0.0107	0.0279
HvAO7	0.0361	0.1104	4.2132	5.0501	11.2914	12.0088	9.7516	7.5458
HvABA8'OH-1	0.0032	0.0041	0.0027	0.0069	0.0017	0.0017	0.0087	0.0110
HvABA8'OH-2	0.0030	0.0041	0.0140	0.0088	0.0160	0.0198	0.0197	0.0188
HvABA8'OH-3	0.0241	0.0209	0.0275	0.0332	0.0169	0.0174	0.0081	0.0077
HvBG1	0.0556	0.0767	0.1958	0.2548	0.1388	0.2524	0.2038	0.1864
HvBG2	1.0626	1.2364	2.1606	2.0127	1.3806	1.6169	0.9468	0.5561
HvBG3	1.4874	1.4521	2.5940	9.3266	12.6924	31.4776	47.3725	49.9350
HvBG4	0.0016	0.0046	0.0249	0.0163	0.1335	0.1388	0.1775	0.1098
HvBG5	0.1646	0.1719	0.0340	0.0112	0.0105	0.0059	0.0110	0.0293
HvBG6	0.3583	0.3172	0.3801	0.6430	0.3466	0.6093	0.3497	0.2345
HvBG7	0.0908	0.1132	0.2901	0.4781	0.7115	1.1848	0.6691	0.7862

HvBG8	0.0042	0.0062	0.0216	0.0563	0.3814	0.5411	1.2044	1.0571
HvBG9	0.0425	0.0351	0.0301	0.0283	0.0493	0.0620	0.1123	0.1163
HvBG10	0.0001	0.0205	0.0222	0.0135	0.0131	0.0180	0.0132	0.0068

B: Expression in flag leaves

Gene	16DAF control	16DAF stress	20DAF control	20DAF stress
HvZEP1	3.8299	5.4648	9.6187	8.3467
HvZEP2	0.0242	0.0463	0.0465	0.0860
HvZEP3	0.0007	0.0007	0.0008	0.0022
HvZEP4	0.1994	0.1102	0.4227	0.2289
HvZEP5	0.0115	0.0026	0.0129	0.0616
HvNCED1	0.0039	0.0147	0.0184	0.0101
HvNCED2	0.0049	0.0300	0.0222	0.0037
HvCCD3	0.0097	0.0058	0.0094	0.0193
HvCCD4	1.7864	1.8574	1.7428	0.5318
HvCCD5	1.9380	0.8109	1.4217	2.1072
HvSDR1	0.0007	0.0016	0.0040	0.0015
HvSDR2	0.0016	0.0022	0.0066	0.0008
HvSDR3	0.2524	0.3561	0.3217	0.4205
HvSDR4	0.2895	0.2222	0.2667	0.0903
HvSDR5	0.0014	0.0006	0.0006	0.0000
HvSDR6	0.0040	0.0020	0.0036	0.0241
HvSDR7	0.0939	0.1541	0.1329	0.1879
HvMCSU1	0.4423	0.3894	0.9208	0.9169
HvMCSU2	2.0367	2.9760	2.7309	3.7857
HvMCSU3	n.d.	n.d.	n.d.	n.d.
HvAO1	0.0004	0.0010	0.0025	0.0027
HvAO2	0.0002	0.0026	0.0012	0.0001
HvAO3	0.0155	0.0133	0.0182	0.0134
HvAO4	4.4604	3.1765	6.2415	8.1300
HvAO5a	3.2830	2.1092	3.7211	5.1448
HvAO5b	3.5660	2.7175	6.3833	5.8375
HvAO6	0.0758	0.0379	0.1132	0.0847
HvAO7	0.0006	0.0037	0.0012	0.0030
HvABA8'OH-1	0.0448	0.1172	0.0477	0.3586
HvABA8'OH-2	0.0076	0.0051	0.0084	0.0221
HvABA8'OH-3	0.0872	0.0795	0.1788	0.1504
HvBG1	0.6383	0.8586	1.1766	1.0527
HvBG2	0.2657	0.1796	0.3597	0.1285
HvBG3	0.0094	0.0264	0.0716	0.1125
HvBG4	2.8717	2.8949	3.8379	1.1554
HvBG5	0.0034	0.0062	0.0020	0.0019
HvBG6	n.d.	n.d.	n.d.	n.d.
HvBG7	1.6853	1.6116	3.2347	4.5612
HvBG8	0.0062	0.0470	0.0117	0.1746
HvBG9	1.3094	1.3243	1.4641	1.2162
HvBG10	0.0348	0.0285	0.0245	0.0336

Supplementary table 4: Correlation of ABA and Starch with the starch metabolism genes;WT-wild type,SN-SN410_8,XN-XN26_5,C-control,S-stress

P< 0.05	P<0.01	P<0.001

WT_SN_XN_C_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.259	0.028
HvAGP_L1	-0.185	0.121	-0.321	0.006
HvAGP_L2	-0.308	0.009	0.144	0.228
HvAGP_S1b	0.180	0.130	0.735	0.000
HvAGP_S2	-0.279	0.018	0.112	0.348
HvAMY1	0.249	0.035	-0.648	0.000
HvAMY2	-0.014	0.908	0.271	0.022
HvAMY3	-0.171	0.151	0.532	0.000
HvAMY4	0.136	0.256	-0.083	0.487
HvBAM1	-0.097	0.419	0.875	0.000
HvBAM2	0.120	0.314	-0.868	0.000
HvBAM3	0.390	0.001	0.245	0.038
HvBAM4	-0.447	0.000	0.385	0.001
HvBAM5	-0.041	0.729	0.484	0.000
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.243	0.040	0.527	0.000
HvCWINV1	-0.303	0.010	-0.147	0.219
HvCWINV2	-0.213	0.072	0.742	0.000
HvCWINV3	-0.140	0.241	0.657	0.000
HvGBSS1a	-0.383	0.001	0.671	0.000
HvGBSS1b	0.258	0.029	-0.238	0.044
HvISA1	-0.507	0.000	0.365	0.002
HvISA2	-0.275	0.019	0.577	0.000
HvISA3	-0.049	0.683	0.792	0.000
HvSBE1	-0.334	0.004	0.933	0.000
HvSBE2a	-0.512	0.000	0.167	0.161
HvSBE2b	-0.403	0.000	0.394	0.001
HvSSI	-0.319	0.006	0.037	0.755
HvSSIIIa	-0.246	0.037	0.344	0.003
HvSSIIIb	-0.119	0.319	0.708	0.000
HvSSIIa	-0.361	0.002	-0.039	0.744
HvSSIIb	0.196	0.098	-0.058	0.629
HvSSIV	-0.197	0.098	0.624	0.000
HvSucSyn	-0.243	0.040	0.144	0.228
HvVacINV	0.265	0.024	-0.073	0.541
starch	-0.259	0.028		

WT_SN_XN_C

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.661	0.000
HvAGP_L1	-0.062	0.721	-0.148	0.388
HvAGP_L2	-0.558	0.000	0.458	0.005
HvAGP_S1b	-0.524	0.001	0.819	0.000
HvAGP_S2	-0.571	0.000	0.415	0.012
HvAMY1	0.732	0.000	-0.780	0.000
HvAMY2	-0.018	0.915	0.287	0.089
HvAMY3	-0.322	0.056	0.626	0.000
HvAMY4	0.514	0.001	-0.240	0.158
HvBAM1	-0.547	0.001	0.913	0.000
HvBAM2	0.800	0.000	-0.931	0.000
HvBAM3	0.389	0.019	0.012	0.945
HvBAM4	-0.069	0.691	0.382	0.022
HvBAM5	-0.059	0.731	0.511	0.001
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.415	0.012	0.651	0.000
HvCWINV1	0.567	0.000	-0.380	0.022
HvCWINV2	-0.468	0.004	0.812	0.000
HvCWINV3	-0.500	0.002	0.864	0.000
HvGBSS1a	-0.638	0.000	0.784	0.000
HvGBSS1b	0.544	0.001	-0.541	0.001
HvISA1	-0.569	0.000	0.652	0.000
HvISA2	-0.558	0.000	0.631	0.000
HvISA3	-0.497	0.002	0.827	0.000
HvSBE1	-0.566	0.000	0.952	0.000
HvSBE2a	-0.529	0.001	0.459	0.005
HvSBE2b	-0.543	0.001	0.697	0.000
HvSSI	-0.474	0.004	0.425	0.010
HvSSIIIa	-0.507	0.002	0.629	0.000
HvSSIIIb	-0.413	0.012	0.768	0.000
HvSSIIa	-0.526	0.001	0.336	0.045
HvSSIIb	-0.150	0.383	0.173	0.312
HvSSIV	-0.460	0.005	0.778	0.000
HvSucSyn	-0.339	0.043	0.216	0.206
HvVacINV	0.221	0.195	0.094	0.586
starch	-0.661	0.000		

WT_SN_XN_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.339	0.043
HvAGP_L1	-0.021	0.901	-0.532	0.001
HvAGP_L2	-0.385	0.020	-0.299	0.077
HvAGP_S1b	0.215	0.209	0.708	0.000
HvAGP_S2	-0.081	0.641	-0.279	0.099
HvAMY1	-0.021	0.901	-0.529	0.001
HvAMY2	0.447	0.006	0.461	0.005
HvAMY3	-0.300	0.075	0.381	0.022
HvAMY4	0.180	0.293	0.262	0.123
HvBAM1	-0.208	0.222	0.880	0.000
HvBAM2	0.379	0.023	-0.914	0.000
HvBAM3	0.148	0.389	0.512	0.001
HvBAM4	-0.418	0.011	0.489	0.003
HvBAM5	-0.219	0.199	0.507	0.002
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.330	0.049	0.503	0.002
HvCWINV1	-0.320	0.057	0.084	0.627
HvCWINV2	-0.411	0.013	0.756	0.000
HvCWINV3	-0.264	0.120	0.639	0.000
HvGBSS1a	-0.695	0.000	0.505	0.002
HvGBSS1b	0.495	0.002	0.240	0.158
HvISA1	-0.491	0.002	0.021	0.905
HvISA2	-0.400	0.016	0.495	0.002
HvISA3	0.077	0.657	0.748	0.000
HvSBE1	-0.435	0.008	0.913	0.000
HvSBE2a	-0.588	0.000	-0.067	0.699
HvSBE2b	-0.469	0.004	-0.029	0.865
HvSSI	-0.353	0.035	-0.423	0.010
HvSSIIIa	-0.412	0.012	-0.107	0.536
HvSSIIIb	-0.424	0.010	0.643	0.000
HvSSIIa	-0.338	0.044	-0.503	0.002
HvSSIIb	0.277	0.103	-0.341	0.042
HvSSIV	-0.411	0.013	0.471	0.004
HvSucSyn	-0.415	0.012	0.106	0.537
HvVacINV	0.401	0.015	-0.300	0.076
starch	-0.339	0.043		

Supplementary table 4: continued.....

P< 0.05	P<0.01	P<0.001

WT_C_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.464	0.022
HvAGP_L1	-0.383	0.065	-0.086	0.689
HvAGP_L2	0.029	0.892	0.355	0.089
HvAGP_S1b	-0.165	0.442	0.824	0.000
HvAGP_S2	-0.276	0.192	0.665	0.000
HvAMY1	0.451	0.027	-0.883	0.000
HvAMY2	-0.130	0.545	0.386	0.062
HvAMY3	0.058	0.787	0.692	0.000
HvAMY4	0.206	0.335	-0.496	0.014
HvBAM1	-0.233	0.274	0.846	0.000
HvBAM2	0.143	0.505	-0.870	0.000
HvBAM3	0.113	0.598	-0.038	0.861
HvBAM4	-0.261	0.219	0.319	0.128
HvBAM5	-0.119	0.578	0.420	0.041
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.115	0.593	0.594	0.002
HvCWINV1	-0.122	0.571	-0.131	0.542
HvCWINV2	-0.212	0.321	0.743	0.000
HvCWINV3	-0.094	0.661	0.623	0.001
HvGBSS1a	-0.164	0.444	0.738	0.000
HvGBSS1b	0.091	0.673	-0.376	0.070
HvISA1	-0.433	0.035	0.479	0.018
HvISA2	-0.405	0.050	0.802	0.000
HvISA3	-0.402	0.051	0.873	0.000
HvSBE1	-0.506	0.012	0.963	0.000
HvSBE2a	-0.597	0.002	0.492	0.015
HvSBE2b	-0.434	0.034	0.465	0.022
HvSSI	0.043	0.840	0.226	0.288
HvSSIIIa	0.042	0.847	0.489	0.015
HvSSIIIb	-0.226	0.288	0.784	0.000
HvSSIIa	0.054	0.804	0.224	0.292
HvSSIIb	0.390	0.060	0.187	0.380
HvSSIV	-0.057	0.792	0.655	0.001
HvSucSyn	0.026	0.905	0.422	0.040
HvVacINV	0.427	0.037	-0.426	0.038
starch	-0.464	0.022		

WT_C

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.871	0.000
HvAGP_L1	0.010	0.975	0.012	0.971
HvAGP_L2	-0.595	0.041	0.651	0.022
HvAGP_S1b	-0.759	0.004	0.885	0.000
HvAGP_S2	-0.708	0.010	0.812	0.001
HvAMY1	0.856	0.000	-0.971	0.000
HvAMY2	0.066	0.838	-0.020	0.950
HvAMY3	-0.743	0.006	0.836	0.001
HvAMY4	0.655	0.021	-0.753	0.005
HvBAM1	-0.803	0.002	0.919	0.000
HvBAM2	0.868	0.000	-0.987	0.000
HvBAM3	0.399	0.199	-0.434	0.158
HvBAM4	0.139	0.667	-0.158	0.624
HvBAM5	-0.124	0.700	0.163	0.614
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.580	0.048	0.655	0.021
HvCWINV1	0.586	0.045	-0.638	0.026
HvCWINV2	-0.695	0.012	0.803	0.002
HvCWINV3	-0.716	0.009	0.833	0.001
HvGBSS1a	-0.748	0.005	0.820	0.001
HvGBSS1b	0.769	0.003	-0.858	0.000
HvISA1	-0.580	0.048	0.632	0.027
HvISA2	-0.673	0.016	0.747	0.005
HvISA3	-0.824	0.001	0.947	0.000
HvSBE1	-0.867	0.000	0.978	0.000
HvSBE2a	-0.509	0.091	0.543	0.068
HvSBE2b	-0.668	0.018	0.731	0.007
HvSSI	-0.518	0.085	0.584	0.046
HvSSIIIa	-0.566	0.055	0.635	0.027
HvSSIIIb	-0.738	0.006	0.873	0.000
HvSSIIa	-0.522	0.082	0.580	0.048
HvSSIIb	-0.426	0.167	0.596	0.041
HvSSIV	-0.739	0.006	0.864	0.000
HvSucSyn	-0.297	0.349	0.311	0.326
HvVacINV	0.549	0.065	-0.584	0.046
starch	-0.871	0.000		

WT_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.659	0.020
HvAGP_L1	-0.223	0.487	-0.292	0.357
HvAGP_L2	-0.065	0.841	-0.323	0.306
HvAGP_S1b	-0.320	0.310	0.867	0.000
HvAGP_S2	-0.417	0.177	0.353	0.260
HvAMY1	0.768	0.004	-0.749	0.005
HvAMY2	-0.248	0.436	0.800	0.002
HvAMY3	-0.425	0.169	0.820	0.001
HvAMY4	0.188	0.559	0.406	0.190
HvBAM1	-0.375	0.230	0.877	0.000
HvBAM2	0.678	0.015	-0.849	0.000
HvBAM3	-0.114	0.724	0.666	0.018
HvBAM4	-0.252	0.430	0.758	0.004
HvBAM5	-0.305	0.334	0.719	0.008
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.267	0.401	0.765	0.004
HvCWINV1	-0.081	0.803	0.600	0.039
HvCWINV2	-0.278	0.381	0.815	0.001
HvCWINV3	-0.246	0.442	0.732	0.007
HvGBSS1a	-0.412	0.183	0.633	0.027
HvGBSS1b	0.088	0.785	0.504	0.095
HvISA1	-0.560	0.058	0.229	0.473
HvISA2	-0.598	0.040	0.916	0.000
HvISA3	-0.387	0.214	0.808	0.001
HvSBE1	-0.547	0.066	0.958	0.000
HvSBE2a	-0.721	0.008	0.512	0.089
HvSBE2b	-0.422	0.172	-0.097	0.765
HvSSI	0.195	0.544	-0.534	0.074
HvSSIIIa	-0.175	0.586	0.086	0.790
HvSSIIIb	-0.147	0.648	0.639	0.025
HvSSIIa	0.114	0.725	-0.544	0.068
HvSSIIb	0.542	0.069	-0.260	0.415
HvSSIV	0.007	0.982	0.394	0.205
HvSucSyn	-0.492	0.105	0.703	0.011
HvVacINV	0.072	0.824	-0.480	0.114
starch	-0.659	0.020		

Supplementary table 4: continued… ..

P< 0.05	P<0.01	P<0.001

SN_C_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.529	0.008
HvAGP_L1	0.381	0.066	-0.589	0.002
HvAGP_L2	-0.195	0.360	0.189	0.377
HvAGP_S1b	-0.125	0.562	0.634	0.001
HvAGP_S2	0.068	0.751	-0.074	0.732
HvAMY1	0.607	0.002	-0.640	0.001
HvAMY2	-0.098	0.649	0.464	0.022
HvAMY3	-0.345	0.099	0.831	0.000
HvAMY4	0.075	0.729	-0.214	0.315
HvBAM1	-0.378	0.069	0.895	0.000
HvBAM2	0.232	0.275	-0.897	0.000
HvBAM3	0.210	0.325	0.204	0.339
HvBAM4	-0.575	0.003	0.407	0.049
HvBAM5	-0.072	0.739	0.514	0.010
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.306	0.145	0.480	0.018
HvCWINV1	-0.148	0.492	-0.085	0.693
HvCWINV2	-0.367	0.078	0.816	0.000
HvCWINV3	-0.452	0.026	0.877	0.000
HvGBSS1a	-0.431	0.035	0.789	0.000
HvGBSS1b	0.119	0.578	-0.655	0.001
HvISA1	-0.327	0.118	0.345	0.099
HvISA2	-0.215	0.313	0.383	0.065
HvISA3	-0.302	0.151	0.601	0.002
HvSBE1	-0.478	0.018	0.945	0.000
HvSBE2a	-0.149	0.487	0.129	0.549
HvSBE2b	-0.295	0.162	0.402	0.051
HvSSI	-0.222	0.296	0.028	0.896
HvSSIIIa	-0.152	0.478	0.391	0.059
HvSSIIIb	-0.134	0.531	0.663	0.000
HvSSIIa	-0.280	0.184	0.092	0.670
HvSSIIb	0.038	0.862	-0.403	0.051
HvSSIV	-0.384	0.064	0.714	0.000
HvSucSyn	-0.204	0.339	0.086	0.690
HvVacINV	0.572	0.003	-0.524	0.009
starch	-0.529	0.008		

SN_C

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.990	0.000
HvAGP_L1	0.390	0.210	-0.409	0.187
HvAGP_L2	-0.555	0.061	0.572	0.052
HvAGP_S1b	-0.790	0.002	0.777	0.003
HvAGP_S2	-0.666	0.018	0.599	0.040
HvAMY1	0.799	0.002	-0.861	0.000
HvAMY2	-0.829	0.001	0.824	0.001
HvAMY3	-0.963	0.000	0.950	0.000
HvAMY4	0.325	0.303	-0.376	0.228
HvBAM1	-0.956	0.000	0.930	0.000
HvBAM2	0.968	0.000	-0.973	0.000
HvBAM3	0.492	0.104	-0.473	0.120
HvBAM4	-0.468	0.125	0.399	0.198
HvBAM5	-0.661	0.019	0.587	0.045
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.541	0.070	0.484	0.111
HvCWINV1	0.651	0.022	-0.694	0.012
HvCWINV2	-0.902	0.000	0.852	0.000
HvCWINV3	-0.906	0.000	0.874	0.000
HvGBSS1a	-0.796	0.002	0.787	0.002
HvGBSS1b	0.918	0.000	-0.923	0.000
HvISA1	-0.745	0.005	0.784	0.003
HvISA2	-0.571	0.052	0.531	0.075
HvISA3	-0.561	0.058	0.602	0.038
HvSBE1	-0.932	0.000	0.935	0.000
HvSBE2a	-0.513	0.088	0.527	0.079
HvSBE2b	-0.637	0.026	0.699	0.011
HvSSI	-0.277	0.383	0.279	0.379
HvSSIIIa	-0.657	0.020	0.665	0.018
HvSSIIIb	-0.864	0.000	0.824	0.001
HvSSIIa	-0.352	0.262	0.361	0.250
HvSSIIb	0.449	0.143	-0.481	0.114
HvSSIV	-0.689	0.013	0.667	0.018
HvSucSyn	-0.381	0.221	0.353	0.260
HvVacINV	0.752	0.005	-0.766	0.004
starch	-0.990	0.000		

SN_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.833	0.001
HvAGP_L1	0.655	0.021	-0.780	0.003
HvAGP_L2	-0.053	0.869	-0.225	0.482
HvAGP_S1b	-0.148	0.646	0.490	0.106
HvAGP_S2	0.601	0.039	-0.725	0.008
HvAMY1	0.588	0.044	-0.616	0.033
HvAMY2	-0.073	0.822	0.155	0.631
HvAMY3	-0.585	0.046	0.631	0.028
HvAMY4	0.056	0.863	0.173	0.591
HvBAM1	-0.627	0.029	0.922	0.000
HvBAM2	0.868	0.000	-0.967	0.000
HvBAM3	-0.288	0.365	0.582	0.047
HvBAM4	-0.582	0.047	0.517	0.085
HvBAM5	-0.278	0.381	0.411	0.184
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.450	0.142	0.480	0.115
HvCWINV1	-0.172	0.593	0.528	0.078
HvCWINV2	-0.656	0.020	0.937	0.000
HvCWINV3	-0.690	0.013	0.943	0.000
HvGBSS1a	-0.727	0.007	0.795	0.002
HvGBSS1b	0.495	0.102	-0.167	0.604
HvISA1	-0.040	0.901	-0.154	0.632
HvISA2	-0.260	0.415	0.230	0.472
HvISA3	-0.280	0.378	0.657	0.020
HvSBE1	-0.713	0.009	0.960	0.000
HvSBE2a	-0.019	0.954	-0.265	0.406
HvSBE2b	-0.248	0.437	0.019	0.953
HvSSI	-0.119	0.714	-0.214	0.504
HvSSIIIa	-0.130	0.687	-0.017	0.957
HvSSIIIb	-0.759	0.004	0.833	0.001
HvSSIIa	-0.047	0.885	-0.229	0.475
HvSSIIb	0.210	0.513	-0.243	0.447
HvSSIV	-0.630	0.028	0.817	0.001
HvSucSyn	-0.171	0.596	-0.089	0.784
HvVacINV	0.581	0.047	-0.505	0.094
starch	-0.833	0.001		

Supplementary table 4: continued.....

P< 0.05	P<0.01	P<0.001

XN_C_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			0.102	0.635
HvAGP_L1	-0.382	0.066	-0.433	0.034
HvAGP_L2	-0.625	0.001	-0.054	0.802
HvAGP_S1b	0.607	0.002	0.735	0.000
HvAGP_S2	-0.619	0.001	-0.243	0.253
HvAMY1	-0.211	0.322	-0.403	0.051
HvAMY2	-0.036	0.866	0.404	0.051
HvAMY3	-0.398	0.054	0.339	0.105
HvAMY4	-0.009	0.966	0.461	0.024
HvBAM1	0.254	0.232	0.929	0.000
HvBAM2	-0.061	0.776	-0.948	0.000
HvBAM3	0.662	0.000	0.463	0.023
HvBAM4	-0.586	0.003	0.420	0.041
HvBAM5	-0.076	0.724	0.693	0.000
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.464	0.022	0.531	0.008
HvCWINV1	-0.803	0.000	-0.230	0.279
HvCWINV2	-0.207	0.333	0.745	0.000
HvCWINV3	-0.213	0.318	0.811	0.000
HvGBSS1a	-0.489	0.015	0.545	0.006
HvGBSS1b	0.569	0.004	0.250	0.239
HvISA1	-0.663	0.000	0.342	0.101
HvISA2	-0.219	0.304	0.476	0.019
HvISA3	0.396	0.055	0.864	0.000
HvSBE1	-0.111	0.604	0.904	0.000
HvSBE2a	-0.580	0.003	-0.102	0.637
HvSBE2b	-0.504	0.012	0.483	0.017
HvSSI	-0.694	0.000	-0.114	0.594
HvSSIIIa	-0.619	0.001	0.248	0.242
HvSSIIIb	-0.040	0.854	0.737	0.000
HvSSIIa	-0.676	0.000	-0.338	0.106
HvSSIIb	-0.019	0.929	-0.104	0.627
HvSSIV	-0.229	0.281	0.588	0.003
HvSucSyn	-0.626	0.001	-0.120	0.577
HvVacINV	-0.093	0.666	0.431	0.036
starch	0.102	0.635		

XN_C

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			-0.342	0.277
HvAGP_L1	0.010	0.975	-0.142	0.660
HvAGP_L2	-0.448	0.144	0.326	0.301
HvAGP_S1b	-0.168	0.601	0.799	0.002
HvAGP_S2	-0.022	0.946	-0.156	0.628
HvAMY1	0.344	0.273	-0.386	0.215
HvAMY2	0.016	0.961	0.512	0.089
HvAMY3	-0.029	0.928	0.484	0.111
HvAMY4	0.062	0.848	0.664	0.019
HvBAM1	-0.120	0.710	0.906	0.000
HvBAM2	0.470	0.123	-0.952	0.000
HvBAM3	0.145	0.652	0.603	0.038
HvBAM4	-0.243	0.447	0.875	0.000
HvBAM5	-0.147	0.648	0.903	0.000
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.159	0.622	0.822	0.001
HvCWINV1	-0.135	0.675	0.371	0.236
HvCWINV2	-0.166	0.606	0.896	0.000
HvCWINV3	-0.217	0.498	0.961	0.000
HvGBSS1a	-0.429	0.164	0.893	0.000
HvGBSS1b	0.327	0.300	0.206	0.521
HvISA1	-0.287	0.365	0.920	0.000
HvISA2	-0.524	0.081	0.558	0.060
HvISA3	-0.211	0.510	0.941	0.000
HvSBE1	-0.190	0.554	0.962	0.000
HvSBE2a	-0.519	0.084	0.450	0.143
HvSBE2b	-0.456	0.136	0.955	0.000
HvSSI	-0.414	0.181	0.576	0.050
HvSSIIIa	-0.180	0.575	0.835	0.001
HvSSIIIb	-0.253	0.428	0.792	0.002
HvSSIIa	-0.632	0.027	0.159	0.622
HvSSIIb	-0.177	0.581	0.346	0.271
HvSSIV	-0.081	0.803	0.834	0.001
HvSucSyn	-0.389	0.211	0.080	0.806
HvVacINV	0.050	0.878	0.653	0.021
starch	-0.342	0.277		

XN_S

	ABA_cor	ABA_P	starch_cor	starch_P
ABA			0.201	0.531
HvAGP_L1	-0.335	0.287	-0.765	0.004
HvAGP_L2	-0.871	0.000	-0.351	0.263
HvAGP_S1b	0.720	0.008	0.754	0.005
HvAGP_S2	-0.838	0.001	-0.336	0.286
HvAMY1	-0.628	0.029	-0.506	0.093
HvAMY2	0.881	0.000	0.495	0.102
HvAMY3	-0.747	0.005	0.155	0.630
HvAMY4	0.121	0.707	0.232	0.469
HvBAM1	0.342	0.277	0.964	0.000
HvBAM2	-0.050	0.878	-0.962	0.000
HvBAM3	0.630	0.028	0.455	0.137
HvBAM4	-0.544	0.067	0.304	0.337
HvBAM5	-0.445	0.147	0.513	0.088
HvBAM6	0.000	1.000	0.000	1.000
HvBAM7	-0.601	0.039	0.335	0.287
HvCWINV1	-0.763	0.004	-0.572	0.052
HvCWINV2	-0.501	0.097	0.655	0.021
HvCWINV3	-0.454	0.138	0.701	0.011
HvGBSS1a	-0.838	0.001	0.236	0.461
HvGBSS1b	0.866	0.000	0.295	0.351
HvISA1	-0.756	0.004	-0.023	0.943
HvISA2	-0.550	0.064	0.370	0.237
HvISA3	0.644	0.024	0.785	0.002
HvSBE1	-0.108	0.738	0.855	0.000
HvSBE2a	-0.815	0.001	-0.404	0.193
HvSBE2b	-0.793	0.002	-0.079	0.808
HvSSI	-0.873	0.000	-0.521	0.082
HvSSIIIa	-0.901	0.000	-0.239	0.453
HvSSIIIb	-0.342	0.277	0.732	0.007
HvSSIIa	-0.788	0.002	-0.641	0.025
HvSSIIb	-0.176	0.585	-0.638	0.026
HvSSIV	-0.592	0.043	0.336	0.285
HvSucSyn	-0.914	0.000	-0.259	0.417
HvVacINV	0.810	0.001	0.134	0.678
starch	0.201	0.531		

9 Acknowledgement

It is my desire to obtain a doctoral degree, but this would not have been possible without the support of various people. I would like to take this opportunity to thank them. The names not mentioned are not that I forgot them. I would initially like to thank my supervisor Dr. Nese Sreenivasulu, Head of Stress Genomics group, for providing me an opportunity in August 2007 to carry out my PhD at the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany. I would also like to thank him for his guidance, discussion during my work and his help in compilation of my thesis. I also express my indebtedness to him and his family in making my initial stay far away from India a comfortable feel like home.

I also thank Prof. Ulrich Wobus, one of my supervisors, for his valuable time in improving my thesis. Without whom it would not have been possible to complete my thesis.

It would not be possible without the help of Rajesh Kalladan to reach this point. I still remember the days when we both came together from India to achieve our dreams of obtaining our doctoral degree at IPK. I thank him from the bottom of my heart for the great support he has extended all during my PhD journey. He has been a great support not only in lab and office but also in my personal life sharing my ups and downs in life during this period. He was always there to boost my moral when I was low.

I thank Dr. Christiane Seiler for her substantial help in communicating with lab members, academic personals and gardeners. She also guided me various molecular techniques and trouble-shooting in lab. I thank her for improving my thesis and German translation. She has been very understanding and helped me understand the letters I received in German.

I would also like to thank Angela Stegmann, Jana Lorenz, Gabi Einhert, Elsa Fessel and Mandy Puffeld for their help in lab and green house. They helped in identifying the right spike developmental stage of barley, sample collection, and reagent preparation for various analyses. I thank Angela Stegmann for teaching me the southern hybridisation and Jana Lorenz for assisting me carrying out most of my bench-work. They both made me very comfortable in lab, and I will always cherish the moments I spent with them in lab and trips.

There are a number of other academic members whom I would like to thank. These people have helped me directly or indirectly in completing my PhD. Dr. Volodymyr Radchuk for teaching me all steps of macroarray hybridization. Dr. Hardy Rolletchek, Dr. Nicolas Heinzel and Rajesh Kalladan for ABA measurement and enzyme assay. Dr. Twan Rutten in documenting spike branching using the scanning electron microscope. Dr. Jochen Kumlehn and Dr. Goetz Hensel for agro-bacterium mediated transformation of barley plants and production of the DH plants. Dr. Marc Stickert and Dr Nese Sreenivasulu, for analysis of macroarray data. Dr. Joerg Fuchs for ploidy measurements.

I thank Dr. Geetha Govind for her constant support, constructive criticism and scientific input from M.Sc till date. It would have not been possible without her help and support to complete my PhD.

I would also like to thank our other lab members, Thiruloga Chandar Venkatasubu, Dr. Sudhakar Palakolanu Reddy, and visiting members, Vincent and Javier, for the friendly atmosphere in office, lab and discussions during tea time.

In addition, I would also like to thank the National Research Council of Canada Plant Biotechnology Institute (Saskatoon, Canada) which was outsourced for measurement of plant hormones including ABA metabolites.

I am also grateful to the green house team of Enk Geyer, Peter Schreiber and their colleagues for maintaining the adequate growing condition in the green house and the phytochamber.

I am great full to financial support received from GABI-GRAIN project funded by BMBF and IPK for my PhD work.

I would also like to thank Dr. Bret Leps for her constant support, understanding and solving problems regarding visa, and in the administration.

I thank all members of the IPK Indian community for making my stay at Gatersleben enjoyable. Words would be less to express my thanks to Raj Kishore Pasam and Rajiv Sharma, who were always there and will be there when I need them. They were also part of the ups and downs of my life in Gatersleben. I enjoyed all the discussion (academic and non-academic) we had together although I am a silent listener. I also thank Pooja,

Chitra, Jey, Sid, Nisha, Ravi, Jahnavi, Sumathi, Siri, Shiva, Anu, Amara, Karthik, Vaishnavi, Daya, Shailendra and Sheebha for the good time I spent with them.

I would also like to thank my friend in India viz., Reddy, Rajendra, Rame, Ramu, Jal, Rani, Shree, Shetty and other members of Department of Crop Physiology, UAS (B), India. I also cherish the special time I spent with Rajendra when he was in Germany.

Lastly I would like to thank my family, my father, mother and two sisters for their constant support and encouragement. I also would like to thank my in-law family members for their support and encouragement. I finally thank my wife, Geetha, for her constant support, encouragement, understanding, love and patience. She has kept me going smooth during rough times and added cheerfulness to my life.

Harshavardhan VT

12.07.12

10 Appendix

Curriculum vitae

Personal Data

Full Name: Harshavardhan Vokkaliga Thammegowda

Gender: Male

Date of birth: 14-12-1981

Place of birth: Madikeri, Karnataka, India

Marital status: Married

Nationality: Indian

Email ID: harshavardhan@ipk-gatersleben.de, harshavardhanvt@gmail.com

Education and employment

August 2007 to present:	PhD student at stress genomics group, molecular genetic department, IPK, Gatersleben.
2006 to August 2007:	Senior research fellow, Department of crop physiology, GKVK, Bangalore.
2003 to 2005:	Master student in the Department of crop physiology, GKVK, Bangalore.
1999 to 2003:	Bachelors student in the University of agricultural science Bangalore.

Publication

Geetha-Govind, **Harshavardhan VT**, Patricia KJ, Dhanalakshmi RI, Senthil-Kumar M, Sreenivasulu N and Udayakumar M. Identification and functional validation of a unique set of drought induced genes preferentially expressed in response to gradual water stress in peanut. Molecular Genetics and Genomics, 281: 591- 605, 2010.

S. Sharma, N. Sreenivasulu, **V.T. Harshavardhan**, C. Seiler, S. Sharma, Z.N. Khalil, E. Akhunov, S.K. Sehgal, M.S. Röder. Delineating the structural, functional and evolutionary relationships of sucrose phosphate synthase gene family II in wheat and related grasses. BMC Plant Biology 10: 134, 2010.

S. Worch, R. Kalladan, **V.T. Harshavardhan**, C. Pietsch, V. Korzun, L. Kuntze, A. Borner, U. Wobus, M.S. Roder and N. Sreenivasulu. Haplotyping, linkage mapping and

expression analysis of barley genes regulated by terminal drought stress influencing seed quality. BMC Plant Biology, 11: 1, 2011.

C. Seiler, **V.T. Harshavardhan**, K. Rajesh, M. Strickert, H. Rolletschek, U.Scholz, U., Wobus and N.Sreenivasulu. ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought stress conditions. Journal of Experimental Botany, 62: 2615-2632, 2011.

Sreenivasulu, N., **Harshavardhan, V.T**., Govind, G., Seiler, C. and Kohli, A. Contrapuntal role of ABA: Does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene. In press (2012).

Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder bei der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg noch bei einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den .... ______________________________