Dedicated to my brothers

RAJAN and DASAN

UNDERSTANDING TERMINAL DROUGHT TOLERANCE IN BARLEY USING AB-QTL ANALYSIS AND AN INTEGRATED OMICS APPROACH

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Herrn Rajesh Kalladan

geb. am: 02.02.1978 in: Palloor, India.

Gutachter /in:

1. Prof. Ulrich Wobus
2. Prof. Klaus Humbeck
3. Prof. Björn Usadel

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1 INTRODUCTION

1.1 Importance of barley as a cereal crop

Barley (*Hordeum vulgare* L.), an annual diploid crop (2n=14) belonging to the tribe Triticeae (Poaceae family) was associated with mankind ever since the domestication began (Smith, 1998; Fischbeck, 2002). Among the food crops, it is the fifth most important crop and ranks fourth among cereal crops in terms of production worldwide after maize, rice and wheat (FAO stat, 2009). The major areas of barley production includes Europe, Mediterranean rim of North Africa, Ethiopia, the Near East, the former USSR, China, India, USA, South America and Australia (Nevo, 1992). According to FAO statistics (2009), European Union contributes to 25% (6.2 Mt) of total 152 Mt of barley produced worldwide annually and Germany stands next to France with a total production of 1.2 Mt in the European region.

Apart from its use as feed and food, barley is widely used as a unique source of malt for beer and whisky production. Due to its wider adaptability and genetic variability along with its true diploid nature, inbreeding characterstics, availability of range of genetic stocks and colinearity with other members of its tribe Triticeae, barley is an excellent model C_3 crop for genetic and physiological studies (Koornneef et al., 1997; Hayes et al., 2003). In addition, barley has also been regarded as a model crop for seed development and germination in poaceae family because of the vast amount of genomic resources available in this crop and due to its close resemblance in seed architecture with other members of the family (Sreenivasulu et al., 2008; Schulte et al., 2009). The seven barley chromosomes encompass the large genome (5100 MB), which is 80% composed of repetitive DNA. Although the gold standard reference genome sequence is far from complete for agronomically important crops such as barley, systematic efforts are under way to unlock the gene content by next-generation sequencing of sorted chromosomes, sequencing gene-rich BAC clones and full-length cDNA collections (Schulte et al., 2009; Mayer et al., 2011). Nevertheless, in comparison to wheat (15.9 Gbp), barley genome is 3 fold smaller (5.1 Gbp) and therefore has been treated as a model crop of Triticeae. The genome sequencing of barley is already under progress (http://barleygenome.org/) and the cultivar Morex has been recommended as a reference genome in the Triticeae (Linde-Laursen et al., 1997; Mayer et al., 2011).

Mediterranean regions represent a significant area of barley production where it is mostly grown under rainfed conditions. These regions are characterized by a long hot and dry summer (coupled with erratic rain fall pattern), alternated with cold, wet and relatively short winters (Loss and Siddique, 1994). Owing to these weather conditions, barley is often exposed to several stress conditions such as drought, high temperature or a combination of both which often coincide with the grain filling period. Barley is known to possess genetic diversity for adaptation to a wide range of environments (particularly wild barley) varying in water supply, temperature and photo period (Forster et al., 1997). Besides this, the ability of inter-fertilization of wild barley with cultivated ones offers a great potential for crop improvement under different challenging environments through advanced breeding programs and genomics-assisted selection. However, increasing climatic disturbances worldwide and forecast for more frequent and intense drought occurrences especially in the arid and semi arid regions of the world would be a greater concern for the production of barley as well (IPCC, 2007; Overpeck and Udall, 2010; Fereres et al., 2011).

1.2 Drought

Among various abiotic stress factors, drought is the single most important factor limiting crop production worldwide (Kramer, 1980). Over the years, plants have evolved different strategies to cope up with this increasing water scarcity which can be broadly categorized as drought escape, avoidance and tolerance mechanism that encompass a variety of morphological, physiological, biochemical and molecular adaptations of plant metabolism (Paleg and Aspinall, 1981; Ingram and Baretls, 1996; Verslues and Juenger, 2011; Sanchez et al., 2012). Drought escape involves the developmental plasticity that enables the plant to shorten its life cycle so as to escape drought, whereas avoidance mechanism maintains the plant water status either through increased water uptake or through the closure of stomata. Drought tolerance is the ability of a plant to withstand reduced cell water potential through osmotic adjustment, anti oxidant capacity and various other means to adjust growth and metabolism under drought. Owing to the complexity of drought (intensity, duration and time of occurrence) and crop responses to drought, development of drought tolerant varieties remains far behind compared to other traits (Yang et al., 2010). In cereals, a major concern under drought is reduction in grain yield and impaired quality, the severity of which depends

on the drought occurrence with respect to a particular stage of a plant development (Dolferus et al., 2011). Figure 1 depicts the effect of drought on various yield components when it coincides with different stages of cereal development.

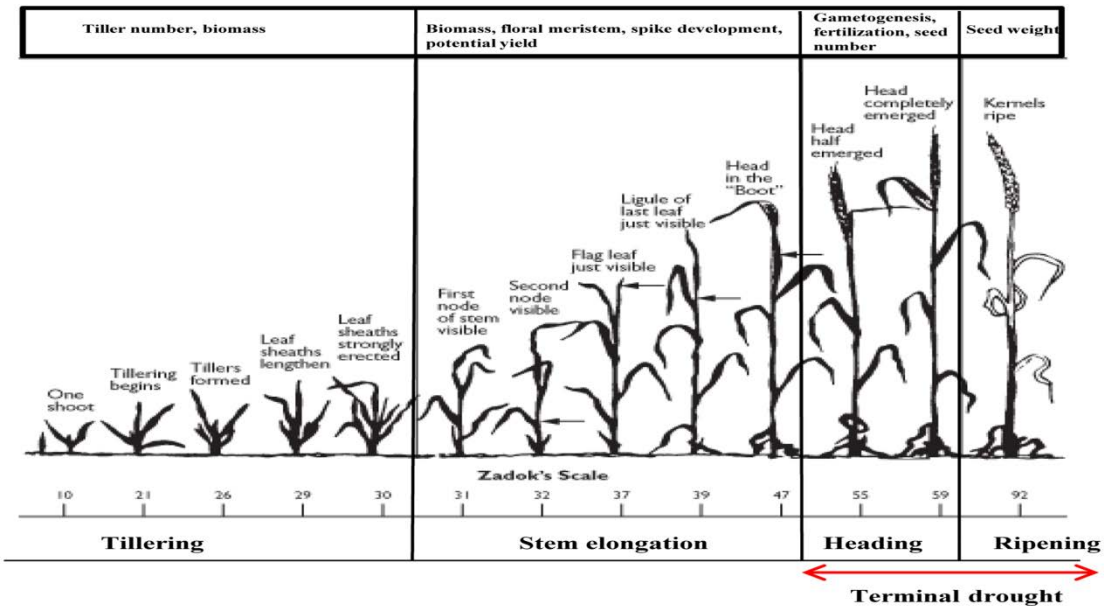


Figure 1. Effect of drought on crop developmental stages and its effect on yield components. Original figure taken from http://www.omafra.gov.on.ca/english/crops/pub811/4planting.htm

Anthesis is one of the most crucial stages of cereal development which determines grain yield. Drought stress at anthesis severely affects both grain yield and quality of the produce (Dolferus et al., 2011). Two important stages with respect to drought occurance during anthesis are anthesis and early seed development. When occurs during anthesis, it reduces fertility and hence seed number which inturn reduces grain yield, whereas drought occurrence during early seed development reduces grain yield mainly through reduction in grain weight (Jamieson et al., 1995; Barnabas et al., 2008). Drought during grain filling (terminal stage a crop cycle) or post-anthesis drought is a major concern for barley when it is grown under available soil moisture from the previous season where they typically encounter with drought during or after flowering period (Blum 2009). The major cause for yield reduction under terminal drought is due to lack of assimilates for developing seeds which is evident from the sucrose feeding experiment in maize. Westgate and Boyer (1985) found that artificial feeding of sucrose to developing kernels could partially reverse the adverse effect of

drought on developing kernels. Hence, availability of assimilates is an important component of crop yield under terminal drought.

1.3 Source of assimilates for grain filling and its nature

Grain filling is the final stage of growth in cereals where a significant portion of assimilates are deposited into developing grains as storage products, which in barley is mainly composed of starch and protein that ultimately determines grain yield. Carbon source required for grain filling is acquired through current photosynthesis and remobilization of reserves stored in different plant tissues, especially in stem (Pheloung and Siddique, 1991; Kobata et al., 1992; Schnyder, 1993). In cereals like barley, major source of assimilates for grain filling under optimal conditions is obtained from current photosynthesis and contribution of stored reserves varies from 10-40% depending on the cultivars (Schnyder, 1993). However, in a number of experiments carried out in wheat and other crops, reduction in photosynthetic capacity of source leaves could not be correlated to corresponding decrease in grain filling indicating the significance of stored reserve pools in stems to grain filling under conditions of limited photosynthesis (Schnyder, 1993).

In temperate cereals like barley, during vegetative and early reproductive growth part of the plant, carbon assimilated is stored in stem and leaf sheaths in the form of various water soluble carbohydrates (WSC) and acts as a long term reserve pool of assimilates for grain filling. WSC constitute up to 50% dry weight of stem in barley at the time of their maximum content, which usually occurs a few days after anthesis and gradually declines as seed filling proceeds. These WSC contribute upto 20 to 80% of the seed assimilate with a concomitant reduction in stem biomass upto 40 to 50% depending on conditions. Among the WSC, fructans and sucrose represent the major portion constituting nearly 85% and 10%, respectively, in wheat and barley (Austin et al., 1977; Wardlaw and Willenbrink, 2000; Ruuska et al., 2006).

Fructans are polymers of fructofuranosyl units starting with sucrose moiety which is present in about 15% of all flowering plant species. Among the five structurally different fructans, barley has the graminan type that is predominantly comprised of β2-6 linked fructosyl units with shorter β2-1 linked branches (Ritsema and Smeekens, 2003; Chalmers et al., 2005; Van

den Ende et al., 2011). The key enzymes involved in the synthesis of fructan are sucrose-sucrose fructosyl transferase (SST), sucrose-fructosyl transferase (SFT), fructan-fructan fructosyl transferase (FFT) and fructan exohydrolase (1-FEH and 6-FEH; Nelson and Spollen, 1987; Pollock and Cairns, 1991; Lasseur et al., 2011). While SST and 1-FEH are the key enzymes involved in synthesis, 1-FEH is also important for hydrolysis of fructan during stem remobilisation (Xue et al., 2008b). Fructan metabolizing enzymes are more closely related to plant acid invertases; single amino acid substitution (Asp-239) of AtcwINV1 by site directed mutagenesis, transforming to 1-FEH suggests that they are phylogenetically more related and might have evolved from β-fructosidase as ancestor (Van den Ende et al., 2000). But in contrast to the location of acid invertase to cell wall and vacuoles, all fructan metabolizing enzymes are localised only in vacuoles. Therefore, the putative site of fructan synthesis, storage and breakdown is restricted to vacuoles (Pollock and Chatterton, 1988; Van Laere and Van den Ende, 2002). Large concentration of fructans in internodes of wheat and barley suggests that, they are present in the parenchyma cells of stems (Evans et al., 1970).

1.4 Terminal drought and seed filling in cereals

In general, an important feature of genotypes tolerant to terminal drought is the ability to provide assimilates for developing grains either through current assimilation or by remobilization of reserves stored in various vegetative tissues. Based on the above feature, some crop genotypes have been categorized either as staygreen or senescing depending on the source of assimilates which they mainly rely on for grain filling, especially under terminal drought. A stay green genotype relies more on current photosynthesis and retains more functional leaf chlorophyll that enables them to provide assimilates for developing seed through current photosynthesis. On the other hand, a senescing genotype relies more on remobilisation of stem reserves which is closely coupled to the process of senescence induction in monocarpic crops like barley (Yang and Zhang, 2005). The relative importance of these two mechanisms for seed filling under terminal drought depends on crop cultivar cultivar and/or severity of stress conditions (Blum, 2005). A general overview of the effect of terminal drought on crop yield and the two mechanisms (stay green and senescence) involved in terminal drought tolerance are depicted in Figure 2.

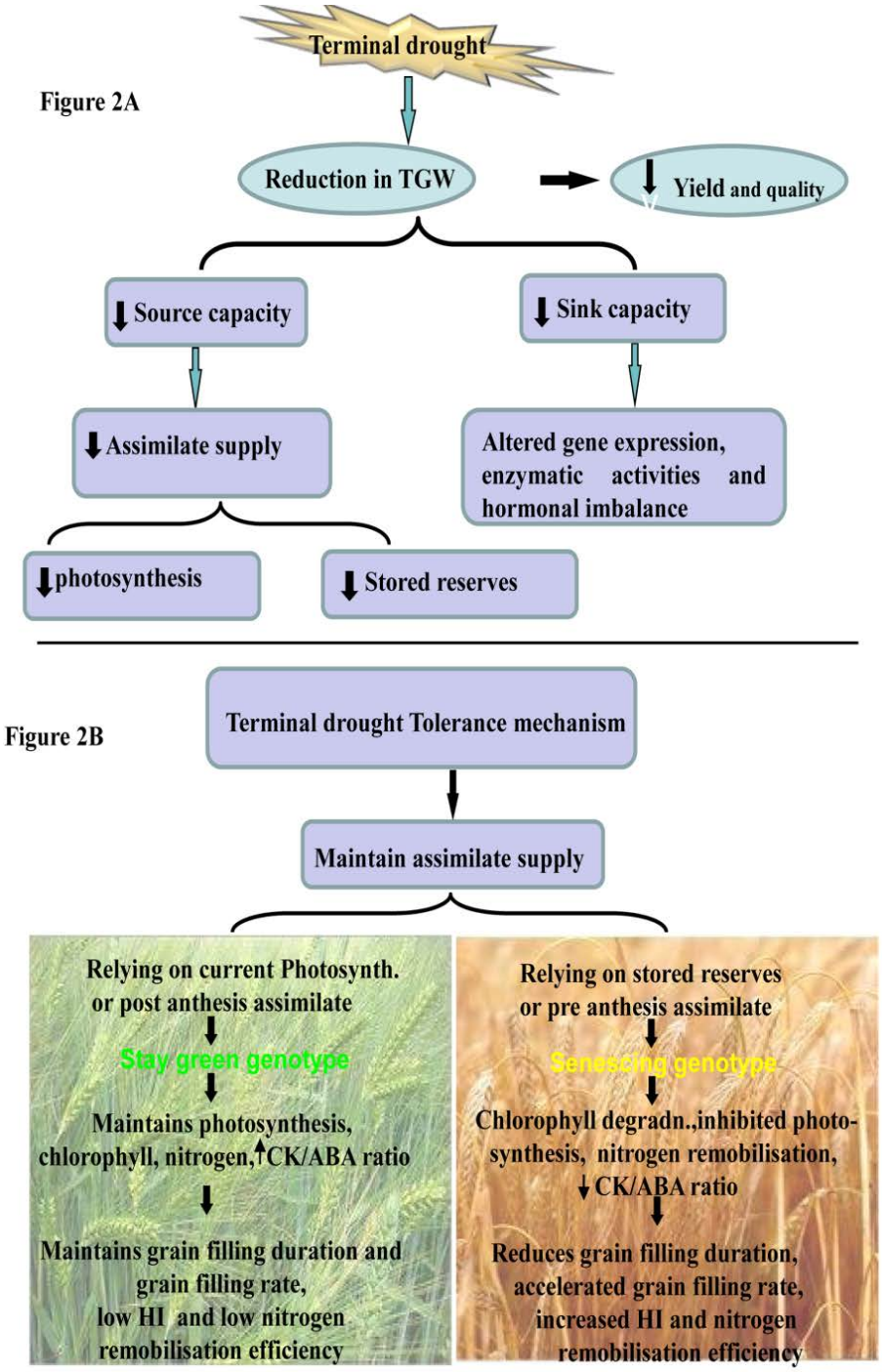


Figure 2: Crop yield under terminal drought.

Effect of terminal drought on various yield components resulting in yield loss (**2A**) and mechanism contributing to terminal drought tolerance observed in crops (**2B**).TGW- thousand grain weight; ABA- abscisic acid; CK- cytokinin; HI- harvest index.

1.5 Importance of stay green under terminal drought

Staygreen is an important secondary trait for crop yield under terminal drought in crops like sorghum, maize and rice (Jiang et al., 2004; Pommel et al., 2006; Harris et al., 2007). Several staygreen QTLs were identified in sorghum by Tuinstra et al., (1998) and grain yield in the mapping population was significantly associated with 'staygreen' character under terminal stress. In wheat, higher yield obtained in genotypes with glaucousness under drought was associated with delayed leaf senescence as a result of cooler leaf temperature (Richards, 1986). The situation was not different for barley, Acevedo (1987) found that under dry land conditions, grain yield in barley was significantly correlated with delayed leaf senescence and the correlation was stronger in the drier of two environments. Significance of staygreen character to drought tolerance is also evident from a number of transgenic plants over accumulating CK (Cytokinin) over ABA (Abscisic acid) through the overexpression of IPT (Isopentenyltransferase; Werner et al., 2010). The enhanced drought tolerance of transgenic plants compared to wild type is a result of extended photosynthetic capacity and maintenance of green leaf area (Ma, 2008; Peleg et al., 2011; Merewitz et al., 2011).

However, there are two important exceptions to the above stay green mechanism; one is staying green to the point of unfavorable advantage and another is the distinction of functional stay green from cosmetic stay green. In the first case, it is reported that unfavourably delayed leaf senescence is becoming a concern for yield in rice and wheat which results in poor grain filling and also leaves large amount of WSC unused in stem (Yang et al., 2002a; Yang and Zhang, 2005). In the second case, staygreen (sgr) mutants have been identified in a number of plant species (Alos et al., 2008; Sato et al., 2009b; Zhou et al., 2011) that belong to class C (Thomas and Smart, 1993; Thomas and Howarth, 2000), where plants retain chlorophyll and stays green but their photosynthetic capacity is severely impaired. In rice, class C stay green mutants identified is related to the enzyme, pheophorbide a oxygenase and the protein suggested to be involved in the regulation of the activity of this particular enzyme (Park et al., 2007; Jiang et al., 2007). Recently, type C locus identified in *Medicago trunculata* and Arabidopsis (SGR) was also found to have influence on nodule senescence and development of disease symptoms, respectively, (Zhou et al., 2011; Mecey et al., 2011) in addition to their role in leaf senescence.

1.6 Importance of senescence under terminal drought

It has also been shown that stay green mechanism would be of little advantage to crops when stress is severe to the point that current photosynthesis is limited (Palta et al., 1994; Plaut et al., 2004). Under such conditions, the gain from the accelerated grain filling as a result of senescence induced remobilization was found to outweigh the advantage of current photosynthesis (Yang and Zhang, 2006). In maize, it is observed that faster reallocation of stem carbohydrate is the major reason for the high grain weight, rather than the 'staygreen' character (Dwyer et al., 1995). A genotype that senesces early and remobilizes stored stem reserves under terminal drought has the advantage of faster grain filling rate with increased harvest index although with a reduced grain filling duration compared to stay green. This was evident from the studies carried out in wheat and barley by Palta et al., (1994), who found that, under terminal drought, photosynthesis was reduced by 57%, while stem remobilization was increased by 36%.

A positive correlation between stem WSC concentration at anthesis and grain weight or yield under terminal drought was observed in a number of studies with wheat and barley (Blum, 1998; Ruuska et al., 2006). A considerable genotypic variation with a high heritability makes stem WSC at anthesis as a useful secondary trait for screening yield potential under terminal drought (Schnyder, 1993; Ehdaie et al., 2006; Pierre et al., 2010; Slewinski, 2012). The significance of stem reserves in crop yield is reflected in the increased grain yields obtained from wheat cultivars of UK and Australia, which is associated with an increase in stem WSC (Van Herwaarden and Richards, 2002; Shearman et al., 2005). However, a study using two varieties of wheat under terminal drought indicated that it is the efficiency of remobilization that is more important to terminal drought tolerance than the WSC content alone and senescence may not be always coupled with remobilization of WSC (Zhang et al., 2009). A positive correlation of *1-FEH w3* with remobilization of WSC was observed in this study and the authors suggest that apart from stem WSC content and green leaf area retention, *1-FEH w3* expression can be a useful indicator for screening terminal drought tolerance.

From the above discussions, it is clear that both staygreen and senescence mechanisms operate in a given crop species and the significance of these two mechanisms could be influenced by either genotype or the severity of stress. In short, traits that contribute

assimilates to developing grains like current photsynthesis (staygreen), capacity for mobilization of stored reserves are some of the important secondary traits that would be useful for maximizing yield under terminal drought (Araus et al., 2002, 2008).

1.7 Factors influencing drought induced senescence

Senescence is an important pre-requisite for mobilization of nutrients to developing grains in monocarpic crops like wheat and barley, and is governed by a large number of genetic and external factors. Among various internal factors, metabolites such as sugars and hormones have been implicated either as a cue or as cause for drought induced senescence in plants (Lim et al., 2007; Thomas et al., 2009; Wingler and Roitsch. 2008; Wingler et al., 2010). Sugar accumulation, in spite of decreased photosynthesis under drought was observed in a large number of studies (Muller et al., 2011). The role of sugar accumulation in plant senescence is a topic of debate; experimental evidences support both contradicting views i.e., sugar accumulation leading to senescence and delaying senescence (Parrott et al., 2005; Pourtau et al., 2006; van Doorn, 2008). However, there is a general consensus that sugar accumulation in relation to nitrogen status of the plant tissue is an important aspect of senescence regulating process; a high carbon to nitrogen ratio was more effective in senescence induction rather than sugar alone in many studies (Ono and Watanabe, 1997; Wingler et al., 2006). Recently, role of sugar accumulation in leaf senescence process was critically evaluated by van Doorn (2008), who suggested that, although sugar accumulation is a common phenomenon in senescing leaves, this alone may not be the cause for triggering senescence process, rather, its complex network with other metabolites and environmental factors could act as a signaling complex involved in senescence process.

Among various phytohormones, ABA and CK are the two major plant hormones having antagonistic effect on plant senescence under abiotic stress (Zeevaart and Creelman, 1988; Peleg and Blumwald, 2011). The role of CKs in delaying senescence is evident from the transgenic plants over expressing IPT gene, an important rate limiting enzyme in the synthesis of cytokinin (Buchanan-Wollaston, 1997; Werner et al., 2010), where as ABA is known to promote senescence (Nooden, 1988b). However, recent evidences suggest that apart from these two hormones, there are also other hormones involved in senescence process and it could be their coordinated action that regulate senescence process (Jaillais and Chory ,

2011; Peleg and Blumwald, 2011). In addition to their role in senescence, ABA and CK are also implicated in seed filling processes of different cereals (Brenner and Cheikh, 1995; Yang and Zhang, 2006; Sreenivasulu et al., 2010; Faix et al., 2012). In a partial soil drying experiment during grain filling in wheat, ABA content in grain was found to positively correlate with enzymes involved in grain filling (Yang et al., 2001, 2003a). On the other hand, ABA is also a well known plant hormone which gets accumulated under stress and mediates drought tolerance mainly by reducing the transpirational loss through stomatal closure (Leung and Giraudat, 1998). Transgenic plants over expressing *NCED*, either constitutively or under drought inducible promoter exhibited enhanced drought tolerance and maintained a better leaf water status, green leaf area and duration compared to the wild type (Iuchi et al., 2001; Thompson et al., 2007). The above observations together with the role of ABA in senescence induction illustrates that, ABA response in plants could be tissue or developmental specific. Hence, for studying the role of hormones in plant development, it is necessary to use conditional promoters driving gene expression at a specific developmental stage or in response to specific environmental stimuli (Peleg and Blumwald, 2011).

1.8 Senescence and nitrogen remobilisation

Apart from its role in carbon remobilization, senescence is an important process involved in remobilization of nitrogen from vegetative tissues to developing caryopsis. In small grained cereals like barley and wheat about 80-90% of the nitrogen assimilated in grain is contributed through remobilization from the vegetative tissues, mainly leaf (Austin et al., 1977; Zhang et al., 2007a; Masclaux-Daubress et al., 2008). During remobilization, a large number of proteolytic enzymes are activated which degrade leaf proteins that are mainly associated with light harvesting complex and RuBisCO into individual amino acids which are eventually transported to the developing grains (Jukanti et al., 2008; Masclaux-Daubress et al., 2008). Large amount of ammonia released during protein degradation is re-assimilated by cytosolic glutamine synthetase, for which the carbon source is provided by the enzyme, glutamate dehydrogenase. Hence, these two enzymes are considered as the marker enzymes of senescence induced remobilization of nitrogen in plants (Pageau et al., 2006; Masclaux-Daubresse et al., 2010). Importance of senescence induced remobilization of nitrogen in crop plants is exemplified by the map based cloning of grain protein concentration (GPC) locus,

NAM-B1 (a NAC transcription factor) originally identified in wheat chromosome 6B. The presence of a functional NAC gene was found to increase the grain protein content as a result of early induction of post-anthesis senescence (Uauy et al., 2006a, b).

A similar gene was also identified in chromosome 6H of barley (HvNAM-1) through QTL analysis, that explained 45% of the heritable variance in protein content of the mapping population (Distelfeld et al., 2008; Lacerenza, 2010). Recently, using near isogenic lines developed for the 6H locus, it was found that, in addition to acceleration of post-anthesis flag leaf senescence, the GPC locus also accelerated the pre anthesis development after transition from shoot apical meristem (SAM) stage (Lacerenza et al., 2010; Parrot et al., 2012). Transgenic wheat lines in which expression of NAM-B1 and its homeologous genes were down regulated using RNAi was characterised by delayed leaf senescence and a lower grain protein, Fe and Zn concentrations (Waters et al., 2009). This illustrates that senescence is an important pre-requisite for remobilizing not just nitrogen but also other nutrients.

1.9 Seed metabolism under terminal drought

Grain yield in cereals is a result of coordinated activities between source and sink tissues. Under optimal conditions, grain growth or seed yield is generally sink limited (Jenner et al., 1991). However, under terminal drought, yield loss in cereals is a result of both source and sink limitations. Yield reduction in barley and other crops even with adequate assimilates made available through artificial feeding to developing grain during terminal drought clearly indicates the role of sink activity in determining yield under terminal drought (Brooks et al., 1982; Westgate, 1994). Starch being the predominant form of storage product in barley grain, activities of various enzymes involved in conversion of sucrose to starch are the major factors determining sink activity and hence crop yield (Duffus, 1992).

Among various enzymes involved in starch synthesis, sucrose synthase (SuSy) which catalyses the conversion of sucrose to fructose and UDP-glucose is considered to be the marker enzyme of sink strength in several crops including cereals (Sun et al., 1992; Wang et al., 1993; Kato, 1995; Jiang et al., 2011). Its activity was found to be a major determinant of seed filling duration in barley and wheat under both optimal and drought stress conditions (Chevalier and Lingle 1983; MacLeod and Duffus 1988). A relatively unresponsiveness of

this enzyme to drought compared to control in a variety of crops during early grain filling and pollination in maize suggests that its activity may not be a limiting factor for starch synthesis under drought (Dorion et al., 1996; Sheoran and Saini 1996). On the other hand, activity of acid invertase, another enzyme involved in the breakdown of sucrose especially during early stages of seed development in barley (Weschke et al., 2003; Weber et al., 2005) was significantly reduced under drought in wheat as well as in maize (Zinselmeier et al., 1995; Dorion et al., 1996). Therefore, fine tuning different sucrose cleavage pathways as per the requirement of stage dependent fashion is an important criteria for regulating seed metabolism under drought.

AGPase (ADP-glucose pyrophosphorylase), an important rate limiting enzyme of starch synthesis catalyzing the production of ADP-glucose was found to be negatively affected by drought stress in wheat and potato (Caley et al., 1990; Geigenberger et al., 1997). Similarly, reduction in activity of this enzyme was also noticed under heat stress in wheat and *in vitro* cultured maize (Duke and Doehlert, 1996; Ahmadi and Baker, 2001). Drought stress had no significant effect on the activity of GBSS (Granule bound starch synthase) in wheat when occurred during the initial stages of seed development but was negatively affected in maize kernels (Caley et al., 1990; Ober et al., 1991). A reduction in SSS (soluble stach synthase) activity in wheat under heat stress was correlated with reduction in starch accumulation (Jenner and Hawker, 1993; Keeling et al., 1993); however, it was little affected by drought in maize (Dorion et al., 1996). A notable exception to all the above results was reported in a controlled soil drying experiment carried out by Yang et al. (2003a, b, and 2004a) in rice and wheat during grain filling period. Here the authors found that activities of SuSase, SSS, SBE (Starch branching enzyme) and AGPase were significantly enhanced under drought and was positively correlated with seed starch accumulation rate and ABA content in grains. Enhanced seed filling under mild drying was attributed to accumulation of ABA which enhanced sink strength and remobilization of stem reserves. Similarly, role of ABA in seed filling under terminal drought was also reported by Seiler et al. (2011) and Govind et al. (2011).

Another important aspect of terminal drought with respect to seed metabolism in cereals is altered protein metabolism. Among many factors, seed protein content is the most important one determining the end use of barley for malting. Generally, a low protein content which is usually less than 11.5% is preferred for malting, as high protein content was found to negatively affect both malt extract and beer quality (Weston et al., 1993). Terminal drought and heat stress are known to increase seed protein content of barley, rendering it unsuitable for malting (Macnicol et al., 1993; Savin and Nicolas, 1996). A major reason for increased seed protein content observed under drought is due to the fact that starch deposition is more sensitive to drought than protein deposition. Hence, increase in protein content observed under drought is not an increase in protein deposition *per se* but rather due to the reduction in starch deposition (Morgan and Riggs, 1981; Brooks et al., 1982).

Among seed storage proteins, prolamin (Hordein) constitutes more than 50% of the seed nitrogen in barley and is classified into four groups namely B, C, D and γ based on their electrophoretic mobilities. Among hordeins, the major fraction is constituted by B (70-80%) and C (10-12%) fractions while D and γ are considered as minor (Shewry et al., 1985). Studies on the effect of hordein fractions on malting quality revealed that hordein fractions in particular B and D fractions are negatively correlated to malting quality (Peltonen et al., 1994; Simic et al., 2007); however, no such correlation was found by Shewry et al. (1980) and Riggs et al. (1983). A general negative correlation observed between hordeins and malt extract is attributed to a relatively low starch content of the grain compared to protein and also to the fact that starch granules are embedded into a hordein matrix, thus restricting the access for amylolytic enzymes during malting (Molina-cano et al., 2000). Further, B and D fractions also reduce the yield of malt extract as they have the tendency to form colloidal aggregates and thus reducing malting quality (Smith and Lister, 1983).

1.10 Integrated omics approach to study drought tolerance

Drought tolerance is a complex trait which involves many molecular, biochemical, physiological, phenological and whole plant responses that enable plants to withstand stress. In agricultural point of view, drought tolerance essentially means yield of the produce, which in cereals is grain yield (Turner, 1979). Because of its complex nature, drought tolerance has to be dissected at different levels to understand genetic basis of tolerance mechanism and to

develop superior genotypes to cope up with the increasing scarcity of water (Fleury, et al., 2010). An upcoming field in plant biology is a systems biology approach which integrates data from different omics such as transcriptomics, metabolomics and proteomics to identify the molecular targets for crop improvement (Kitano, 2002). “Transcriptomics” refers to the expression profiling analysis of all the expressed sequences of both coding and non coding RNAs while “metabolomics” refers to the identification and analysis of wide array of metabolites using a variety of techniques which is often coupled with a mass spectrometer. “Proteomics” is a large scale study of protein structure, function, protein interactions and a variety of modifications that proteins undergo inside the cell. Such an integrated approach enables to study different processes at their component levels and to unravel complex interplay or cross talk between different components in mediating dynamic activities of a tissue/organ/organism to different environments (Cramer et al., 2011).

Currently, plant stress responses are studied using either one or a combination of two approaches, mainly transcript and metabolite analysis. Transcriptome analysis is increasingly used to study stress responses in different crops as the technology is more advanced, easy to perform and due to its high throughput nature to uncover genome wide expression patterns. It enables to identify differentially expressed genes, co-expressed genes and to find the master genes through network analysis (Cramer et al., 2011). Some of the examples of identification of key genes using transcriptome analysis that have been validated under field conditions includes a SNAC1 (Stress responsive NAC) and LEA (late embryogenesis abundant) genes in rice (Hu et al., 2006; Xiao et al., 2007). Transgenic plants over expressing these genes were found to have increased drought tolerance under field conditions. Another transcription factor identified using transcriptomics under drought is NF-YB1 (Nuclear transcription factor Y subunit B-1), and the increased drought tolerance contributed by this transcription factor was validated both in Arabidopsis and maize through transgenic approach. The transgenic maize line over expressing ZmNF-YB2 had higher chlorophyll content, stomatal conductance and photosynthesis resulting in higher yield due to increased drought tolerance (Nelson et al., 2007). In barley, transcriptome approach has been employed to study spike responses to light, drought and other metabolites (Abebe et al., 2010; Mangelsen et al., 2010).

Although transcriptome analysis is widely used in plant system to study various abiotic stress responses, a poor correlation of transcripts with protein profiles or enzyme activities urged the need to combine the transcriptomics with other approaches such as metabolomics or proteomics. A number of studies have been carried out in different crops combining transcriptome with metabolite analysis (Armengaud et al., 2009; Osorio et al., 2011; Kang et al., 2011). Integrated analysis of transcriptome and metabolite profiling in Arabidopsis under drought revealed the significance of ABA accumulation under dehydration and its positive correlation with other genes responsible for accumulation of various amino acids and sugars (Urano et al., 2009). In another study, comparative analysis of metabolites produced under heat and cold shock in Arabidopsis revealed that majority of the metabolites produced in response to these treatments overlapped, hinting some common mechanism of plant responses to temperature (Maruyama et al., 2009; Salekdeh, 2009). With the development of proteome analysis, all three components of the system biology are beginning to be used in plant biology (Armengaud et al., 2009; Hummel et al., 2010; Osorio et al., 2011). An integrated analysis of transcripts, metabolites and enzyme activities under potassium (K) deficiency in Arabidopsis revealed that carbon and nitrogen metabolism was altered under K deficiency and the metabolic disorder observed under K deficiency was mainly due to pyruvate kinase activity and not its transcription (Armengaud et al., 2009). Another study in Arabidopsis using all three approaches combined with growth parameters showed that plants maintained a positive carbon balance under drought through a relatively large reduction in rosette expansion compared to reduction in photosynthesis, while root growth was promoted (Hummel et al., 2010).

1.11 Use of wild relatives in crop improvement

A pre-requisite for any successful breeding programme in a crop is the genetic diversity available for crop improvement. However, genetic diversity available in most of the present cultivated crops including barley is limited due to selection processes mainly targeted towards yield under favourable conditions (Harlan, 1976; Tanksley et al., 1996). This is well exemplified in the findings of Russell (Ellis et al., 2000), who reported that only 40% of the wild alleles are found in cultivated barley. Hence, it has become imperative for breeders to look for favourable alleles in wild ancestors of a particular crop species and transfer them to

cultivated ones to broaden their performance under different challenging environments (Tanksley and McCounch, 1997). Although wild species are inferior to cultivated varieties for a number of agronomic traits, AB-QTL analysis in different crops showed that, about 30 to 50% of the favorable alleles in breeding lines are contributed by wild relatives (Xiao et al., 1998; Thomson et al., 2003; Pillen et al., 2003; Frary et al., 2004; Von Korff et al., 2006; Swamy and Sarla, 2008; Nevo and Chen, 2010).

Many QTL studies carried out using wild barley as a donor parent for various agronomic and malting quality traits indicated that wild barley harbours many favourable alleles for these traits (Nevo, 1992; Volis et al., 2000; Pillen et al., 2004; Li et al., 2005, 2006). A notable example of cultivar improvement using wild barley is the development of Mlo resistance to powdery mildew (Jorgensen, 1992; Thomas et al., 1998) and leaf rust (Moseman et al., 1990). In addition, *H. spontaneum* was also found to possess positive alleles for abiotic stresses such as drought and salt tolerance (Talame et al., 2004; Suprunova et al., 2007; Ceccarelli, 2007; Lakew et al., 2011; Lakew et al., 2012). Because of the quantitative nature of yield *per se* and its complex interaction with various environmental factors, trait enhancement of the cultivated barley using wild accessions through traditional breeding was usually difficult and slow in the past as little information was available about the chromosomal regions controlling the trait, its nature and their interaction (Swamy and Sarala, 2008). The development of dense linkage maps in barley and other crops using modern molecular markers and sophisticated mapping softwares have enabled the identification of various QTLs influencing a trait and its interaction with other genomic regions influencing the trait (Collard and Mackill, 2008; Tester and Langridge, 2010).

1.12 Importance of QTL mapping

Major hindrances to utilization of wild species in crop improvement using conventional breeding is the quantitative nature of most of the agronomic traits and linkage drag of undesirable genes present in wild species (Wang and Chee, 2010). With the advent of various molecular markers and linkage maps, studies on the effect of individual loci controlling quantitatively inherited traits and its genomic location was made possible through QTL analysis. This has also enabled faster breeding process in the development of new varieties through implementation of marker assisted selection (MAS) (Patterson, 1998; Tanksley,

1993). The principles of QTL analysis were first applied to map a QTL for seed size in bean (Sax, 1923). Several types of population such as F_2 and BC_1 are used for QTL analysis as they are easy to develop. However, its temporary nature in the sense that segregating unit is an individual plant makes it difficult to study complex traits which are quantitative in nature. In addition, the above population is inferior in detecting favourable QTLs when epistatic interactions existed between QTLs and other donor genes in early generations (Pillen et al., 2003).

Recombinant inbred lines that are developed through repeated selfing of the progenies resulting from F_2 progenies were the means of solution to the low heritability and high experimental error associated with the QTL study using F_2 or BC_1. In addition, this population remains permanent for analysis of heterosis and QTL study (Hua et al., 2002). However, development of such population is time consuming and dominant effect cannot be detected, as each locus is homozygous in this population. When either of the above population structure mentioned above is used for QTL analysis in breeding programmes involving a wild genotype, this would substantially delay the development of a new cultivar, because QTL discovery and transfer of the detected QTLs to commercial cultivars through repeated back crossing are two separate processes (Tanksley and Nelson, 1996; Tanksley and McCouch, 1997). Hence, Tanksley and Nelson (1996) proposed advanced back cross QTL (AB-QTL) as the potential solution to the above problems.

1.13 Advanced back cross-QTL method

AB-QTL method using a wild species involves an initial hybridization of an elite cultivar with a wild species followed by a repeated back crossing of the resulting progenies to the elite cultivar until the BC_2 or BC_3 generation. The plants with undesirable traits as a result of linkage drag are discarded at the BC_1 or BC2 itself and the mapping population is usually BC_2F_2, BC_3 or BC_4 generations. This method enables the precise measurement of the individual QTLs as the undesirable effect associated with the genetic background of the wild species is reduced through successive back crossing with the recurrent parent. Since then AB-QTL analysis was successfully employed in different crops and in barley, it was first reported by Pillen et al. (2003) using a BC_2F_2 population developed between the cultivar Apex and the wild accession, ISR101-23 for various agronomic and malting qualitities.

1.14 Significance of the present study

Many studies in the past under terminal drought clearly established that traits contributing to terminal drought differ from genotype/crop and the severity of stress. In barley, most of these studies addressed only either source or sink using physiological or biochemical or molecular techniques and in some cases combination of aforementioned techniques. Drought tolerance being a more complex trait and development of drought tolerant varieties lagging much behind compared to other traits, is a clear indication that, in order to understand the basis of drought tolerance and to develop superior genotypes, a clear understanding of plant responses to drought have to be studied at both source and sink using a combination of different techniques (Fleury et al., 2010). Another issue concerned with drought response studies using integrated omics techniques is the sample collection and processing. Many of the studies using integrated omics approach use different samples for transcripts, proteins and metabolite measurements which can induce substantial errors resulting in poor correlations between the components (Weckwerth et al., 2004; Martins et al., 2007).

Considering the above facts, the present study was undertaken to understand terminal drought tolerance in barley using two complementary approaches; AB-QTL analysis and an integrated omics approach. AB-QTL analysis of an introgression line (IL) population (67lines) developed using the German spring barley cultivar, Brenda (*Hordeum vulgare* ssp. *vulgare*) and the wild accession, Hs584 (*Hordeum vulgare* ssp. *sponatneum*) was carried out to study the exotic regions influencing seed yield and quality under terminal drought. In addition, the above population was also used for studying various seed morphological traits (seed length, breadth and shape) which in general contribute to grain weight. To the best of our knowledge, this is the first AB-QTL study addressing seed morphological traits under terminal drought in barley. In another part of the present study, senescence and stay green mechanisms of terminal drought tolerance in barley were analyzed in great detail using elite breeding lines by an integrated omics approach combined with various physiological parameters to address seed yield and quality under terminal drought.

With this back ground, the major objective of the present study was to understand the mechanism of terminal drought tolerance in barley using,

(1) AB-QTL analysis of a BC_3-DH population developed between cultivated barley, Brenda and the wild accession, Hs584 to identify genomic regions influencing seed yield and quality under terminal drought.
(2) An integrated omics approach to understand the importance of stay green/senescence mechanism for terminal drought tolerance using contrasting elite genotypes.

A brief overview of the main objectives is as follows:

Approach 1:

1. Screening of the mapping population for yield, thousand grain weight, and seed quality parameters under terminal drought.
    I. Under field condition by spraying chemical desiccant (KI).
    II. Under green house conditions by withholding water at 10 DAF.
2. QTL analysis for the identification of genomic regions influencing various traits under both control and drought conditions.
3. Identifying the importance of C/N ratio as determinant factor of seed quality to assess drought tolerance.

Approach 2:

1. Selection of contrasting drought sensitive and tolerant genotypes under terminal drought and to characterize them using various physiological, biochemical and molecular techniques.
2. Understanding the source sink relationship in these contrasting genotypes in terms of carbon and nitrogen metabolism.

2 MATERIALS AND METHODS

Various chemicals, materials, instruments and buffers used during the experiment are given in appendix.

2.1 Plant material

Experimental material consisted of elite breeding lines (LP) and an introgression line (IL) population that were used for characterising terminal drought tolerance mechanism and QTL study respectively. LP lines (*Hordeum vulgare* ssp. v*ulgare*) comprised of 10 elite breeding lines from a German seed company, KWS-Lochow GmbH and IL was comprised of 67 lines from a BC_3-DH population derived from a cross between German spring barley cultivar 'Brenda' (*Hordeum vulgare* ssp. *vulgare*) as the recurrent parent and the wild accession 'Hs584' (*H. vulgare* ssp. *spontaneum*) as the donor parent (Li et al., 2006).

2.2 Experimental location and drought treatment

Experiments were conducted at two different locations in Germany, one at a plant breeding station Nordsaat (Böhnshausen), GmbH and another at IPK, Gatersleben. A schematic representation of the various growing and traits scored during the study are given in the **supplementary figure 1**. Screening of all genotypes for drought tolerance at Nordsaat was carried out during two consecutive years, 2007 and 2008 under both field and green house conditions. Terminal drought in the field and green house (GH) was imposed by spraying with potassium Iodide (0.4%) and by withholding water, respectively, at 10DAF. In the case of introgression line population, screening was carried out under both field and green house conditions; whereas, LP lines were screened only under GH condition during both years. In the field, all genotypes were planted as three-row plots per entry with two replications in randomized blocks where as in GH, all genotypes were planted as two-row plots per entry with two replications in randomized blocks. Under both conditions (field and green house), drought was imposed at 10 DAF for the drought treatment group, whereas, control plants were continued to irrigate until seed maturity. Yield and thousand grain weight (TGW) were determined from matured plants harvested from the respective treatments under both GH and field conditions. The data obtained from Nordsaat screening experiments was used for QTL

analysis of IL population and for selection of contrasting LP lines for further characterization.

At IPK, the selected contrasting LP lines were further grown under phytochamber and green house conditions (during 2009-2010) for source sink studies (leaf and seed) and stem remobilization, respectively. The IL population was grown under both GH and field conditions and various traits related to yield, seed quality and seed morphological traits under terminal drought were scored for QTL analysis (**Supplementary figure 1**).

2.3 Growing condition and sample collection at IPK

In green house, plants were grown in individual pots with a 16 hours light/20°C and 8 hours dark/15°C cycle. Irrigation was achieved through an automatic irrigation system (Delta T devices) that consisted of a large number of soil moisture sensors (SM200) connected to a central device (DL2 data logger) that is capable of recording, storing of soil moisture content and irrigating according to the conditions defined. Control plants were maintained at 40% soil moisture content (100% field capacity) from sowing to until maturity, whereas, stress plants were maintained at 10-15% (25-30% field capacity) from 10 DAF to until maturity. Figure 3 is a representative example for soil moisture content maintained during the experiment. Each genotype had 10 plants (5 plants/treatment) and in order to have spikes of various developmental stages, the date of flowering for individual spikes were tagged upon anthesis. Flag leaf and seeds from the tagged spikes were harvested at 25 DAF and analyzed for starch and nitrogen content. Yield and contributing characters such as TGW and other seed morphological characters such as seed length and breadth were analyzed using a digital seed analyzer, Marvin®. Seed length to breadth ratio which indicates seed shape was calculated from the primary data obtained for seed length and breadth.

In phytochamber, growing conditions were similar to green house except that irrigation was done manually after measuring the soil moisture on a daily basis using SM 200. Apart from various physiological studies, samples were collected from both leaf and developing seed at 12, 16, 20 and 25 DAF which were subsequently used for various biochemical and gene expression studies.

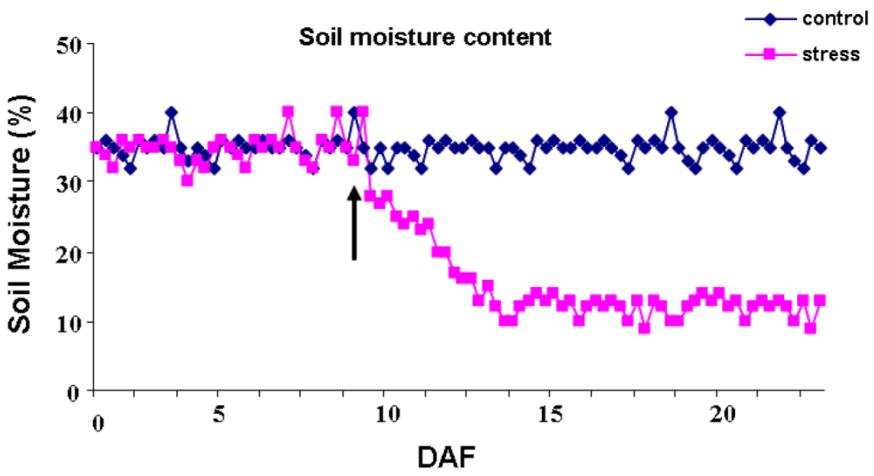


Figure 3. Soil moisture content recorded during the experimental period.
Soil moisture content recording and irrigation was achieved through an automatic irrigation system (DL2) and the control and drought treatments were maintained at 40 and10% respectively until maturity after the drought was imposed from beginning 10 DAF.

2.4 Drought screening indices for selection of contrasting LP genotypes

Selection of contrasting LP lines for further characterization was based on various drought screening indices calculated from the TGW data obtained from the Nordsaat study. Various drought screening indices were calculated as follows,

Drought susceptibility Index (DSI),

DSI= {(Gc- Gds)/ Gc}/ DII

Drought Tolerance Index (DTI)

DTI= (Gc* Gds)/ $(Xc)^2$

Percentage Kernal Injury (%KI)

(Gc- Gds)*100/ Gc

Where Gc= TGW under control; Gds= TGW under drought; Xc= mean TGW of all genotypes under control; Xds=mean TGW of all genotypes under stress; DII= Drought intensity index = (Xc-Xds)/ Xc.

2.5 Isotopic labeling with ^{13}C

In order to quantify post anthesis stem remobilisation in the selected contrasting LP lines, stable isotope labeling with $^{13}CO_2$ was carried out with plants grown under green house conditions at 5 days before flowering. Each treatment (control labeled, control unlabeled, stress labeled) consisted of 4 replications (3plants/replication). For ^{13}C labeling, whole plant was covered in a transparent plastic bags (5 plants each, diameter 59.5 cm, height: 150 cm, LDPE-foil, 150 µm; Roundliner GmbH, Forst, Germany) and pulse labeled with ^{13}C barium carbonate (99 atom%, CAMPRO Scientific GmbH Berlin, Germany) for 60 minutes. $^{13}CO_2$ was generated by injecting 5 mL of 2 M $HClO_4$ into a beaker with 1 g $Ba^{13}CO_3$. The ^{13}C label were measured by isotope ratio mass spectrometry with the tracer mass 20-20, SerCon, Crewe, UK. An initial sampling was carried out at 8 DAF after which the stress treatment was initiated and the final harvest was made at 25 DAF. Samples were collected from stem, leaves and grains of the main tiller and immediately oven dried at 70°C. All ^{13}C data are expressed on excess basis, which means tracer minus background isotope, and total ^{13}C was calculated. Percentage distribution and remobilization of ^{13}C was calculated using the formula given below and expressed per organ.

Percentage distribution (%) of isotopes: Control (8 DAF) = (control labeled 8 DAF – control unlabeled 8 DAF); Control 25 DAF = (control labeled 25 DAF – control unlabeled 25 DAF); Stress 25DAF = (stress labeled 25 DAF– control unlabeled 25 DAF).

Remobilization efficiency under well watered condition (%) =

[Amount ^{13}C 8 DAF ww / [^{13}C amount 8 DAF ww + ^{13}C amount 25 DAF ww)]* 100

Remobilization efficiency under water deficit (%) =

[Amount ^{13}C 8 DAF ww / [^{13}C amount 8 DAF ww + ^{13}C amount 25 DAF wd)]* 100

ww = well watered or control and wd = water deficit or stress

2.6 Relative leaf water content

Relative leaf water content (RWC) is the ratio of current water content of the sampled leaf to the maximum water it can hold at its full turgidity expressed in percentage. It is one of the most extensively used parameter for various stress experiments especially under drought to quantify and study various physiological consequences of water deficit in plants (Barr and Weatherley, 1962). Flag leaf from the main tiller was cut and fresh weight (W) was recorded

immediately followed by incubating at 4°C overnight by floating on deionised water in a petri plate. Turgid weight (TW) was recorded next day after gently wiping with a tissue paper. Finally, samples were oven dried at 80°C for 24 h to obtain the dry weight (DW). Sample collection was carried out early in the morning before the onset of light period. RWC was computed using the below formula,

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

Where, W=Sample fresh weight; TW – Sample turgid weight; DW – Sample dry weight.

2.7 Chlorophyll estimation

Leaf chlorophyll was estimated using acetone method according to the protocol of Porra et al. (1989). In brief, about 100-150 mg of leaf tissue was ground using a pre cooled pestle and mortar with buffered aqueous acetone (80%). The contents were transferred to a 2mL eppendorf tube and centrifuged at 13000g for 10 min. The supernatant obtained after centrifugation was used for chlorophyll estimation. Absorbance was recorded at 663, 645 nm and chlorophyll content (µg/mL) was estimated using the following formula,

Chla= 11.24A663 - 2.04A645 and **Chlb**= 20.13A645 - 4.19A663.

2.8 Photosynthetic measurements

The principle of gas exchange is based on IRGA (Infra Red Gas Analysis) i.e., heteroatomic molecules such as CO_2, H_2O, NO, and NH_3, absorb infra-red radiation at a particular infra red wave bands. The main absorbance band for CO_2 is at 4.25 µm with secondary peaks occurring at 2.66, 2.77 and 14.99 µm .Photosynthetic gas exchange measurements at single leaf level were carried out using a portable photosynthetic instrument, LCPro+ at two different time points after stress induction (4 and 8 days after stress). The instrument was stabilized for 30 min. before the actual measurements were carried out. A constant supply of 400 ppm of CO_2 with a flow rate of 200 µmol/sec was obtained using a CO_2 catridge (4gm CO_2 per catridge) 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. All photosynthetic measurements were carried out in the morning between 10.00 and 13.00 in a fully emerged flag leaf of a main tiller. Different parameters like net assimilation rate (A), stomatal conductance (g_s), internal CO_2

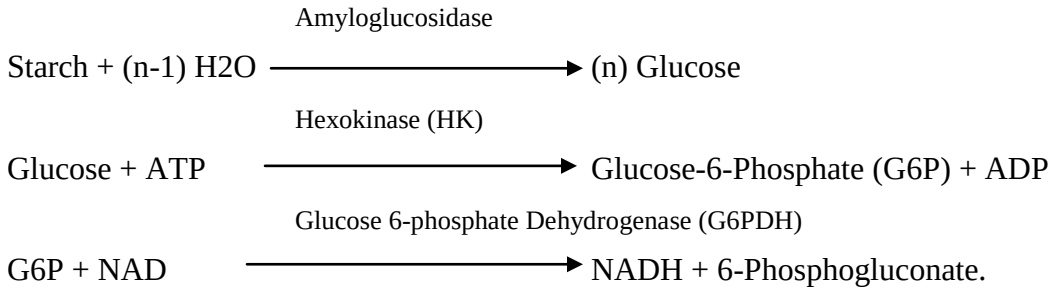
concentration (Ci) etc., were recorded with two readings per plant and in a total of 5 plants/treatment. All measurements were recorded once a stable Ci was obtained (2-3 min. after mounting the leaf to leaf chamber head).

2.9 Determination of osmolality

After freezing and thawing leaf tissue in closed 1.5 mL eppendorf tubes, it was centrifuged at 5000g for 5 min at 4°C. Osmolality of the resulting supernatant was measured using an osmometer (Wescor Vapor Pressure Osmometer, model 5500).

2.10 Enzymatic method of starch estimation

Starch estimation



Starch was estimated by measuring the NADH absorption at 340 nm which was generated during the conversion of glucose 6 phosphate to 6-phosphogluconate by the enzyme, glucose 6 phosphate dehydrogenase (Ernst and Arditti, 1972). The pellet obtained (from 15-20 mg of seed or leaf) after ethanolic extraction (details in amino acid estimation section) was used for starch estimation either by HCl (Hydrochloric acid) or treatment with amyloglucosidase. In HCl method, the pellet was dissolved in 2N HCl (1.5 mL) and incubated at 95°C for 1 h. The resulting mixture was directly used for glucose estimation after centrifugation at 13000g for 5 min. In enzymatic method, the pellet was dissolved in 0.5 M KOH (1.5 mL) and incubated at 95°C for 1 h followed by adjusting the pH of the solution approximately to 5-7 using 5N HCl. Finally, 5 µL of extract was incubated with 100 µl of amyloglucosidase (7.9 units) at 55 to 60°C for 30 min.

Glucose resulting from the above methods was estimated according to the scheme depicted above. Briefly, a mixture of 750 µL Imidazole buffer (pH 6.9) consisting of 2 mM NAD and 1 mM ATP was incubated at room temperature for 10 min. in a disposable plastic cuvette

along with 5-10 µl of the extract and 2 µL of glucose 6 phosphate dehydrogenase (2 units). After recording the initial absorbance of the mixture at 340 nm, 10 µL of hexokinase (8 units) solution was added to the mixture and incubated for further 25 min and the absorbance was recorded at 340 nm. A standard curve was prepared using starch from maize kernel as standard.

2.11 Ion chromatography for estimation of soluble sugars (HPAEC-PAD)

Soluble sugars were analysed by ion chromatography, HPAEC-PAD (High Performance Anion Exchange Chromatography- Pulsed Amperometric Detection, Lee, 1990). Chromatographic analysis was conducted with a Dionex IC system consisting of an autosampler AS 50, a gradient pump GP 50, and an electrochemical detector ED 40 equipped with a thin-layer-type amperometric cell. The cell comprised of a gold working electrode and an Ag/AgCl reference electrode. Data acquisition and processing were accomplished with the Dionex Chromeleon 6.70 software. Chromatographic separation was carried out with the analytical column, CarboPac PA 20 in conjunction with a guard column and an Ion Pac trap guard column. Column temperature was maintained at 35°C in a column oven (STH-585). Analytes were separated with isocratic elution using 50% A (150 mM NaOH) and 50% B (water) as eluents at a flow rate of 0.3 mL min^{-1} for 15 min. Analyte detection was achieved by applying a quadrupole-potential waveform on the gold electrode (E1 = 0.1 V from 0 to 0.4 ms; E2 = 2.0 V from 0.41 to 0.42 ms; E3 = 0.6 V from 0.42 to 0.43 ms; E4 = –0.1 V from 0.4 to 0.5 ms). The analytical data quality was controlled by standard addition methods. A sample chromatogram from the analysis is shown in Figure 4.

2.12 Estimation of amino acids by HPLC

Sample extraction:

Lyophilised powdered plant sample was extracted thrice with 80% ethanol by incubating at 60°C for 30 min. in a thermomixer. The supernatant obtained after centrifugation at 13000g for 10 min. at 4°C was evaporated to dryness using a centrifuge vaccum evaporator. The dried material was redissolved in deionized water and vortexed thoroughly. Contents were then filtered (Ultrafree-MC Membranes; Millipore) and the filtrate obtained was used for estimation of either amino acids or sugars or can be stored at -20 °C until analysis.

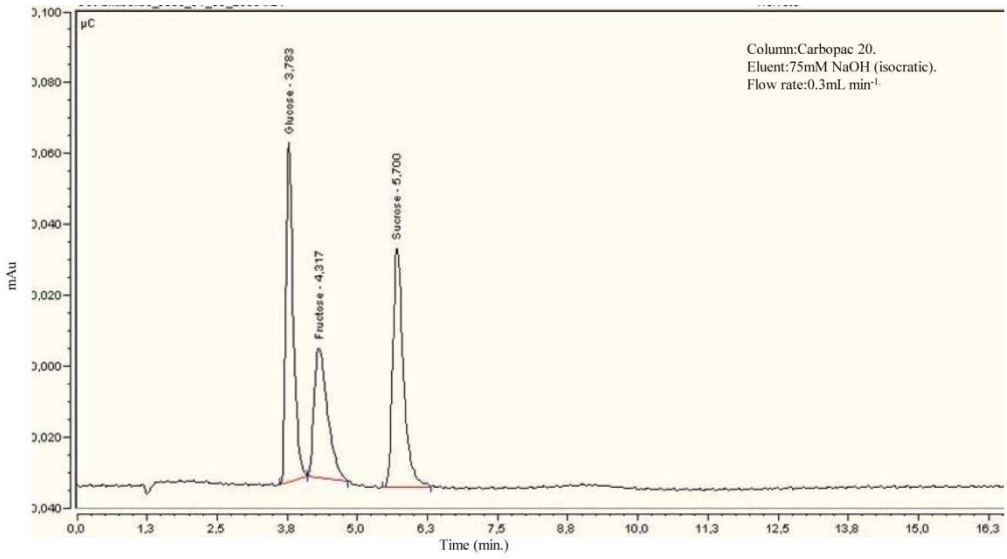


Figure 4. A representative chromatogram from the analysis of standard mixture of glucose, fructose and sucrose by HPAEC-PAD.

Reagent preparation:

The reagents and solutions required for sample derivatization was available in the kit provided by Water (AQC dry powder, acetonitrile for dissolving the reagent and borate buffer). Derivatization was carried out according to the instructions provided in the manual, AccQ-Tag method. Briefly, AQC reagent powder was dissolved in 1 mL of acetonitrile which was approximately 3.0 mg/mL, vortexed thoroughly and incubated at 50°C for 10 min. A mixture of standard amino acids except asparagine and glutamine was available from sigma (0.5 mM in 0.01 M HCl). A working solution of 50 pmol/µL of each amino acid was made using 0.01 M HCl after adding asparagine and glutamine separately. The details of chemistry involved in the amino acid analysis by AccQ-Tag method can be found in Cohen, 2005 and Meyer et al., 2008.

Sample derivatization:

About 10 µL of the fluorescent dye reagent was added to a small eppendorf (0.5mL) containing 10 µL of sample and 80 µL of borate buffer (0.2M, pH 8.8). The contents were thoroughly mixed immediately and incubated at 50°C for 10 min. and analyzed by HPLC. Similarly, standard was prepared by derivatizing with different volumes of the working standard solution. Unused reagent could be stored at -20 °C for several weeks.

Chromatography analysis:

Before the chromatographic analysis, the system was equilibrated with 100% eluent A (140 mM sodium acetate and 7 mM triethanolamine) and the column temperature was set to 37°C. Fluorescence detector was set at 248 nm wavelength for excitation and 395 nm for absorbance. Chromatography was carried out using a Dionex HPLC system (Summit) consisting of a gradient pump (P680), a degasser module, an autosampler (ASI-100) and a fluorescent detector (RF 2000). Data acquisition and processing was accomplished with Dionex Chromeleon 6.70 software. The gradient was accomplished with eluent A, B and C representing buffer, acetonitrile and water, respectively. Analytes were separated on a reversed-phase analytical column (AccQ Tag) coupled to a guard column (Nova-Pak C18). The column temperature was maintained at 37°C throughout the measurement and the flow rate to 1 mL/min. The gradient was produced by the following concentration changes, t=0, 100%A; t=0.5 min, 99%A and 1%B; t=27.5 min, 95%A, and 5%B; t=28.5min, 91%A and 9%B; t=44.5min, 82%A, 18%B; t=47.5min, 60%B and 40%C; t=50.5min, 100%A and t=60 min, 100%A. During the whole run, the gradient curve was always maintained at 6. A representative chromatogram from the analysis is shown in the Figure 5.

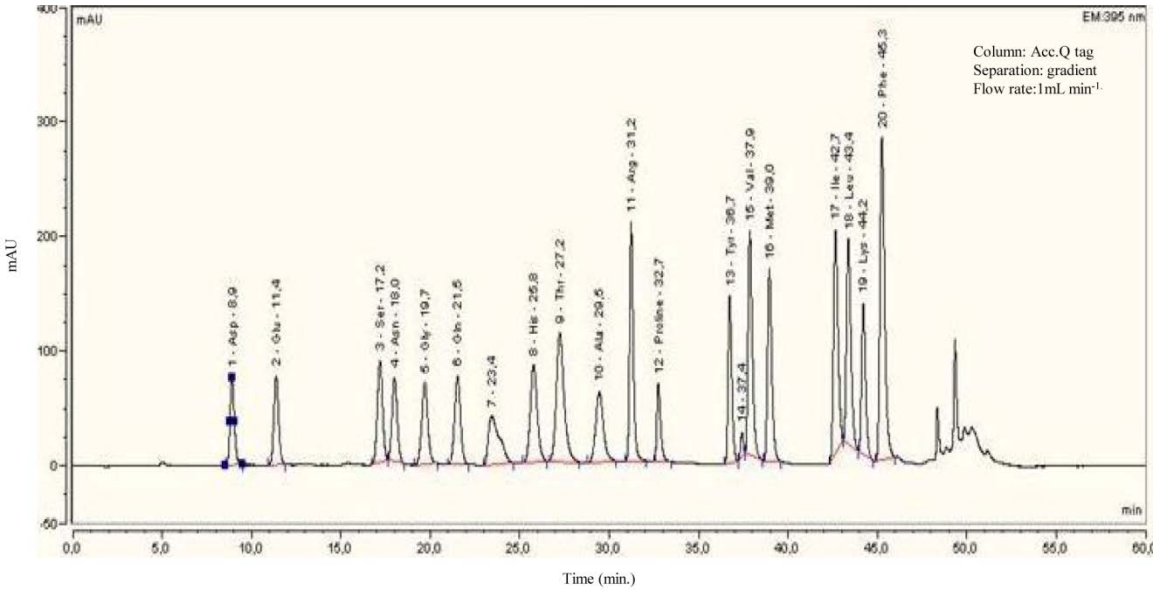


Figure 5. A representative chromatogram from the analysis of standard mixture of amino acids by HPLC.

2.13 Extraction and analysis of ABA (LC-ESI-MS-MS)

ABA was extracted from lyophilised plant material using ethyl acetate (100 %). Isotopically labeled D6-ABA was used as an internal standard and added to each sample during the extraction procedure. Extraction was carried out twice with 1 ml of ethyl acetate at 4°C. The supernatant collected after centrifugation (x13000g, 10 min., and 4°C) was evaporated using a vacuum concentrator to dryness at room temperature. The dried samples were redissolved in acetonitrile: methanol (1:1) and filtered using 0.8µm filter (vivaclear). The filtrate (10µL) was used for subsequent quantification using LC-MS/MS (Dionex Summit coupled to Varian 1200L). Chromatogram acquisition and data processing was accomplished with the Varian software, "Work station". Chromatographic separation was carried out on a C18 column (4 µm, 100 mm; GENESIS; Vydac/USA). The solvent system used was 0.1% acetic acid (A), 100% acetonitrile (B) and distilled water (C) with the following gradient: starting at 0 min with 70%B and 29.2%C, within 4 min to 89% B and 10.2% C; 4 to 4.5 min 99.2% B and 4.5 to 5 min 70%B and 29.2%C. Solvent A was held constant at 0.8%. Solvent flow rate was 200 µl/min. The MS was operated with ESI in the negative mode with the following parameters: collision gas 1.7 mTorr, API drying gas 20 psi, 250 °C, API nebulizing gas 51 psi, needle -5000 V, shield -200 V, detector 1500 V. MRM and quantification was done using the mass traces 263/153 for ABA and 269/159 for D6-ABA. The validity of the extraction and measurement procedure was checked by recovery experiments (approx. 82-95 %). Quantification was based on calibration with known ABA standards and individual recovery rates for the samples.

2.14 Carbon and nitrogen analysis

Carbon and nitrogen analysis was carried out with elemental analyzer (vario EL *III*), which can also be used for measuring hydrogen, sulphur and oxygen (Rezl, 1978).

Principle:

The principle of elemental analysis is based on the fact that all atoms prefer to be in their oxidation states. In pure oxygen, at high temperatures, all available carbon will easily burn to become carbon dioxide, all hydrogen will burn to become water and all nitrogen will become various nitric oxides which are subsequently separated and detected.

Methodology:

Instrument was switched on about 3-5 hours before actual analysis and the measurement was carried out in CN mode. About 3 to 4 mg of oven dried sample was weighed in an aluminium capsule, folded and placed in the autosampler. During measurement, the capsule enclosing the sample falls into a combustion chamber with excess oxygen kept at 900°C, where it is mineralized with the help of some catalysts. Various gases formed (CO_2, H_2O und NO_x) then passes through a silica tube packed with copper granules held at about 500°C (reduction tube) where the remaining oxygen is bound and nitric/nitrous oxides are reduced to N_2. The leaving gas stream includes analytically important CO_2, H_2O, N_2 and SO_2. All gases are removed at appropriate traps leaving the analytically important CO_2 and N_2 which are subsequently detected with a thermal conductivity detector. High purity helium (Quality 5.0) is used both as a carrier and reference gas. Blank values are obtained from empty aluminium capsules and calibration is done by elemental analysis of standard substances supplied by the instrument's manufacturer.

2.15 LC-MS based metabolite profiling

Extraction of metabolites for LC-MS based detection was carried out according to Rolletschek et al. (2011). About 50 mg of powdered lyophilised seed material was taken in a 2mL eppendorf tube to which 1.2 mL of ice cold 1:1 (v/v) methanol/chloroform containing the internal standard (^{13}C Succinate) was added and vortexed thoroughly. To this, 750uL of water was added and again vortexed thoroughly for about 30-45 sec. The contents of the eppendorf were vortexed thoroughly and centrifuged for 2000g for 2 min. at 4°C. About 500uL of the upper aqueous/methanol phase obtained after centrifugation was transferred to a Vivaclear centrifugal filter (0.8um pore size) and filtered by centrifuging at 2000g for 2 min. The flow-through could be either used immediately or can be kept frozen at -80°C until the measurement. The samples were analysed in an LC-MS/MS based system (Dionex Ulimate 3000 RSLC and API 4000 (Applied Biosystems) according to Rolletschek et al. (2011) and the ionization was achieved through electron spray method (LC-ESI-MS-MS). To obtain a better resolution of the various metabolites, separate measurements were carried out either by coupling ion chromatography to MS in negative mode or liquid chromatography coupled to MS either in the positive or negative mode. Various compounds separated using different

methods and corresponding MS parameters are given in supplementary Tables S1, S2 and S3. The compounds listed in supplementary Table S1 were measured using Ion chromatography (ICS-3000) coupled to an electrospray ionization triple quadrupole mass spectrometer (API 4000) in negative mode. Chromatographic separation was performed using an IonSwift MAX-100 (1 × 250 mm; Dionex) with a constant column temperature of 40°C and a column flow of 150 µL/min. The suppressor was set to a value of 38 mV. As sodium hydroxide eluent, we used the following gradient: t = 0 min (5 mM); t = 10 min (5 mM); t = 16 min (12 mM); t = 28 min (25 mM); t =32 min (100 mM); t = 38 min (100 mM); t = 42 min (5 mM); t = 56 min (5 mM).

All other metabolites were measured by coupling of LC (Ultimate 3000) with the mass spectrometer either in the positive or negative mode. The chromatographic separation was carried out using an aminopropyl column (Phenomenex Luna NH2; 250 mm × 2 mm, particle size 5 µm) that was mentioned in Bajad et al. (2006). The compounds determined in the negative mode are listed in supplementary Table S2. The eluents used for the chromatographic separation are: solvent A, 20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45; solvent B, acetonitrile. The following gradient using the above mentioned eluents were used for LC separation in the negative mode: t = 0, 85% B; t = 15 min, 0% B; t = 38 min, 0% B; t = 40 min, 85% B; t = 50 min, 85% B. The compounds determined in the positive mode is listed in the supplementary Table S3 and the gradient was achieved as following: t = 0, 85% B; t = 15 min, 0% B; t = 28 min, 0% B; t = 30 min, 85% B; t = 40 min, 85% B. Data were normalized to plant milligrams of dry weight and to an internal standard added during extraction (^{13}C-succinate; Cambridge Isotope Labs).

2.16 RNA isolation and quality checking

Total RNA was isolated from flag leaves using the TRIZOL reagent (Invitrogen GmbH, Karlsruhe, Germany) and RNAeasy columns (Qiagen, Hilden, Germany). About 1 mL of TRIZOL reagent was added to 100 mg of the homogenized leaf material and incubated at room temperature for 5 min. The supernatant obtained after centrifugation (10,000 rpm for 10 min.) was transferred to a new eppendorf tube to which 200uL of chloroform was added and incubated at room temperature for 2–3 min. Samples were again centrifuged as described above and the aqueous supernatant was transferred to the Qia shredder column and

centrifuged for 30 s at 10,000 rpm. To the flow-through obtained in the above step, 350 ul of RLT buffer (plus beta-mercaptoethanol 10ul/ml of RLT buffer) and 250 ul of absolute ethanol were added 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 (Qiagen Hilden, Germany) digestion. The quality of the RNA obtained was checked using NanoDrop photometer (Peqlab) and Agilent 2100 bioanalyzer according to manufacturer's protocol.

2.17 Probe preparation and affymetrix barley1 genechip analysis

Gene expression analysis of flag leaf harvested at 20DAF was carried out using the 22K Affymetrix barley1 gene chip. Probe synthesis, labeling, and hybridization were carried out according to the manufacturer's protocols (Affymetrix) and the arrays were scanned on a GeneChip Scanner 3000 (Close et al., 2004). Data processing and analysis was carried out as mentioned in Sreenivasulu et al. (2008). Briefly, the extracted genes were normalized (RMA) by applying a linear model via the the limma package using R/Bioconductor functions in Robin software. After normalization, $\log_2$ expression values were derived to generate fold differences between non-stressed and drought stressed treatments and the p value was corrected using the Benjamini-Hochberg ($P \leq 0.05$) to recognize significant differences in expression levels. The clustering groups obtained after subjecting the $\log_2$ transformed expression data to hierarchical clustering. The differentially expressed genes were functionally assigned according to Sreenivasulu et al. (2008) and the heat maps were generated using Genesis software (Sturn et al., 2002). Functionally overrepresented gene categories have been calculated by Fisher's exact test with a P-value cut off 0.01 and the differentially expressed genes were functionally assigned using Mapman and PageMan softwares as described in Sreenivasulu et al. (2008).

2.18 QTL analysis and Statistics

QTL analysis of the introgression line population for various traits was carried out using the data obtained from both Nordsaat and IPK experiments (**Supplemental figure 1 and Table 1**). QTL analysis and correlation between the traits were carried out using Qgene 3.0 program (Nelson, 1997). QTL regions were identified based on the linkage map developed by Worch et al. (2010) for the same population using various EST based SNPs and SSR markers. QTL

analysis was performed for control and stress treatments separately for each environment. To determine the effect of a particular marker on each trait, initially a single marker regression was carried out and the markers lying close to each other was considered to be in a QTL region. The regions of the genome were identified as QTL only if a particular marker/QTL has appeared in atleast two environments with an LOD≥3.0 for yield, thousand grain weight and their tolerance indices. For other traits (seed length, breadth, length to breadth ratio, seed starch content and nitrogen content), QTL was considered if it had an LOD≥3.0 and appeared in at least one treatment (control or drought) as these traits were recorded only in one environment (IPK, Gatersleben). QTL regions are represented on a consensus linkage map developed using EST based SNP markers based on three population (Worch et al., 2011). Significant difference between the treatment groups (control and drought) were carried out using students t-test in Microsoft office Excel 2003 and the significant levels are indicated by * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$).

3 RESULTS

3.1 AB-QTL ANALYSIS TO IDENTIFY THE GENOMIC REGIONS INFLUENCING TERMINAL DROUGHT TOLERANCE IN BARLEY FOR SEED YIELD AND QUALITY

Wild barley is a valuable source for variety of traits including various agronomic characters, seed quality, and resistance to both biotic and abiotic factors. The present study was aimed at the identification of exotic QTLs conferring terminal drought tolerance in terms of yield and seed quality using a BC_3-DH IL developed between the cultivated parent, Brenda and the wild accession, Hs584 (Li et al., 2006). Genotyping of this population was previously carried out using various SSR and SNP markers (Li et al., 2006; Worch et al., 2011). Details of the various experiments are given in Table 1 and Supplementary Figure 1.

Table 1: Details of the experiments along with various traits scored.

Traits	Nordsaat_2007		Nordsaat_2008		IPK_2010-11	
	Field	GH	Field	GH	Field*	GH
Yield (g)	X	X	X	X	-	-
DTI-Y	X	X	X	X	-	-
TGW (g)	X	X	X	X	X	X
DTI-TGW	X	X	X	X	X	X
Seed starch (mg/seed)	-	-	-	-	-	X
Seed nitrogen (N%)	-	-	-	-	-	X
Seed breadth (mm)	-	-	-	-	X	X
Seed length (mm)	-	-	-	-	X	X
Seed L/B ratio	-	-	-	-	X	X
No: genotypes	67	67	67	67	65	61

Table 1: Details of the experiments along with various traits scored.
At all locations experiments included two treatments (control and stress) except at IPK field where only control condition was carried out (*). At Nordsaat in the field and green house, drought was imposed by potassium iodide spray and water withholding, respectively. At IPK, drought was imposed by water withholding. If the trait was scored under particular environment, it is indicated by X symbol and if not, by -. Unit of measurements for the various traits are given in brackets followed by the trait in the traits column. Abbreviations: **GH**-green house, **L/B**-length to breadth ratio. **DTI-Y**- drought tolerance index of yield, **DTI-TGW**- drought tolerance index of TGW.

3.1.1 Comparison between parents

As an initial study, the parents of the introgression line population were grown separately under phytochamber conditions and various traits were recorded under both control and drought conditions. Thousand grain weight (TGW) was severely affected in Brenda

compared to Hs 584 under stress (Figure 6A). Brenda had 12% reduction in TGW under stress over control while it remained almost unaffected in Hs 584. Starch, a major component of matured barley seed also exhibited a similar trend as that of TGW with a severe reduction observed in Brenda compared to Hs 584 under stress (Figure 6B). However, TGW and seed starch content were generally higher in Brenda compared to Hs 584 under control conditions. Seed nitrogen content (N%) was significantly higher in Hs 584 compared to Brenda under both control and drought conditions. Drought significantly increased seed N% in Brenda from 1.7% under control to 1.9% under drought; whereas in Hs 584, drought had little effect on seed N% (3.6% under both conditions; Figure 6C). Apart from the above characters, parents also differed in seed morphological characters such as seed length (SL), breadth (SB) and length to breadth ratio (L/B) that influence TGW (Figures 6D and 6E). Seed breadth was significantly lower in Hs 584 (3.3mm) compared to Brenda (4.3 mm) whereas SL was higher in Hs 584 (12.2mm) compared to Brenda (9.9mm). Another notable difference between the parents was in plant height; Hs 584 was significantly taller (185 cm) compared to Brenda (100cm; Figure 6F).

3.1.2 Analysis of trait variation

The comparative analysis of the DH and the parental data indicated that, in most cases, DH mean was intermediate to the parental values (Supplemental Tables S4 and S5) and were generally normally distributed. Transgressive segregation was visible in yield, drought tolerance for yield (DTI-Y), TGW and drought tolerance for TGW (DTI-TGW).

There was a large variation in both morphological (Figures 7A to 7C) and quality traits (Figures 8A and 8B) analyzed in the present study. Within the IL population, seed length ranged from 9.8 mm in ILHS 19 to 12.1 mm in ILHS 5 (Figure 7A); whereas breath ranged from 3.7 mm in ILHS 58 to 4.4 mm in ILHS 60 (Figure 7B). Seed length to breadth ratio ranged from 2.33 in ILHS 19 to 2.96 in ILHS 5 (Figure 7C). A large set of introgression lines such as IL7, IL42, IL60, IL22 and IL23 etc., with similar or lower grain width compared to Brenda had significantly higher TGW which could be a result of increased seed length.

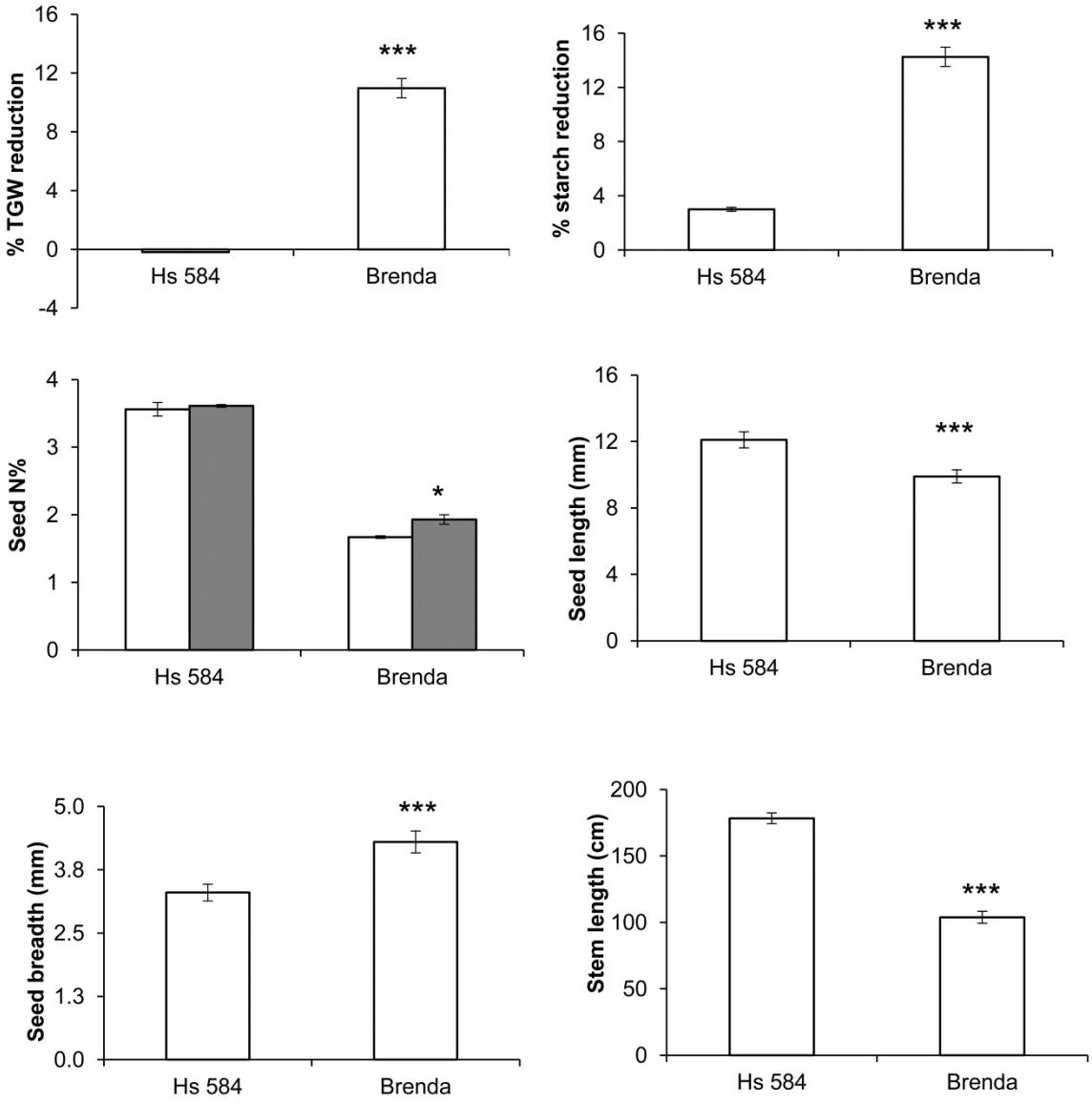


Figure 6. Terminal drought decreased the grain yield but increased the protein content of Brenda.

(A) 1000 grain weight was significantly reduced in Brenda while it remained almost unaffected in Hs 584.

(B) Seed starch content was significantly reduced in Brenda compared to Hs 584.

(C) Seed N% content was increased in Brenda while it remained unaffected in Hs 584. In general, Hs 584 had higher seed N% compared to Brenda.

(D) Seed length was higher in Hs 584 compared to Brenda under control condition.

(E) Seed breadth was higher in Brenda compared to Hs 584 under control condition.

(F) Stem length was significantly higher in Hs 584 compared to Brenda under control condition.

Significant differences between the treatments/parents are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

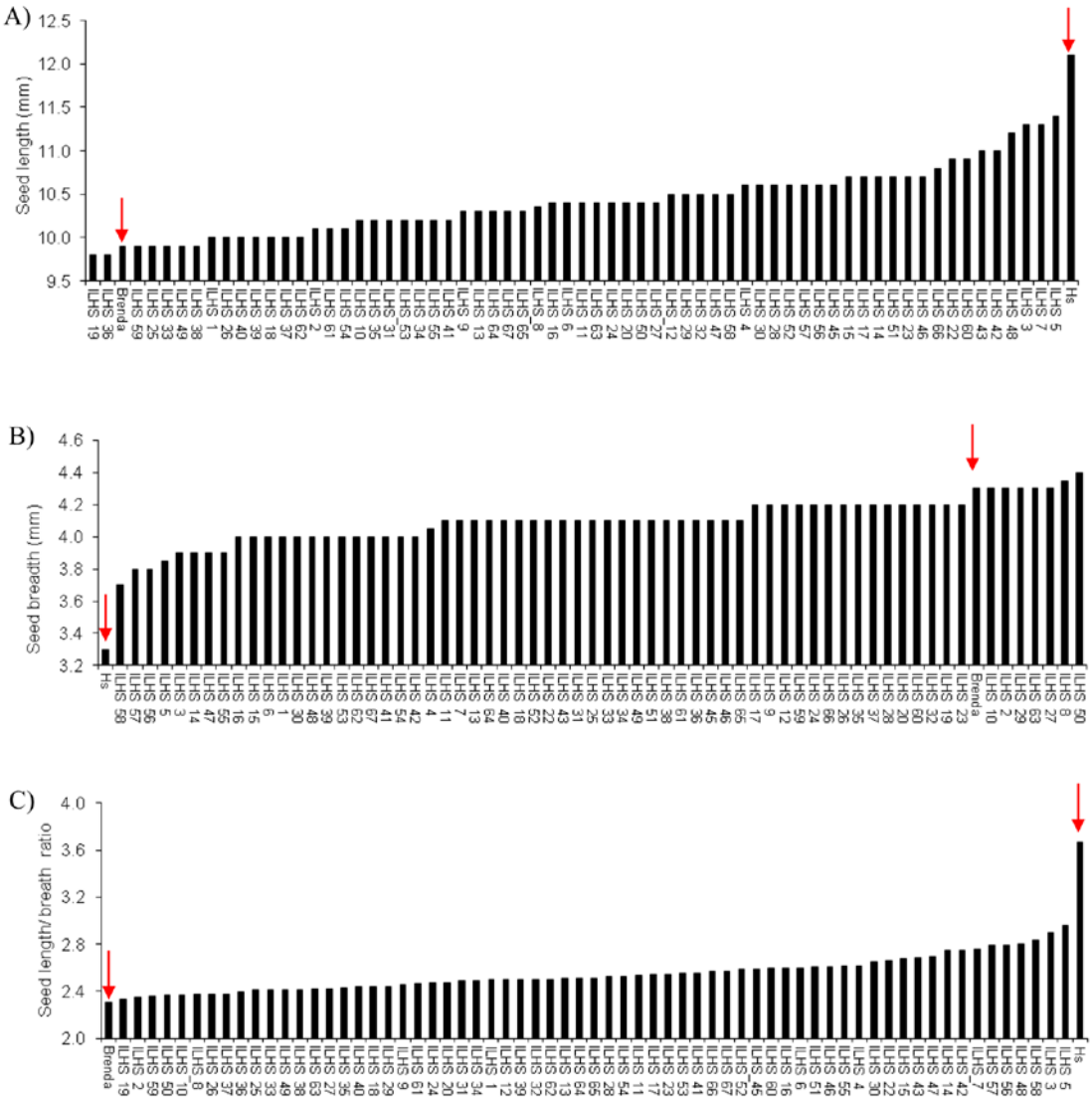


Figure 7. Variation in different seed morphological and quality traits scored across the IL population under control condition.

(A) Variation in seed length with parents representing the extreme values.
(B) Variation in seed breadth with some of the lines showing higher values than Brenda.
(C) Variation in seed L/B ratio with parents representing the extreme values.
The population is arranged in ascending order of the values of the traits and the parents are indicated with a red arrow.

This is a clear indication of a positive effect of grain length on TGW. Within the IL population, starch content ranged from 8.2 mg/seed in ILHS 50 to 33.9 mg/seed in ILHS 9 under drought while seed N% ranged from 1.25% in IL37 to 2.98% in IL57 (Figures 8A and 8B). An increase in seed starch content observed in some of the genotypes under drought

could probably due to reduction in seed number as drought might have coincided with their flowering time. In general, all the trait values were higher under control condition except seed nitrogen content, which was higher under drought.

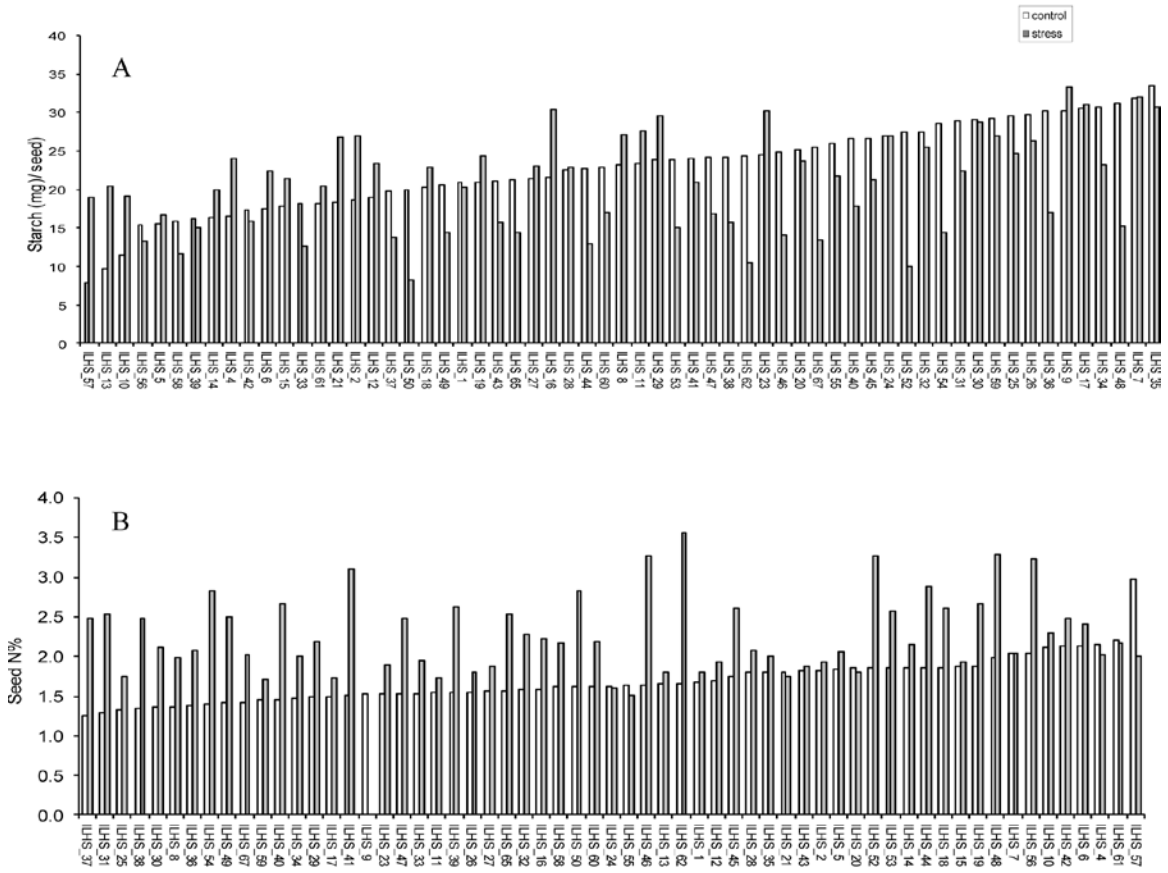


Figure 8. Altered seed quality traits under terminal drought.

(A) Effect of terminal drought on seed starch content.
(B) Increased seed nitrogen content under terminal drought

3.1.3 Trait correlations within and between the environments

Correlations of different traits within an environment are summarized in Tables 2, 3 and Supplemental Table S6. Among various traits, correlation between treatments for yield and TGW was significant in most of the environments indicating that, relative ranking of the genotypes remained similar under both control and drought conditions. Interestingly, TGW and yield were significantly correlated in all the green house experiments (3) but not in any of the field experiments (2). Similarly, DTI-Y and DTI-TGW were significantly correlated ($p<0.001$) to their respective traits in all the environments calculated. Among seed

morphological traits, SB was positively correlated to both starch content and TGW under both conditions; however, no such correlation was found with seed length (Table 3). On the other hand, while a negative trend was observed for seed breadth with seed nitrogen content, seed length and nitrogen content were positively correlated. Interestingly, SLB ratio was negatively correlated to TGW.

Correlation of a particular trait across different environments is an indication of stability of a trait. Among all traits scored, TGW was correlated significantly between the different environments in most cases, which is consistent with the high heritability of TGW observed in cereals compared to other traits. Supplementary tables S7 to S12 summarize the correlation of a particular trait between different environments.

3.1.4 QTL analysis

QTL analysis for each environment and treatments (control and drought/KI) was carried out separately. Considering all traits and treatments together, a total of 124 QTLs were detected, and out of these, 71 and 53 QTLs were present under control and drought conditions, respectively (Table 4). Majority of the QTLs detected were found to be common for both the treatments. Brenda contributed 52% and 62% of the total QTLs obtained under control and drought conditions, respectively. On an average, 8 and 6 QTLs were obtained per trait under control and drought, respectively. Among all traits, TGW had the highest number of QTLs detected under both conditions (11 each), where as for yield, 11 and 8 QTLs were detected under control and drought, respectively.

Majority of the QTLs detected for yield under control condition was contributed by Hs 584 (73%), whereas, under drought, Brenda contributed the most (62%). A total of 9 QTLs were detected for DTI-Yield and more than 50% of it was contributed by Hs584. For TGW and its DTI, about 80% of the QTLs detected under both conditions were contributed by Brenda. All the QTLs for seed length and nitrogen content were derived from Hs 584, whereas, seed breadth and starch content QTLs were derived from Brenda. For seed LB ratio, Brenda contributed 2 out of 10 QTLs detected under control, while under drought, all four QTLs detected were contributed by Hs 584 (Table 4).

Table 2: Correlation between traits within the environment at Nordsaat.

Nordsaat field 2007	**1**	**2**	**3**	**4**	**5**	**6**
1_Y_C_F_NS_07						
2_Y_S_F_NS_07	0.642***					
3_TGW_C_F_NS_07	0.165	0.014				
4_TGW_S_F_NS_07	-0.101	0.145	0.345**			
5_DTI-Y_F_NS_07	0.88**	0.883***	0.071	0.063		
6_DTI-TGW_F_NS_07	0.023	0.102	0.783***	0.852***	0.082	
Nordsaat green house 2007	**1**	**2**	**3**	**4**	**5**	**6**
1_Y_C_GH_NS_07						
2_Y_S_GH_NS_07	0.488***					
3_TGW_C_GH_NS_07	0.448***	0.312*				
4_TGW_S_GH_NS_07	0.273*	0.244*	0.698***			
5_DTI-Y_GH_NS_07	0.839***	0.841***	0.385**	0.191		
6_DTI-TGW_GH_NS_07	0.119	0.022	0.659***	0.549***	0.016	
Nordsaat field 2008	**1**	**2**	**3**	**4**	**5**	**6**
1_Y_C_F_NS_08						
2_Y_S_F_NS_08	0.664***					
3_TGW_C_F_NS_08	0.017	-0.107				
4_TGW_S_F_NS_08	-0.196	0.061	0.569***			
5_DTI-Y_F_NS_08	0.822***	0.937***	-0.051	-0.014		
6_DTI-TGW_F_NS_08	-0.139	-0.021	0.833***	0.927***	-0.048	
Nordsaat green house 2008	**1**	**2**	**3**	**4**	**5**	**6**
1_Y_C_GH_NS_08						
2_Y_S_GH_NS_08	0.811***					
3_TGW_C_GH_NS_08	0.393***	0.316**				
4_TGW_S_GH_NS_08	0.426***	0.557***	0.599***			
5_DTI-Y_GH_NS_08	0.941***	0.886***	0.282*	0.357**		
6_DTI-TGW_GH_NS_08	0.421***	0.444***	0.903***	0.875***	0.327**	

Abbreviations are Y- Yield, TGW-Thousand grain weight, Y-DTI- Drought Tolerance Index for Yield, TGW-DTI- Drought Tolerance Index for TGW, C- control, S- Stress, GH- Green House, F- Field, NS- Nordsaat, 07 and 08 represent years 2007 and 2008 respectively. Karl Pearson correlation significance at $p<0.05$, $p<0.01$ and $p<0.001$ are represented by *, ** and *** respectively. Significant correlations are shaded red.

Table 3: Correlations between different traits for the experiment carried out in green house, IPK

Traits	1	2	3	4	5	6	7	8	9	10	11	12	13
1_Yield_C_GH_IPK													
2_Yield_S_GH_IPK	0.068												
3_TGW_C_GH_IPK	0.771***	0.164											
4_TGW_S_GH_IPK	0.01	0.721***	0.204										
5_N%_C_GH_IPK	-0.45***	-0.122	-0.42***	0.046									
6_N%_S_GH_IPK	0.237	-0.54***	0.234	-0.49***	0.062								
7_Starch_C_GH_IPK	0.658***	0.273*	0.816***	0.211	-0.52***	0.023							
8_Starch_S_GH_IPK	0.079	0.801***	0.187	0.871***	-0.057	-0.59***	0.329**						
9_SB_C_GH_IPK	0.681***	0.114	0.853***	0.183	-0.29*	0.135	0.701***	0.181					
10_SB_S_GH_IPK	-0.097	0.539***	0.101	0.811***	0.108	-0.40**	0.145	0.628***	0.196				
11_SL_C_GH_IPK	0.103	0.151	0.13	0.151	0.269*	-0.004	-0.024	0.066	0.199	0.175			
12_SL_S_GH_IPK	-0.191	0.07	-0.017	0.243	0.246*	0.03	-0.065	-0.019	0.052	0.627***	0.433***		
13_S L/B_C_GH_IPK	-0.354**	0.086	-0.41**	0.011	0.436***	-0.106	-0.44***	-0.057	-0.4***	0.04	0.765***	0.372**	
14_S L/B_S_GH_IPK	-0.113	-0.48***	-0.108	-0.59***	0.17	0.488***	-0.223	-0.71***	-0.138	-0.325*	0.338**	0.523***	0.411**

Length, L/B- Length to Breadth ratio, C- control, S- Stress, GH- Green House, F- Field, NS- Nordsaat, 07 and 08 represent years 2007 and 2008 respectively. Karl Pearson correlation significance at $p<0.05$, $p<0.01$ and $p<0.001$ are represented by *, ** and *** respectively. Signifiant positive, negative and no correlations are indicated by pink, blue and green colours respectively.

Table 4: Total Number of QTLs detected for various traits

Traits	No. of envmts.	Control			Stress		
		Tot. QTLs	Brenda	Hs584	Tot. QTLs	Brenda	Hs 584
Yield	5	11	3	8	8	5	3
DTI-Y	5	9	4	5	9	4	5
TGW	6	11	9	2	11	8	3
DTI-TGW	5	10	8	2	10	8	2
Seed breadth	2	6	6	0	5	5	0
Seed length	2	4	0	4	1	0	1
Seed L/B	2	10	2	8	4	0	4
Seed starch	1	5	5	0	3	3	0
Seed nitrogen	1	5	0	5	2	0	2

Abbreviations: **L/B**-length to breadth ratio. **DTI-Y**- drought tolerance index of yield, **DTI-TGW**- drought tolerance index of TGW.

3.1.5 Stable QTLs

One of the major hindrances to the identification of stable QTLs in a QTL study is the effect of environment or large GxE interactions. Fulton et al. (1997) suggested that QTLs that are consistent over different environments are more valuable than those with higher significance found in a single environment. Taking this into account, a QTL was considered to be stable in the present study, only if it has appeared in at least two environments (For yield, TGW, DTI-Y and DTI-TGW) with an LOD≥3 in each environment. Only stable QTLs were considered for our further data interpretation and analysis. Details of the various stable QTLs along with their parental source and appearance in a particular environment is given in Tables 5, 6, 7 and 8. A total of 57 stable QTLs were obtained for 8 traits studied, and 60% of it was derived from Brenda. Among stable QTLs, a total of 42 and 24 QTLs were detected under control and drought conditions, respectively. Among these, 18 QTLs were found to be common to both conditions and a total of 24 and 6 QTLs were found to be specific for control and drought, respectively.

3.1.6 Grain yield (qYLD)

There were altogether 5 stable QTLs obtained for yield that mapped to 1H (3), 2H (1) and 3H (1). Among these, 3 QTLs were found to be stable under control and 3 under drought (Table 5). The most stable QTL was qYLD1.3 which has appeared in 4 environments followed by

Table 5: Details of the stable QTLs for yield and DTI-Y under different environmental conditions.

				LOD -control						LOD -stress						Appearance	
Traits	**QTL Name**	**Source**	**Marker**	**1**	**2**	**3**	**4**	**5**	**R^2**	**1**	**2**	**3**	**4**	**5**	**R^2**	**cont.**	**str.**
Yield	qYLD1.1	Brenda	GBS3171		7.4	4.6	**7.5**		54.0		5.0	4.1	**8.6**		49.1	3	3
			Bmac90														
	qYLD1.2	Brenda	GBS3286		3.1	**4.9**	3.3		29.7		**3.2**	3.0			20.7	3	3
			GBMS17														
	qYLD1.3	Brenda	GBS3269	3.1		**8.8**	4.7		46.4	**6.3**	5.1	5.3	3.7		37.6	4	4
			GBS3272														
	qYLD2.1	Brenda	GBS3273			3.2		**6.6**	42.4		4.9				31.5	2	1
			GBS3279														
	qYLD3.1	Hs 584	GBS3192	5.9					33.5	**3.7**				3.0	22.3	1	2
DTI-Yield	qDTI-Y1.1	Brenda	GBS3171						24.4		4.6	4.0	**4.7**		24.4		3
			Bmac90														
	qDTI-Y1.2	Brenda	GBS3269						45.8	3.4	3.8	**6.3**	3.1		45.8		4
			GBS3272														
	qDTI-Y1.3	Hs 584	GBS3178						32.4				4.3	**5.2**	32.4		2
			GBMS184														
	qDTI-Y2.1	Brenda	GBS3273						23.1		**3.4**			3.0	23.1		2
			GBS3279														

The numbers 1 to 5 in the table heading corresponds to different environmental conditions (1- Nordsaat field 2007; 2- Nordsaat green house 2007; 3- Nordsaat field 2008; 4- Nordsaat green house 2008; 5-IPK green house 2010). **R^2** corresponds to the marker correlation to a particular QTL. The LODs marked in bold corresponds to the highest LOD observed for a particular QTL among different environments. Abbreviations are cont.- control; str.- stress

qYLD1.1 and qYLD1.2 which appeared in 3 environments. All three QTLs mentioned above were expressed under both control and drought conditions. Two QTLs, qYLDd2.1 and 3.1 were found to be specific for control and drought, respectively. Among all yield QTLs, only qYLDd3.1 was obtained from Hs 584. Major QTLs for yield, qYLD1.1, qYLD1.3 and qYLD2.1 were all derived from Brenda and presence of Hs 584 segments at these regions have significantly reduced grain yield under both conditions.

3.1.7 Drought tolerance index of yield (qDTI-Y)

A total of 4 stable QTLs were obtained for drought tolerance index that mapped to 1H (3) and 2H (1). Among these, the most stable QTLs were qDTI-Y1.2 and qDTI-Y1.1 which appeared in 4 and 3 environments, respectively. All qDTI-Y were derived from Brenda except qDTI-Y1.3 which appeared in 2 environments (Table 5).

3.1.8 Thousand grain weight (qTGW)

Among all traits, the highest numbers of stable QTLs were detected for TGW (11) which mapped to all 7 linkage groups except 4H and 5H. Majority of the QTLs (except qTGW3.1 and qTGW3.2) were contributed by Brenda. The most stable QTLs, qTGW1.1, qTGW1.3 and qTGW2.1 were detected under both control and drought conditions. The qTGW3.2 contributed by Hs584 and qTGW7.2 contributed by Brenda and were found to be specific to control and drought conditions, respectively (Table 6).

3.1.9 Drought tolerance index of thousand grain weight (qDTI-TGW)

A total of 5 QTLs were detected for DTI-TGW that mapped to 1H (3), 2H (1) and 6H (1) and were all derived from Brenda. Among these, the most stable one was qDTI-GW6.1, which appeared in 4 environments (Table 6).

3.1.10 Seed breadth (qSB)

For seed breadth, a total of six QTLs were detected that mapped to all 7 linkage groups except 4H and 5H. Interestingly, all the breadth QTLs were contributed by Brenda and 3 QTLs, qSB3.1, qSB6.1 and qSB7.2 were drought specific, while other were detected under both conditions (Table 7).

Table 6: Details of the stable QTLs for TGW and DTI-TGW under different environmental conditions.

Traits	QTL Name	Source	Marker	LOD- control						R^2	LOD- stress					R^2	Appearance	
				1	2	3	4	5	6		1	2	3	4	5		cont.	str.
TGW	qTGW1.1	Brenda	GBS3171 Bmac90	6.1	5.4		**6.6**		3.3	37.5		7.8	**15.6**			48.8	4	2
	qTGW1.2	Brenda	GBS3286 GBMS17			3.1	**3.9**			24.6			**3.0**			22.3	2	1
	qTGW1.3	Brenda	GBS3269 GBS3267	**7.6**	3.4	3.5	5.6		4.3	41.7	3.7		**7.4**	6.4		39.9	5	3
	qTGW2.1	Brenda	GBS3273 GBS3279	5.8	5.4		4.2	**6.7**	4.8	40.5	**4.9**	3.1				30.1	5	2
	qTGW3.1	Hs584	GBS3186 Bmac67	4.4		3.0			**5.2**	31.0	**3.6**					22.7	3	1
	qTGW3.2	Hs584	Bmag225					3.1	**3.3**	19.4							2	0
	qTGW6.1	Brenda	GBMS222 Bmag613	3.4	3.5	4.2		4.4	**6.8**	38.3			**3.2**			19.6	5	1
	qTGW7.1	Brenda	GBS3128 GBS3159	3.3	3.0	**4.4**				26.2			**3.0**			18.4	3	1
	qTGW7.2	Brenda	GBS3152 GBS3120										**3.1**		3.1	19.6	0	2
	qTGW7.3	Brenda	Bmac187 Bmac31										**3.2**		3.1	19.9	0	2
	qTGW7.4	Brenda	Bmag516										**3.2**		3.0	19.9	0	2
DTI-TGW	qDTI-TGW1.1	Brenda	GBS3171 Bmac90								3.2			**10.4**			53.4	2
	qDTI-TGW1.2	Brenda	GBS3286 GBMS17										6.0	**6.1**			35.5	2
	qDTI-TGW1.3	Brenda	GBS3269 GBS3267								6.8		**7.2**	6.6			39.2	3
	qDTI-TGW2.1	Brenda	GBS3273 GBS3279								**7.2**	3.0		4.1	4.5		42.4	4
	qDTI-TGW6.1	Brenda	GBMS222 GBMS125								3.2		**4.3**				25.4	2

The numbers 1 to 5 in the table heading corresponds to different environmental conditions (**1**- Nordsaat Field 2007; **2**- Nordsaat green house 2007; **3**- Nordsaat Field 2008; **4**- Nordsaat green house 2008; **5**-IPK green house 2010; 6- IPK field 2011). $\mathbf{R^2}$ corresponds to the marker correlation to a particular QTL. The LODs marked in bold corresponds to the highest LOD observed for a particular QTL among different environments. Abbreviations are cont.- control; str.- stress. TGW at IPK field was scored only under control condition.

3.1.11 Seed length (qSL)

A total of four QTLs were detected for seed length that mapped to 1H (2), 2H (1) and 5H (1). The only QTL which was common to both conditions was qSL2.1 and the rest were control specific (Table 4). All seed length QTLs were contributed by Hs 584 which had the higher value for this trait among parents (Table 7).

3.1.12 Seed length to breadth ratio (qSLB)

A total of 10 QTLs were detected for seed L/B ratio that mapped to all 7 linkage groups except 4H. The two QTLs derived from Brenda (qSLB5.1 and qSLB7.2) were found to be control specific. Among the Hs derived QTLs, qSLB1.1, qSLB1.2, qSLB5.2, and qSLB6.1 were found to be control specific and the rest were present under both conditions. No QTL was found to be drought specific (Table 7).

3.1.13 Seed starch content (qSS)

A total of five QTLs were detected for seed starch content that mapped one each to chromosomes 1H, 2H, 3H, 6H and 7H. Two QTLs, qSS2.1 and qSS3.1 were specific to control and the rest were present in both conditions. All starch QTLs were derived from Brenda and QTLs with highest significance (LOD) were qSS2.1 and qSS7.1 which were found to be specific to control and drought conditions, respectively (Table 8).

3.1.14 Seed nitrogen content (qSN)

For seed nitrogen content, a total of 7 QTLs were detected that mapped one each to 1H, 2H and 6H and two each to 3H and 7H. Of all the QTLs, qSN7.1 and qSN3.2 were found to be stress specific and the rest were control specific. No QTL was detected that was common to both conditions. The QTL with highest LOD (7.54) was qSN1.1. Like seed seed length, all the seed nitrogen QTLs were contributed by Hs584 (Table 8).

3.1.15 Co-location of traits

Many of the crop agronomic characters are correlated to one another or could be dissected into individual components; so, it is natural to observe a particular QTL detected for two or more different traits. In the present study, there were many QTLs that was detected for different traits, and are herein referred to as “hot-spot QTLs” (qHS)**.**

Table 7: Details of the stable QTLs for various seed morphological characters.

Traits	QTL Name	Source	Marker	LOD-cont.			LOD-stress		Appearance	
				1	2	R^2	1	R^2	cont.	str.
Seed breadth	qSB1.1	Brenda	GBS3276		9.6	50.3	3.0	19.2	1	1
	qSB2.1	Brenda	GBS3273 / GBS3279	4.2	**9.8**	50.1	6.3	38.3	2	1
	qSB3.1	Brenda	GBS3145		4.6	29.6		40.3	1	0
	qSB6.1	Brenda	GBMS222 / GBMS125	4.3	**6.1**	35.0	4.3	20.4	2	1
	qSB7.1	Brenda	GBS3128 / GBS3159	**5.4**	4.9	29.5	5.4	29.5	2	1
	qSB7.2	Brenda	GBS3275		5.5	34.9	3.9	24.3	1	1
Seed length	qSL1.1	Hs584	GBS3171	2.8	**3.1**	16.4			2	0
	qSL1.2	Hs584	GBS3269 / GBS3267	3.4	**6.2**	36.1			2	0
	qSL2.1	Hs584	GBS3273 / GBMS230	**4.2**	3.9	29.4	3.3	22.9	2	1
	qSL5.1	Hs584	GBS3134	**4.4**	4.0	20.4			2	0
Seed L/B	qSLB1.1	Hs584	GBS3171 / Bmac90		4.1	25.9			1	0
	qSLB1.2	Hs584	GBS3276	**3.9**	13.5	62.6			2	0
	qSLB2.1	Hs584	GBS3273 / GBS3279	**9.4**	5.6	52.4	5.6	36.3	2	1
	qSLB3.1	Hs584	GBS3145	3.6	**4.6**	29.9	4.5	31.1	2	1
	qSLB5.1	Brenda	GBMS32		3.6	22.3			1	0
	qSLB5.2	Hs584	GBS3165		3.1	21.0			1	0
	qSLB5.3	Hs584	GBS3169		3.8	24.6	3.9	26.5	1	1
	qSLB6.1	Hs584	GBMS222 / GBMS125	**6.1**	5.1	37.2			2	0
	qSLB7.1	Hs584	GBS3128 / GBS3159	**4.4**	3.3	28.4	4.0	25.9	2	1
	qSLB7.2	Brenda	GBMS226	3.0		18.5			1	0

The numbers 1 and 2 in the table heading corresponds to IPK green house 2010 and IPK field 2011, respectively.**R^2** corresponds to the marker correlation to a particular QTL. The LODs marked in bold corresponds to the highest LOD observed for a particular QTL among different environments. Abbreviations are cont.- control; str.- stress, L/B-length to breadth ratio.

Table 8: Details of the QTLs for grain quality identified from IPK green house experiment.

Traits	QTL Name	Source	Marker	LOD		LOD		Appearance	
				cont.	R^2	str.	R^2	cont.	str.
Seed starch	qSS1.1	Brenda	GBS3276	3.8	25.2	5.1	32.3	1	1
	qSS2.1	Brenda	GBS3273 GBS3279	7.4	43.6			1	0
	qSS3.1	Brenda	GBS3145	3.5	25.0	3.6	23.2	1	0
	qSS6.1	Brenda	GBMS222 Bmag613	4.0	26.1	4.3	27.3	1	1
	qSS7.1	Brenda	GBS3128 GBS3159	4.9	33.1	7.6	44.0	1	1
Seed nitrogen	qSN1.1	Hs584	GBS3276 GBS3267	7.5	44.0			1	0
	qSN2.1	Hs584	GBS3273 GBS3279	4.7	30.1			1	0
	qSN3.1	Hs584	GBS3145	3.8	26.5			1	0
	qSN3.2	Hs584	Bmag225			4.0	25.9	0	1
	qSN6.1	Hs584	GBMS222 GBMS125	3.1	21.1			1	0
	qSN7.1	Hs584	Bmac31 GBMS111			3.5	23.2	0	1
	qSN7.2	Hs584	GBS3275	3.9	27.4			1	0

R^2 corresponds to the marker correlation to a particular QTL. Abbreviations are cont.- control; str.- stress.

There were altogether 9 such qHS which were distributed to all 7 linkage groups except on 4H and 5H (Figure 9). The maximum number of qHS was detected on chromosome 1H (3) and followed by 2 each on 3H and 7H. Among the qHS, qHS1.3 and qHS2.1 had the highest number of traits co-located (9). The major 6 hot-spot QTLs (qHS1.1, qHS1.3, qHS2.1, qHS3.1, qHS6.1 and qHS7.1) together harbours more than 80% of the stable QTLs detected in the present study.

3.1.16 Selection of contrasting lines

Selection of contrasting lines was carried out based on 7 major qHS, which are summarized in Table 9. In addition to these qHS, qYLD3.1 and qTGW3.1 were also considered during selection as they were two important Hs derived QTLS for yield and TGW, respectively.

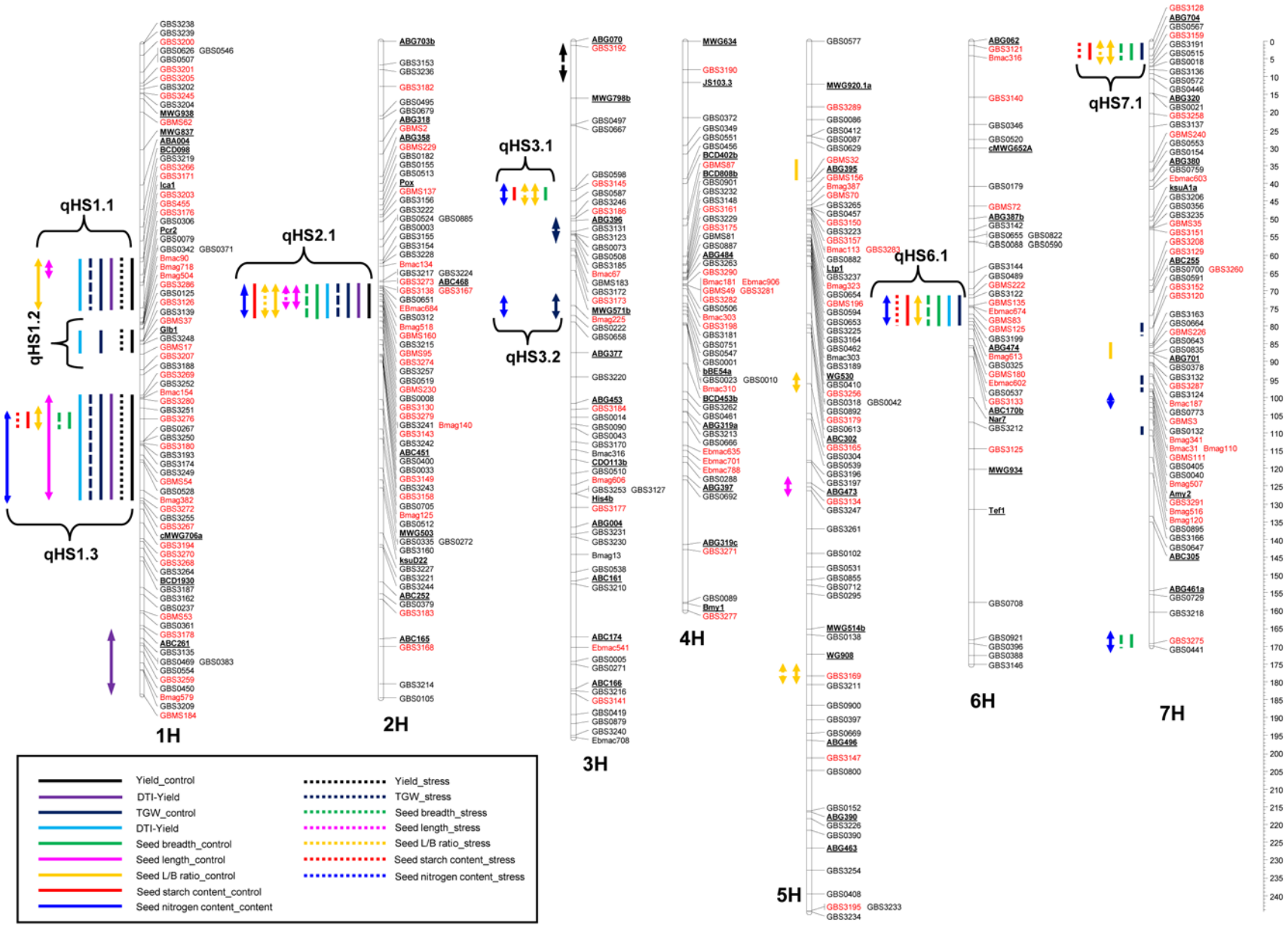


Figure 1. QTLs identified for various traits under control and drought conditions. QTLs contributed by the wild accession is indicated by arrow head. qHS refers to hot spot QTLS.The linkage map was adopted from Worch et al. (2011).

Table 9: Details of the hot-spot QTLs

QTL name	marker	Coll. QTLs	Coll.traits	source	control	stress
qHS1.1	GBS3171	qYLD1.1	Yield	Brenda	Yes	Yes
		qDTI-Y1.1	DTI-Y	Brenda	Yes	NA
		qTGW1.1	TGW	Brenda	Yes	Yes
	Bmac90	qDTI-GW1.1	DTI-TGW	Brenda	Yes	NA
		qSL1.1	Seed length	Hs 584	Yes	No
		qSLB1.1	Seed L/B	Hs 584	Yes	No
qHS1.3	GBS3269	qYLD1.3	Yield	Brenda	Yes	Yes
		qDTI-Y1.2	DTI-Y	Brenda	Yes	NA
		qTGW1.3	TGW	Brenda	Yes	Yes
		qDTI-GW1.3	DTI-TGW	Brenda	Yes	NA
		qSLB1.2	Seed L/B	Hs 584	Yes	No
	GBS3272	qSL1.2	Seed length	Hs 584	Yes	No
		qSB1.1	Seed breadth	Brenda	Yes	Yes
		qSS1.1	Seed starch	Brenda	Yes	Yes
		qSN1.1	seed nitrogen	Hs 584	Yes	No
qHS2.1	GBS3273	qYLD2.1	Yield	Brenda	Yes	No
		qDTI-Y2.1	DTI-Y	Brenda	Yes	NA
		qTGW2.1	TGW	Brenda	Yes	Yes
		qDTI-GW2.1	DTI-TGW	Brenda	Yes	NA
		qSLB2.1	Seed L/B	Hs 584	Yes	Yes
	GBS3279	qSL2.1	Seed length	Hs 584	Yes	Yes
		qSB2.1	Seed breadth	Brenda	Yes	Yes
		qSS2.1	Seed starch	Brenda	Yes	Yes
		qSN2.1	seed nitrogen	Hs 584	Yes	No
qHS3.1	GBS3145	qSB3.1	Seed breadth	Brenda	Yes	Yes
		qSS3.1	Seed starch	Brenda	Yes	Yes
		qSN3.1	seed nitrogen	Hs 584	Yes	No
		qSLB3.1	Seed L/B	Hs 584	Yes	Yes
qHS3.2	Bmag225	qTGW3.2	TGW	Hs 584	Yes	No
		qSN3.2	seed nitrogen	Hs 584	No	Yes
qHS6.1	GBMS222	qTGW6.1	TGW	Brenda	Yes	Yes
		qDTI-GW6.1	DTI-TGW	Brenda	Yes	NA
		qSLB6.1	Seed L/B	Hs 584	Yes	Yes
	Bmag613	qSB6.1	Seed breadth	Brenda	Yes	Yes
		qSS6.1	Seed starch	Brenda	Yes	Yes
		qSN6.1	seed nitrogen	Hs 584	Yes	No
qHS7.1	GBS3128	qTGW7.1	TGW	Brenda	Yes	Yes
		qSB7.1	Seed breadth	Brenda	Yes	Yes
	GBS3159	qSS7.1	Seed starch	Brenda	Yes	Yes
		qSLB7.1	seed nitrogen	Hs 584	Yes	Yes

Presence or absence of a QTL under a particular condition is indicated by yes and no, respectively. NA- not applicable.

Introgression of Hs segments at the hot-spot QTLs have been found to significantly reduce the trait value for important traits like yield, TGW, seed starch and seed breadth. Hence, the selection was based on the presence of Hs 584 QTLs improving the trait values, especially yield and TGW (qYLD3.1, qTGW3.1, qHS3.2). In general, IL lines having Hs 584 introgressions at major Brenda QTLs for yield and TGW were found to be inferior genotypes. Based on the above criteria, genotypes, IL 21, 22, 23 and 24 have been categorized as superior lines while IL 5, 44, 56, 57 and 58 as inferior (Figure 10). Among the superior genotypes, IL 22 was the only genotype which had all the beneficial Hs segments (QTLyld3.1, QTLtgw3.1 and qHS3.2). IL 23 and 24 did not have Hs introgression at qYLD3.1 whereas ILHS 21 did not have Hs introgression at qHS3.2. In addition, all the superior genotypes also had beneficial Hs introgression at qTGW3.1. Another notable feature among the superior genotypes was that IL23 had an Hs introgression at a major Brenda hot-spot QTL, qHS6.1. Among the inferior lines, IL 5 and 44 had Hs introgression at the important Brenda QTLs, qHS1.1, qHS1.3 and qHS2.1 while IL 57 had introgression at qHS1.3, qHS2.1 and qHS6.1. IL 56 and 58 had Hs introgression at Brenda QTLs, qHS1.3, qHS2.1, qHS6.1 and qHS7.1. A schematic representation of the selected contrasting lines along with the QTLs considered for their selection is shown in Figure 10.

Figure 10. Schematic representation of the selected genotypes. The introgression of Hs segments are shown in shaded colours. Red and green colours represent the Hs introgression having negative and positive influence on most of the traits, respectively.

3.1.17 Comparison of the contrasting genotypes

Comparison of selected contrasting genotypes for different traits (Yield, TGW, seed starch, seed nitrogen and seed C/N ratio) is shown in Figures 11A to 11D. The average of yield and

TGW data obtained from four different experiments carried out at Nordsaat during 2007 and 2008 indicated that inferior lines were characterized by lower yield and TGW compared to superior ones (Figures 11A and 11B). The poor performance of the ILs 5 and 44 among the inferior lines could be attributed to the fact that they had Hs introgression at all three major Brenda yield QTLs (Figure 10) while others were devoid of introgression at qHS1.1. Similarly, seed starch content obtained from the IPK green house experiment showed that inferior lines were generally low in starch content and were more susceptible to drought compared to the superior lines (Figure 11C).

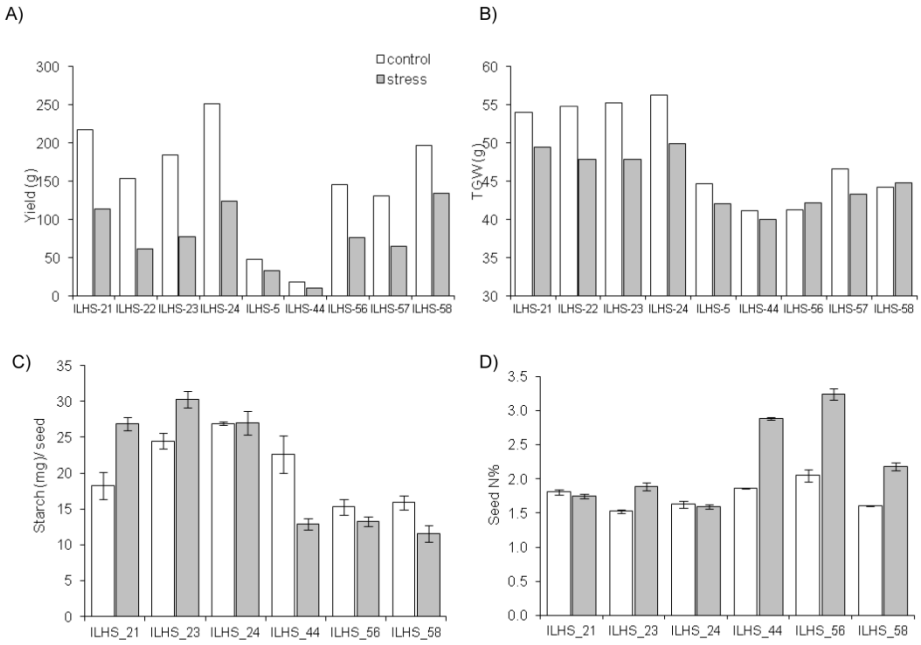


Figure 11. Effect of Hs introgression for yield and quality traits in the contrasting genotypes.

(A) Comparison of yield (combined across 4 locations) under control and drought for the selected contrasting genotypes.
(B) TGW (combined across 4 locations) was less affected by drought in the inferior lines.
(C) Drought significantly reduced the seed starch content in the inferior lines.
(D) Drought significantly increased the seed nitrogen content in the inferior lines

There was no major difference between the contrasting groups in terms of seed nitrogen content under control condition; however, drought stress had significantly increased the seed nitrogen content of the inferior lines over their control compared to the superior lines (Figure 11D). A significantly reduced C/N ratio of inferior genotypes under drought over their control compared to superior ones indicates that starch and nitrogen metabolism of the contrasting groups were differentially affected by drought (Figure 12).

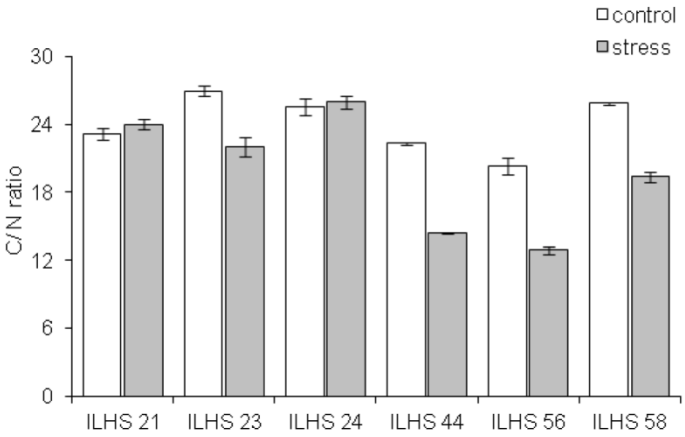


Figure 12. Carbon to nitrogen ratio of selected contrasting ILs under terminal drought. Carbon to nitrogen ratio was significantly reduced in the inferior compared to the superior lines.

3.2 CHARACTERIZATION OF THE IMPORTANCE OF STAYGREEN/ SENESCENCE PHENOTYPES UNDER TERMINAL DROUGHT

Stay green and senescence are the two important components of terminal drought tolerance in cereals. The present study was undertaken to study these mechanisms in barley using a panel of 10 elite breeding lines (LP 101 to LP 110) for their seed yield and quality under terminal drought. An initial screening of these genotypes for yield under terminal drought was carried out during 2007 and 2008 and the selected contrasting genotypes were further grown to study in detail using various physiological, biochemical and molecular techniques. As grain filling is a finely coordinated activity between source (leaf) and sink (seed) tissues, both these components were studied simultaneously using integrated omics approaches.

3.2.1 Screening and selection of contrasting genotypes for terminal drought

Screening for stable yield (TGW) under terminal drought was carried out at Nordsaat company, Germany by imposing drought beginning at 10DAF. A significant correlation in TGW between the years under both conditions (control and drought) indicates that variation in TGW was largely genotypic (Figures 13A and 13B). Selection of contrasting lines were based on different drought screening indices like drought susceptibility index (DSI), drought tolerance index (DTI) and percentage kernel injury (Fischer and Maurer, 1978; Fernandez, 1992) of the average TGW data obtained from the two independent years (Figure 14; Supplemental Table S13 and Supplemental Figure S1). Under control condition, TGW ranged from 55.2 to 68.7g in LP 108 and 105, respectively. Drought significantly reduced TGW in all genotypes and the lowest grain weight was found in LP 108 and 110 (48.7g) while the highest was in LP 104 (62.2g).

DSI, a better indicator of drought susceptibility was highest in genotypes LP 106 and LP 109 (1.54); while tolerance indicator (DTI) was highest in LP 104 and LP 105. Percentage kernel injury was highest in LP 106 and LP 109 (16.9 and 17 respectively), whereas the lowest kernel injury was found in LP 107 and 104 (6.5 and 7.0 respectively). Considering the above, genotypes LP 104 and 106 were selected as the most tolerant and susceptible genotypes, respectively, under terminal drought. Although genotypes 104 and 107 were similar in

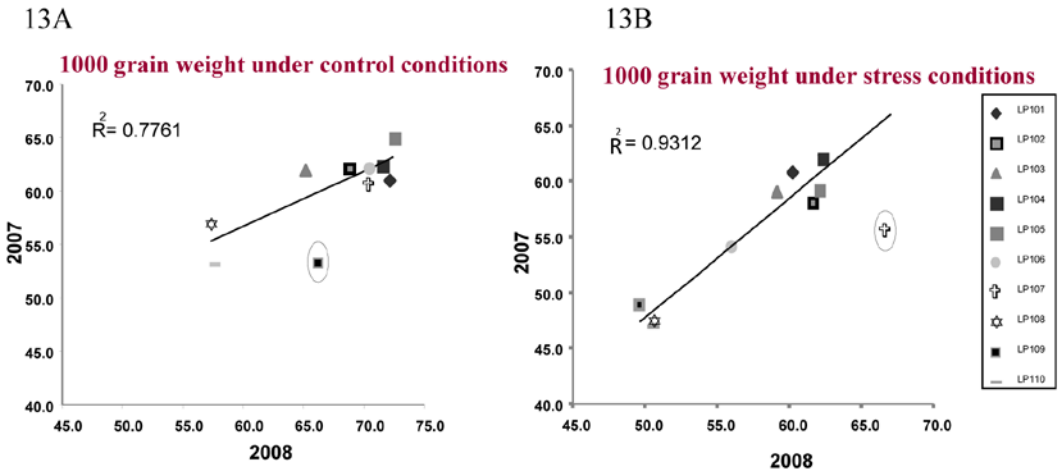


Figure 13. Stability of 1000 grain weight under post anthesis drought.
Relationship between TGW between the two independent experiments carried out in 2007 and 2008 shown separately for control (A) and drought stress conditions, (B). Correlations under both conditions are shown after removing LP 109 in control and LP 107 under stress (encircled points in the graphs). Both correlations are significant at $p<0.01$. Correlations would be 0.64 under control and 0.69 under drought, if LP 109 and LP 107 are included in the respective conditions.

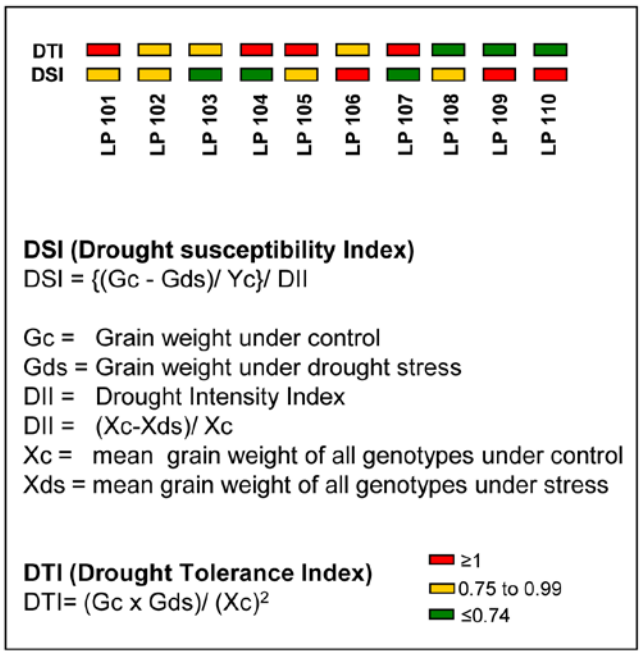


Figure 14.Schematic representation of the DSI and DTI of the genotypes under terminal drought.
For calculation of DSI and DTI, average of TGW obtained from the two independent years were used. DSI-drought susceptibility index, DTI-drought tolerance index.

performance, LP 104 was chosen over 107 because of its stable performance under stress (Larger standard deviation in TGW was observed in LP 107 under stress; Supplemental Figure S1).

The two selected genotypes exhibited contrasting phenotypes under stress; LP 104 was stable yielding and exhibited an early senescing phenotype while LP 106 yielded low but maintained a higher green leaf area under drought. An overview of these two genotypes at 25 DAF (17 days after stress) under drought when grown in green house is shown in Figure 15. In addition to the above two genotypes, LP 110, which also exhibited senescence phenotype under terminal drought was included for various studies carried out at the flag leaf level; however, a detailed study (stem remobilisation and seed filling) was carried out using only contrasting genotypes, LP 104 and LP 106. At many places in the text (results and discussion), LP 104 and 110 are referred to as senescing genotypes and LP 106 as stay green; to distinguish LP 104 from LP 110 (both senescing genotypes), LP 104 has often been referred to as remobilizing genotype.

Figure 15. Overview of the contrasting genotypes, senescing and stay green at 25 DAF (17 DAS). Plants were grown under green house conditions at IPK and stress was imposed beginning 8 DAF.

General phenotypic features of the contrasting genotypes (LP 106 and LP 104) recorded at the time of maturity under control condition are given in Table 10. Two genotypes had little difference in number of tillers and spikes per plant, although spike length, number of seeds per spike, plant height and plant biomass were significantly different. Spike length and plant height were higher in LP 104 (25.4 cm and 110 cm, respectively) compared to the stay green, LP 106 (22.2 cm and 101 cm, respectively). On the other hand, number of seeds per spike (22.9) and plant biomass (19.5 g) were significantly higher in stay green genotype compared to LP 104 (26.5 and 17.47g, respectively). The selected contrasting genotypes were further grown under both green house and phytochamber conditions for studying various physiological, biochemical and molecular aspects of their responses to terminal drought.

Table 10. General phenotypic characters of the selected contrasting genotypes

Genotype	No: tillers	No:Spikes	Ear length (cm)**	seeds/Spike **	Plant height (cm)*	Plant biomass (g)*
LP 104	16 (1)	14.4 (1.6)	25.4 (0.9)	26.5 (0.9)	110 (3)	17.5 (1.4)
LP 106	16.1 (1.4)	15.3 (2)	22.2 (0.3)	29 (0.8)	101 (5)	19.5 (2)

Plants were grown under green house conditions at IPK and all the below characters were recorded at the time of maturity. Values are mean from 10 individual plants except for the grains/spike, for which spikes from 3 main tillers/plant; a total of 10 plants were considered. Characters differing significantly between the genotypes are marked with * and ** for $p<0.05$ and 0.01 respectively. The numbers in bracket represents the S.D for the corresponding values.

3.2.2 Parameters measured on flag leaf

Drought mediated response of various physiological parameters

In order to characterize the extent of senescence under terminal drought, various physiological parameters were measured at the flag leaf level during 12 and 16 DAF (corresponding to 4 and 8 DAS, respectively). Drought significantly reduced flag leaf relative water content (RWC) in all genotypes (Figure 16A). The reduction over control was significantly higher in both the senescing genotypes, LP 104 and 110 compared to stay green genotype, LP 106 during both stages of measurement. Both senescing genotypes had similar RWC at 12 DAF; however, at 16 DAF, RWC of the remobilizing genotype was lower

compared to senescing genotype (12.5% and 9%, respectively). The effect of drought on the primary photosynthetic pigments, both chlorophyll a and b was similar across the genotypes (Figure 16B and 16C). At 12 DAF, chlorophyll content was little affected by drought; however, at 16 DAF, a severe reduction was observed in both the senescing genotypes compared to stay green, and there was little difference among the senescing genotypes.

Similarly, photosynthetic parameters were also severely impaired by drought in all three genotypes, and were more severe in the two senescing genotypes compared to stay green. Stomatal conductance (g_s) was severely reduced by drought in both the senescing genotypes compared to stay green (Figure 16D) and a similar response was also evident in assimilation rate (A). The remobilizing genotype had the lowest g_s and A during both stages of measurement compared to the other senescing genotype (Figure 16E). In accordance with the above observations, the severity of drought stress on the remobilizing genotype was evident from internal CO_2 concentration (Ci), which is governed by both stomatal and non stomatal factors. All three genotypes exhibited different Ci response to drought. While Ci of the stay green and the remobilizing genotypes were respectively increased and decreased under drought over its control; it was little affected by drought in the senescing genotype (Figure 16F).

One of the strategies adopted by plants to withstand stress is through the accumulation of various osmolytes. Drought significantly increased flag leaf osmolality of both the senescing genotypes compared to stay green during both stages of measurement (Figure 16G). Proline, a universal osmolite which gets accumulated under drought was significantly increased under drought in all genotypes across various stages. In general, both absolute amount and accumulation under drought over control were higher in the senescing genotype LP 110 compared to the other two. There seemed to be no difference between the stay green and remobilising line in terms of absolute amount of proline content under drought (Figure 16H). In short, various physiological parameters measured at the flag leaf level indicated that the two senescing genotypes, LP 104 and 110 were more susceptible to drought compared to the stay green and among the senescing genotypes, LP 104 was more susceptible to drought.

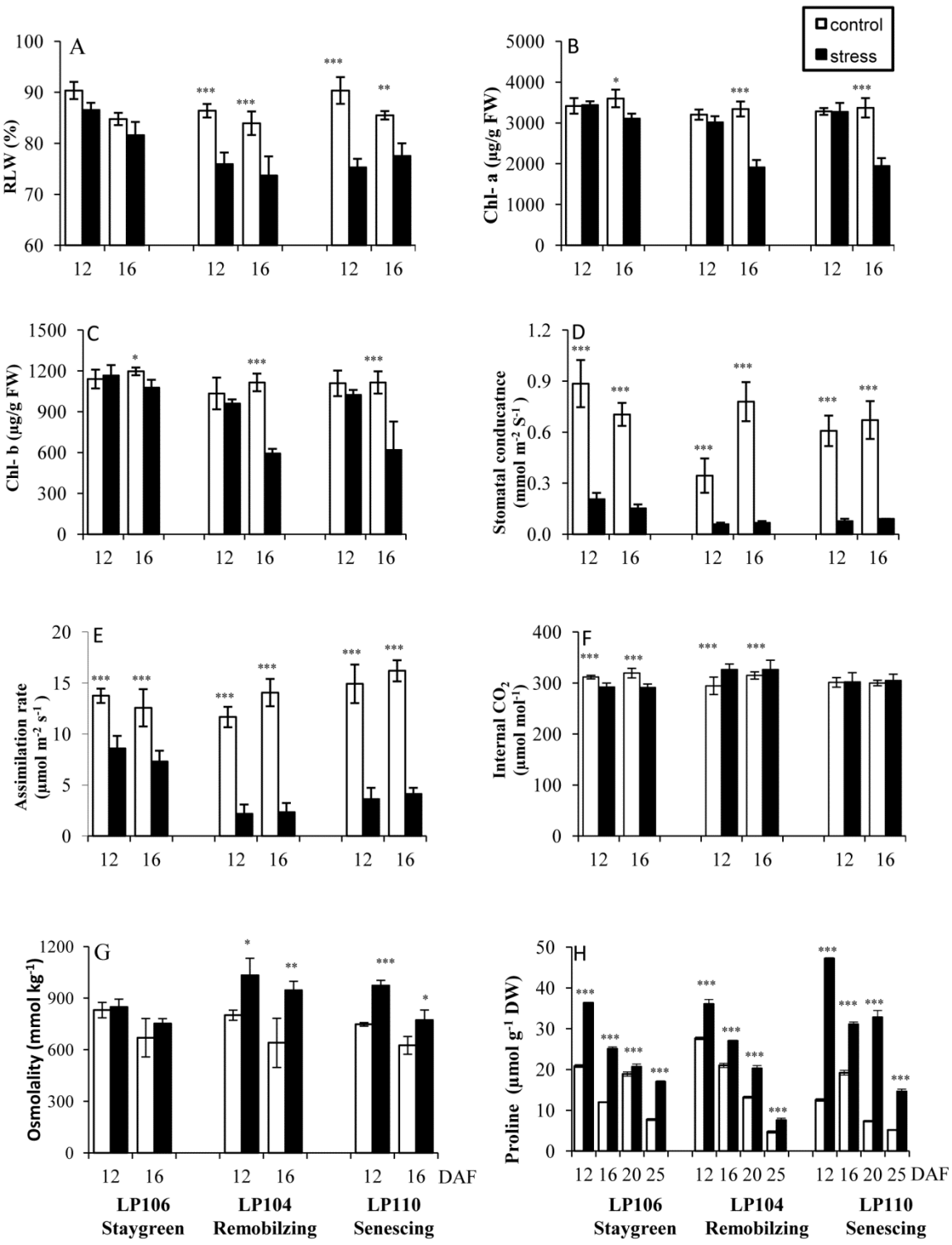


Figure 16. LP 104 and LP 110 were more susceptible to drought compared to LP 106. Effect of drought on mean ± SD of relative water content (A, n=5), chlorophyll a and b(Band C, respectively. n=4), assimilation rate (D, n=6), stomatal conductance (E, n=6)and internal carbon dioxide concentration (F, n=6), flag leaf osmolality (G, n=5), proline content (H, n=3). Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

3.2.3 Drought mediated response of the flag leaf carbohydrate metabolism

To study the effect of drought on primary photosynthetic metabolites and its significance under drought, starch and various soluble sugars were quantified across various developmental stages under both conditions. Flag leaf starch content was significantly reduced under stress in all three genotypes but the reduction over control was higher in the stay green genotype compared to both the senescing genotypes (Figure 17A).

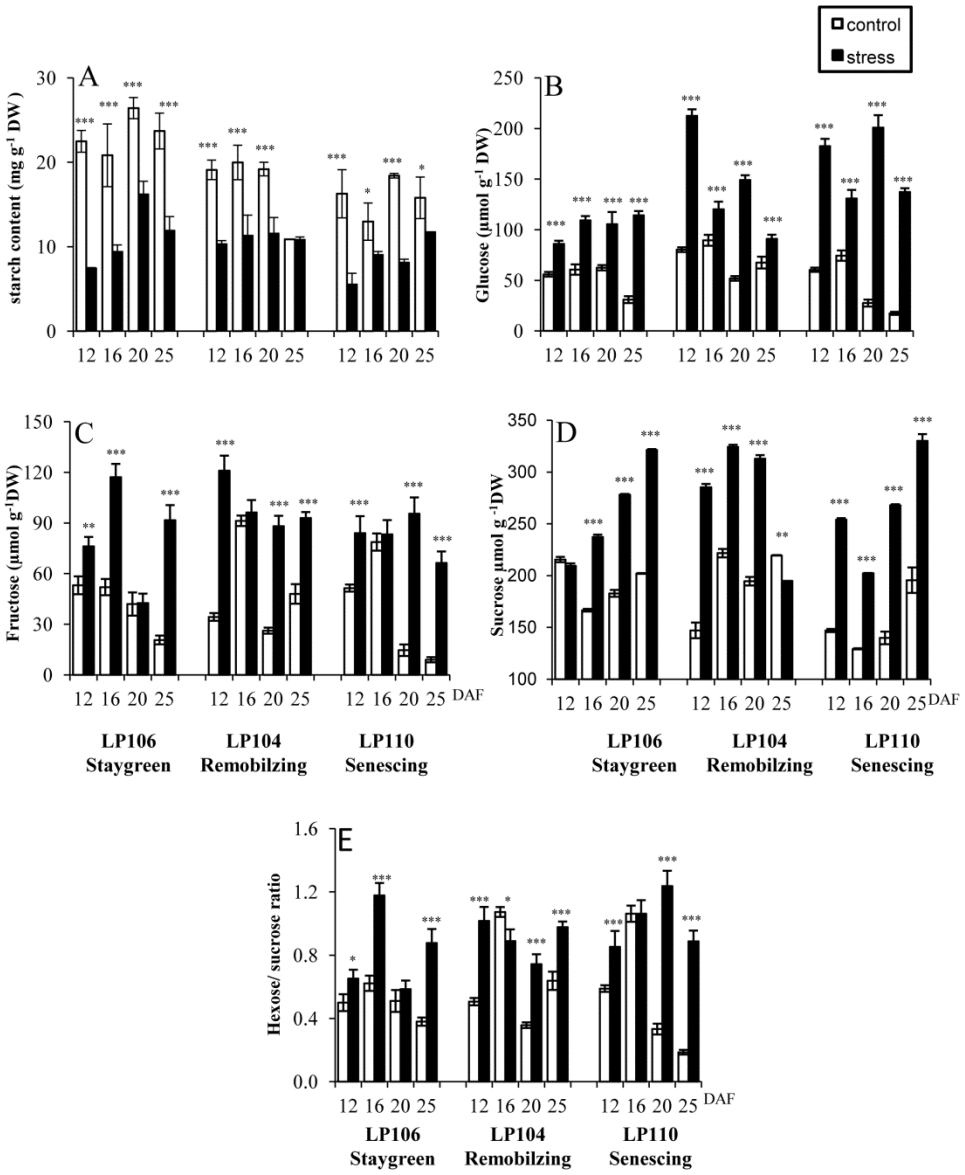


Figure 17. Drought induced changes of carbohydrates in LP 104, LP 106 and LP 110. Effect of drought on starch (A), glucose (B), fructose (C), sucrose (D) and hexose to sucrose ratio (E). Values are represented as mean ± SD (n=3) and expressed as µmol/g dry weight. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Quantitation of various soluble sugars, glucose (Figure 17B), fructose (Figure 17C) and sucrose (Figure 17D) indicated that all three sugars were significantly increased under drought in all genotypes across the developmental stages. Both the senescing genotypes had a similar pattern of accumulation compared to the stay green. A notable difference in sugar accumulation was observed at 12 DAF where all three sugars were significantly increased under drought over control in the senescing genotypes compared to stay green. However, a reverse trend observed at 25 DAF, where stay green genotype accumulated more sugars compared to senescing genotype probably indicates at the late initiation of drought response in the stay green.

Among the WSC, sucrose was the major constituent in all genotypes across various developmental stages under both conditions (Figure 17D). A higher hexose to sucrose ratio especially during the early stage (12 DAF) was found in the remobilizing genotype compared to the other two (Figure 17E). In addition to soluble sugars, we have also measured various sugar alcohols, namely, glycerol, erythritol and inositol which did not exhibit any genotypic or treatment specific effect; hence, were not considered for further analysis (data not shown).

3.2.4 Drought mediated response of flag leaf nitrogen metabolism

Nitrogen is one of the essential elements required for the plant growth. In cereals like barley and wheat, most of the nitrogen required for grain growth is obtained through remobilization of nitrogen stored in vegetative tissues upon senescence induction. Leaf being the major reservoir of nitrogen present in vegetative tissues, the amount of nitrogen available in leaf at the time of flowering has often been used to estimate the nitrogen available for remobilization; and is termed as apparent N remobilization (Masclaux-Daubresse et al., 2008). Drought significantly reduced leaf N% in all genotypes and the pattern was similar in stay green and the senescing genotype LP 110 (Figure 18). Nitrogen remobilisation (calculated by difference in N% between 12 and 25 DAF) was higher in the remobilising genotype, LP 104 compared to other two genotypes. Another important aspect of plant development is the maintenance of carbon to nitrogen balance in plant. Drought significantly increased flag leaf C/N ratio over control in all genotypes across various stages (Figure 19). Compared to stay green and LP 110, C/N ratio under drought was much higher and induced early in the remobilizing genotype, LP 104.

Mobilisation of nitrogen from vegetative tissues during grain filling essentially involves degradation of proteinaceous components of leaf cells to amino acids, which are eventually transported to developing grains. Estimation of free amino acid content of leaf showed a decreasing trend with developmental stages in LP 104 under both control and drought conditions (Figure 20A). The reduction in total free amino acid content under drought over control was much higher in LP 104 compared to other two genotypes which exhibited a simi-

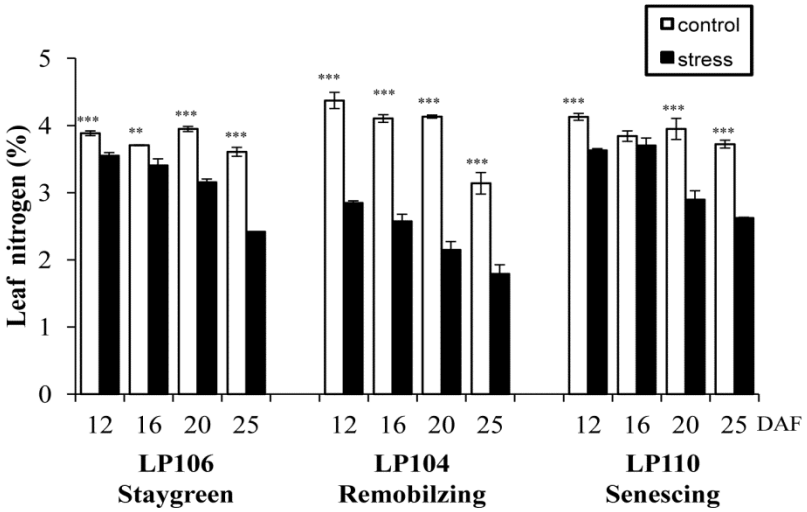


Figure 18. Leaf N remobilisation was enhanced by drought in LP 104 compared to other two genotypes. Values are mean ± SD, n=4 and expressed as percentage. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

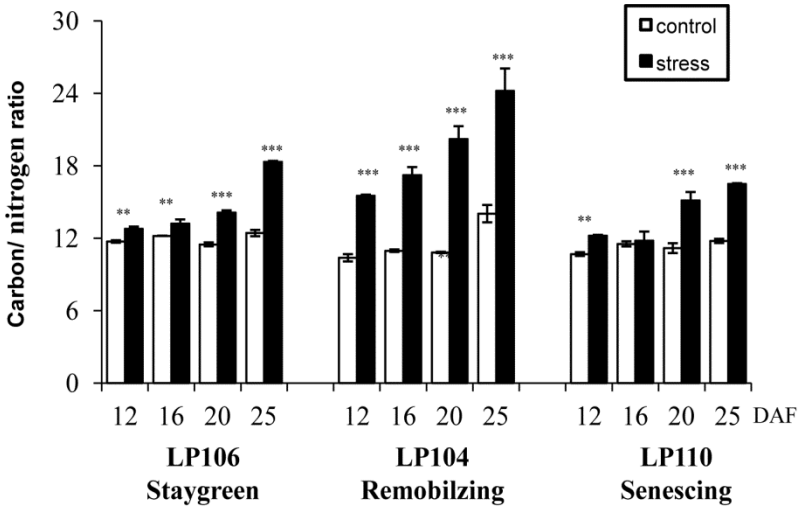


Figure 19. C/N ratio was significantly enhanced by drought in LP 104 compared to other two genotypes. Values represented are mean ± SD (n=4). Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

-lar pattern of accumulation. In general, among various amino acids, proline constituted the highest percentage (about 25 to 30%) of total free amino acid content in all genotypes during most of the stages under both conditions.

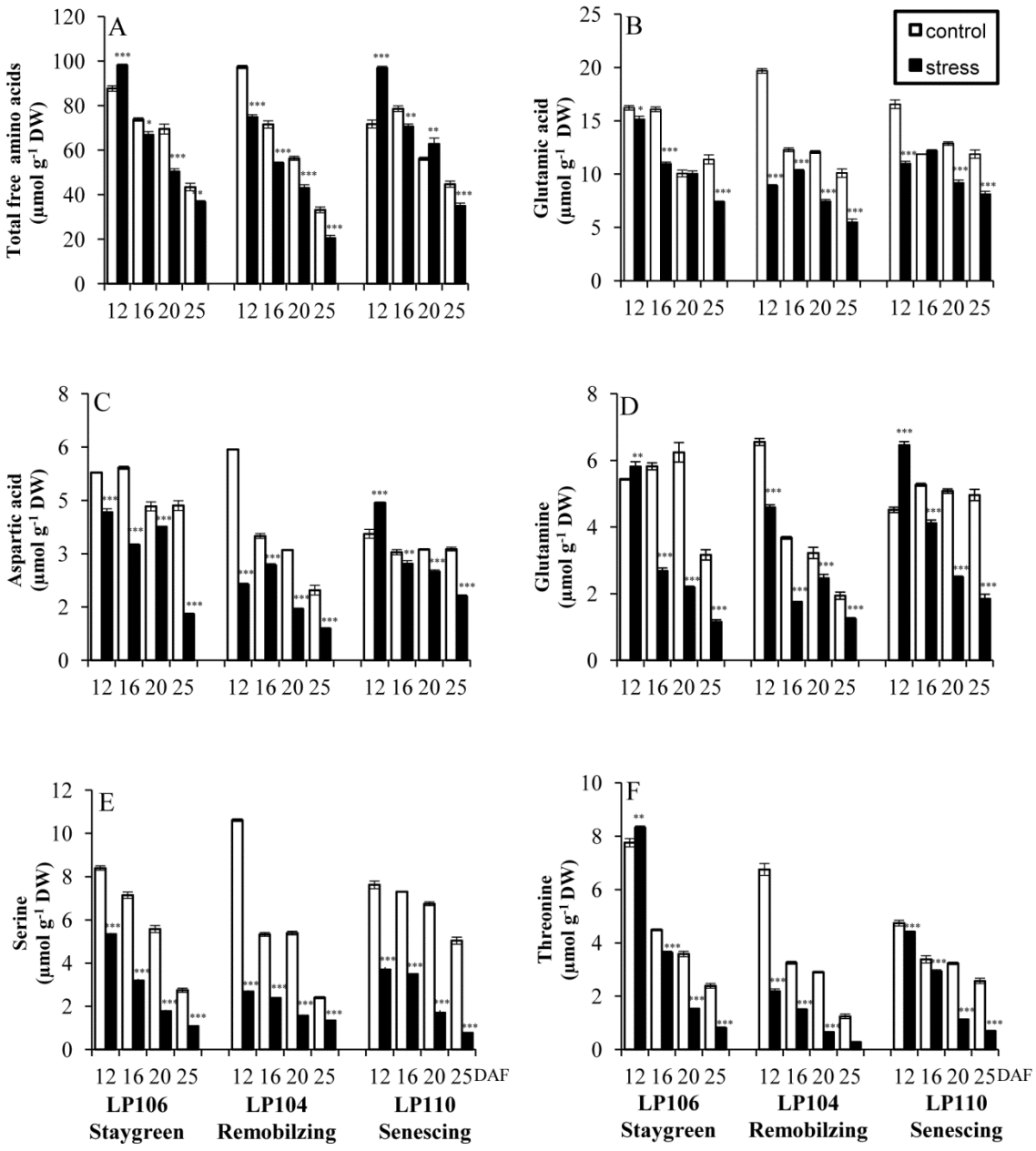


Figure 20. Drought caused reduction in flag leaf free amino acid content

Total free amino acid content (A), Glutamic acid (B), Aspartic acid (C), Glutamine (D), Serine (E), Threonine (F). Values are mean ± S.D, n=3 and expressed as µmol per gram dry weight. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

In barley, major phloem translocated amino acids are glutamate followed by varying levels of aspartate, glutamine, serine and threonine. In general, these amino acids together with proline constituted about 70 to 80% of the total free amino acid content of leaf in all genotypes. All these transported amino acids had a similar trend as observed with total free amino acid content of the leaf. A major difference between the genotypes was observed at 12 DAF, where drought significantly reduced the levels of these major transported amino acids in LP 104 over its control compared to other two genotypes (Figures 20B to 20F).

Reduction in availability of CO_2 leads to increased oxygenation reaction of RuBisCO, leading to higher photorespiration. Glycine to serine ratio has been used as one of the parameters to measure the extent of photorespiration in plants (Diaz et al., 2005). In both senescing genotypes, Gly/Ser ratio was significantly increased under drought over control compared to stay green. At 12 DAF, it was more pronounced in the remobilizing genotype compared to other two genotypes (Figure 21).

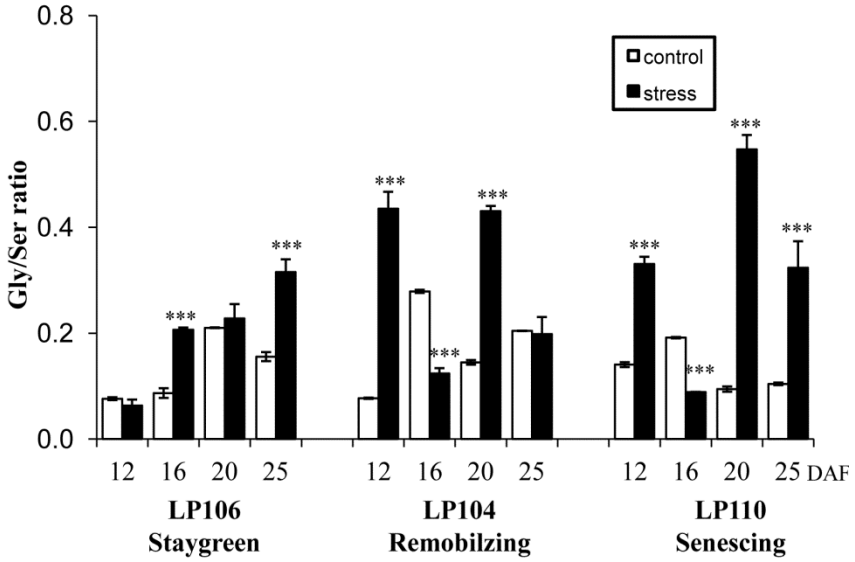


Figure 21. Drought induced higher accumulation of Gly/Ser ratio in the senescing genotypes. Values are mean ± S.D, n=3.

From the above results, it can be summarized that drought caused an early senescence induced remobilisation of nitrogen in the remobilising genotype LP 104 compared to the

other two, and the two genotypes LP 106 and LP 110 behaved similar with respect to total leaf nitrogen and accumulation of various free amino acids unlike the physiological parameters.

3.2.5 Effect of drought on flag leaf ABA content

Abscisic acid (ABA) is a universal plant hormone which gets accumulated under stress. Drought stress significantly increased the leaf ABA content in all genotypes across various stages (Figure 22). The peak of ABA accumulation under drought in different genotypes, LP 104, LP 110 and LP 106 corresponded to 12, 20 and 25 DAF, respectively.

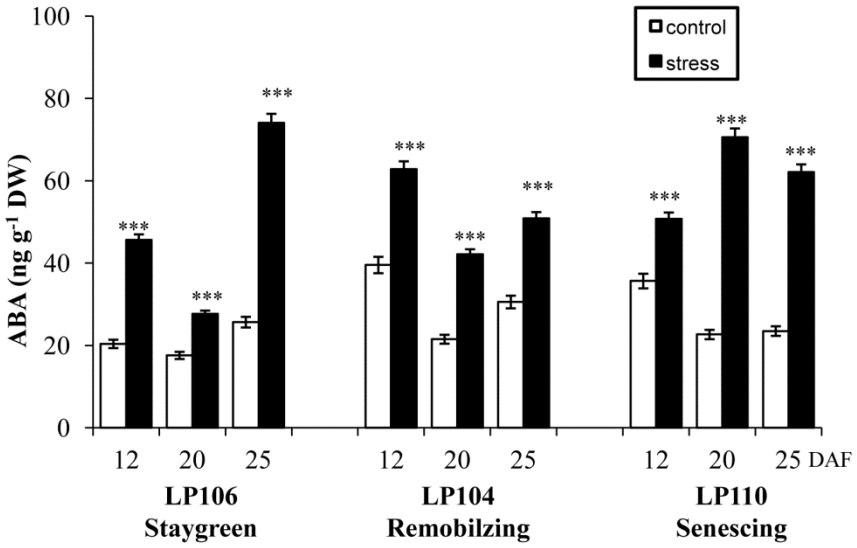


Figure 22. Drought induced accumulation of ABA in flag leaf

Values are mean ±S.D, n=3 and expressed as ng per gram dry weight. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

3.2.6 Flag leaf Transcriptome of the contrasting genotypes under terminal drought

Drought mediated transcriptome response of flag leaf at 20 DAF (12 DAS) was monitored in the 3 selected genotypes using 22K Affymetrix chips. We used PageMan and MapMan software tools to explore transcriptome data, identified the overrepresented functional groups of drought regulated genes in flag leaf and inferred the metabolic pathway alterations of central metabolism (Figures 23A and 23B) and also identified key regulators influencing senescence coupled remobilization (Figure 24).

3.2.7 Carbohydrate and nitrogen metabolism

Consistent with the physiological data, transcriptome data revealed that photosynthesis was severely affected in the two senescing genotypes compared to stay green. Photosystem II polypeptide subunits, electron carriers from the light reaction and the rubisco small subunits were all down regulated in both the senescing genotypes, while it remained unaltered in the stay green (Figures 23A and 23B). Similarly, genes involved in tetrapyrrole synthesis (magnesium chelatase, protochlorophyllide reductase, urogen III methylase) and regulation were also down regulated in both the senescing genotypes compared to stay green; and could be correlated to the chlorophyll data obtained in these genotypes. Starch degrading enzymes, alpha and beta amylase were prominently upregulated in all three genotypes under drought, possibly indicating starch as a carbon source under conditions of limited photosynthesis. Drought also induced the expression of various cell wall invertase related transcripts in all three genotypes. Although they differed in the particular members of the gene family induced by drought. Another sucrose cleaving related transcript, vacuolar invertase was highly induced by drought in the stay green compared to LP 110; while it was unaltered in the remobilizing genotype. Hence, the observed increase in hexose content in these genotypes under drought (Figures 17B and 17C) in general could be the result of induction of the above two sucrose cleaving enzymes. Interestingly, in both remobilizing and senescing lines fructose 2,6-bisphosphatase (Fruc2,6BisPase) and cytosolic Fru-1,6-bisphosphatase (cyt-FBPase) were dramatically down regulated under drought.

Some of the other transcripts upregulated under drought includes the ones involved in glycolysis (pyrophosphate-fructose-6-P phosphotransferase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate mutase), TCA cycle (aconitase, isocitrate dehydrogenase, succinate-semialdehyde dehydrogenase, mitochondrial and cytosolic malate dehydrogenase, malic enzyme, pyruvate dehydrogenase, E1 and E3 subunits) and mitochondrial electron transport system (Figures 23A and 23B), indicating the activation of mitochondrial respiratory activities. The intermediates of glycolysis and TCA cycle also serve as the precursors for synthesis of various amino acids. Specifically in the senescing line, amino acid metabolism genes involved in synthesis of aspartate-derived methionine,

threonine, homoserine; glutamate-derived proline; aromatic amino acids such as chorismate, phenylalanine, tyrosine, tryptophan were prominently induced under drought (Figures 23A and 23B). Also, transcripts of glutamine synthase involved in converting ammonium (NH_4^+) to glutamine and glutamate dehydrogenase members involved in converting glutamate to alpha-oxoglutarate were preferentially upregulated in senescing line under stress. Surprisingly, in the senescing line LP 110, hexoses were probably channeled towards production of cell wall and lignin as we noted an enrichment of the functional bins of several cell wall precursors and lignin biosynthesis in this genotype (Figures 23A and 23B).

Intriguingly, in the remobilizing line, amino acid biosynthesis pathway was not activated, instead, amino acid degradation pathway was prominently upregulated together with induction of several classes of proteases under drought stress (Supplemental Figure S3). Among protease families, typical senescence associated genes belonging to cysteine proteases (SAG12, papain-like, RD21A thiol protease and cathepsin-B), serine proteases (serine carboxypeptidase i, ii and iii types, cucumisin-like ARA12, ATP-dependent clp protease), aspartate protease (CND41) potentially involved in chloroplast degradation, autophagy and AAA-type protease were prominently upregulated in remobilizing line under stress, indicating the trigger of nitrogen remobilization through trigger of plastidial and cytosolic proteolysis (Supplemental Figure S2). Congruently, several amino acid, oligo-peptide ABC transporters were found to be predominantly upregulated in remobilizing line and certain distinct member in senescing line under drought (Supplemental Figure S3) implying the importance of enhanced N reallocation. Another major difference between the genotype was in terms of sucrose-fructan 6-fructosyltransferase, an important enzyme of fructan biosynthesis that was up regulated in the remobilizing line and remained unaltered in the other two (Supplemental Figure S3).

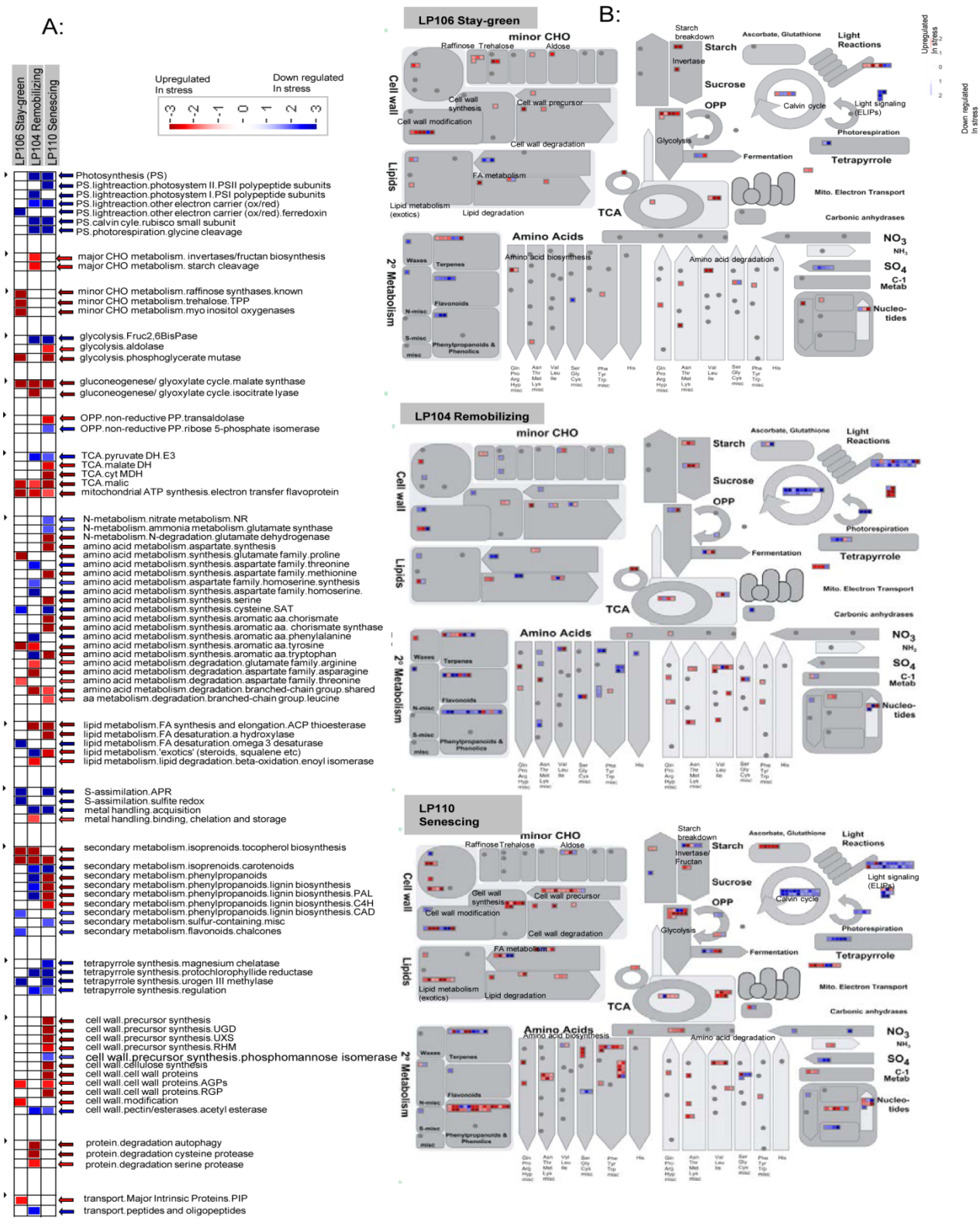


Fig 23. Drought induced differential expression of transcripts involved in carbon and nitrogen metabolism analysed by Mapman.

3.2.8 Hormones, signaling and regulators

ABA is a universal plant hormone which gets accumulated under stress. Although ABA was induced under drought in all genotypes, the transcripts related to ABA synthesis (NCED) and degradation (ABA 8-hydroxylase) were differentially affected by drought in three genotypes (Figure 11). NCED was upregulated in stay green and down regulated in LP 110, while hydroxylase was upregulated only in LP 110. Both these transcripts remained unaffected by

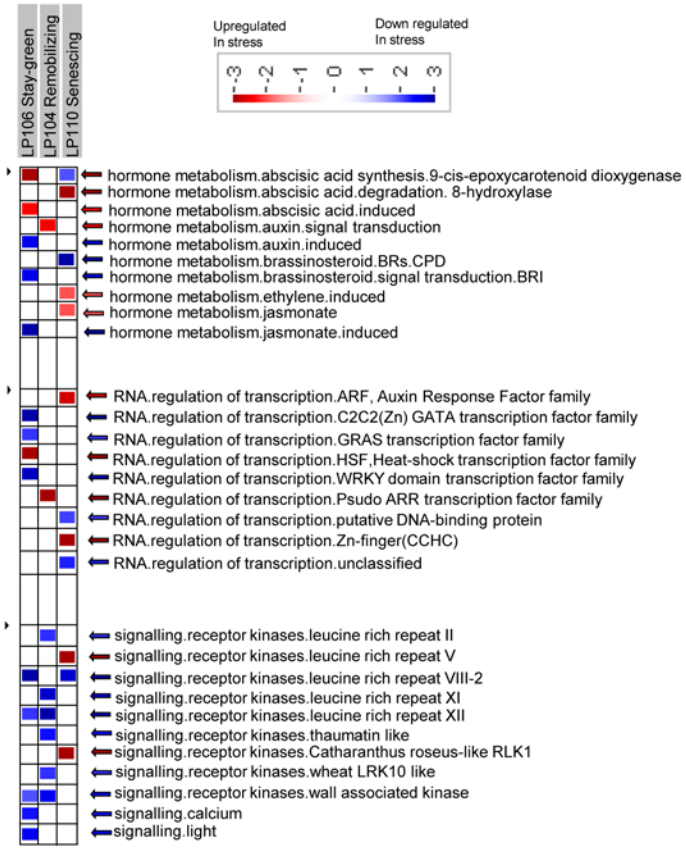


Figure 24. Effect of drought on the transcripts involved in hormones, signaling and transcription factors in the selected contrasting genotypes

drought in the remobilizing genotype. In addition to ABA, some of the transcripts related to auxin, brassinosteroids (BR), ethylene and jasmonate were also differentially expressed under drought. One each of the transcript related to BR was down regulated under stress in both stay green and LP 110 while in the remobilizing genotype it remained unaffected. On the other hand, transcripts related to ethylene and Jasmonate were both induced under drought in LP 110 while it remained unaffected in the other two genotypes.

Receptor kinases are an important component of various signal transduction pathways in plants. Most of the transcripts related to various receptor kinases were all either repressed or unaffected in the stay green and remobilizing genotypes whereas in LP 110, it was little affected except leucine rich repeat V and catharanthus roseus-like RLK1 which were up regulated under drought (Figure 24). Calcium and light dependent signaling transcripts were both repressed in stay green while in other two genotypes, it remained unaffected by drought. Detailed characterization of the effect of post anthesis drought on stem remobilisation and seed filling was carried out using only two contrasting genotypes, LP 106 (stay green) and LP 104 (remobilising).

3.2.9 Parameters measured on seeds

Drought mediated response of seed carbohydrate and nitrogen metabolism

Grain filling is an important phase of cereal crops that determines grain yield. One of the reasons for reduction in yield observed under terminal drought is due to reduced grain filling duration. Seed moisture content of the remobilising genotype was always lower compared to the stay green under both conditions (Figure 25A). Drought significantly reduced seed moisture content of both genotypes over their controls and it was more rapid and pronounced in LP 104, indicating drought induced early seed maturity of the remobilizing genotype. Accordingly, seed starch accumulation pattern was in good agreement with the seed moisture data; starch content was significantly higher in the senescing genotype compared to stay green under both conditions (Figure 25B). Drought induced early maturity of the remobilizing genotype is evident from Figure 26, which shows that, at 20 DAF, spikes of the stay green genotype were still green under both conditions while in the remobilizing genotype, it already started yellowing. In general, drought caused an increase in seed starch content during the initial stage of development (12 DAF). Similarly, it also caused an earlier cessation of starch accumulation in both genotypes which was evident from the plateauing trend observed after 20 DAF compared to their controls. Adverse effect of drought on seed starch accumulation during grain filling was evident from the decrease in seed starch content observed in the matured seeds of these genotypes under stress compared to their controls (Figure 27A).

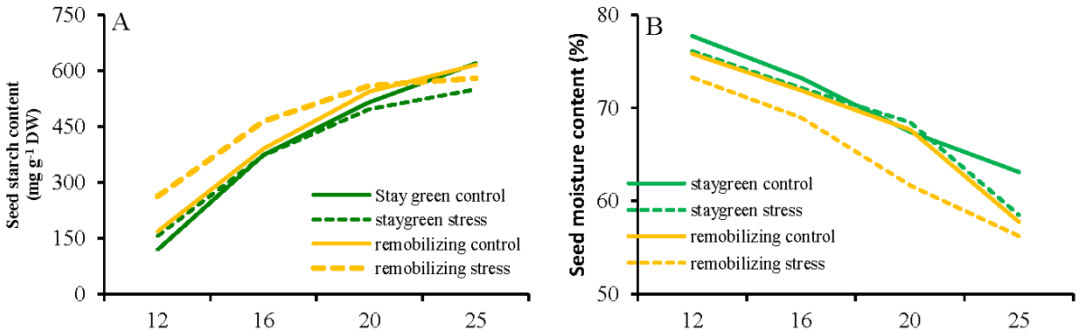


Figure 25. Drought induced early seed maturation of the senescing genotype
A. Drought caused faster reduction in the seed moisture content of the senescing genotype. Values are mean ±S.D, n=3 and expressed as %.
B.Drought caused faster accumulation of seed starch in the senescing genotype. Values are mean ±S.D, n=4 and expressed as mg/g dry weight.

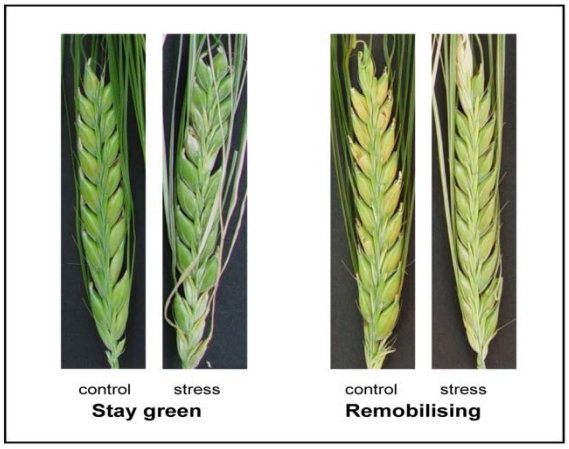


Figure 26. The remobilising genotype was characterised by an early seed maturity under both control and drought conditions. The image was taken at 20 DAF in the green house.

Reduction over control was higher in the stay green genotype (25%) compared to senescing (7%); although they had similar amount of starch under control condition. Starch being the major component of matured barley seed, reduction in seed starch content under drought has resulted in a corresponding decrease in TGW of these genotypes; with a severe reduction observed in the stay green compared to senescing genotype (Figure 27B). Hence, overall reduction in grain yield in these genotypes could primarily be attributed to reduction in TGW as there was little difference in seed number between the treatments in both genotypes (Supplemental Figure S4). In short, above results indicate that terminal drought has caused a faster grain filling and seed maturation in the senescing genotype resulting in lesser reduction in yield compared to stay green under drought.

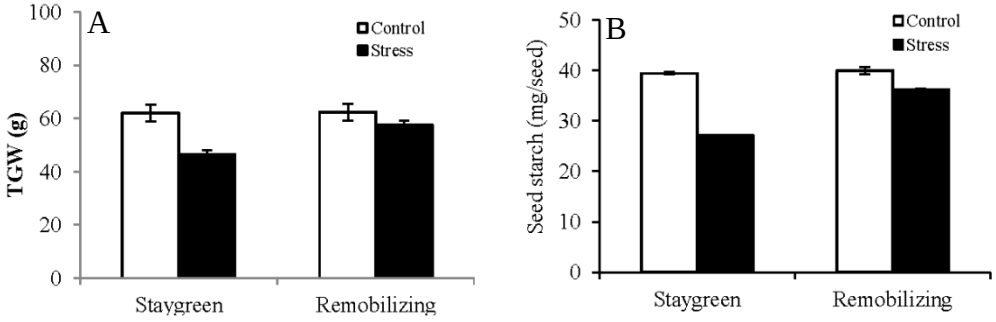


Figure 27. Drought caused higher yield reduction in the stay green genotype.
A. TGW was less affected in the senescing genotype compared to stay green under drought. Values are mean ±S.D, n=12plants/treatment.
B. Starch content was less affected in the senescing genotype compared to stay green under drought. Values are mean ±S.D, n=6. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

3.2.10 Effect of drought on AGPase and SuSY

Active metabolism of seed during itsdevelopment is a concerted action of different enzymes involved in synthesis of the major storage product, starch. Two important enzymes involved in starch synthesis are AGPase, a rate limiting enzyme of starch biosynthesis and SuSy, that is involved in cleavage of the incoming sucrose especially during the mid phase of grain filling period. Activities of both of the above enzymes were generally high in the senescing genotype and had a similar pattern in both genotypes under both conditions (Figures 28A and 28B). Drought stress reduced AGPase activity in both genotypes except at 12DAF, where it was significantly increased in the senescing genotype and remained unaffected in the stay green over their controls. Unlike AGPase, SuSYase activity was increased under drought in both genotypes except at 25 DAF.

3.2.11 Effect of post anthesis drought on seed nitrogen metabolism

A large fraction of protein N present in mature seeds of barley is derived through remobilisation from senescing leaves in the form of amino acids. In both genotypes, total seed free amino acid content gradually decreased with developmental stages under both conditions, probably hinting at the utilization of amino acids for storage protein synthesis

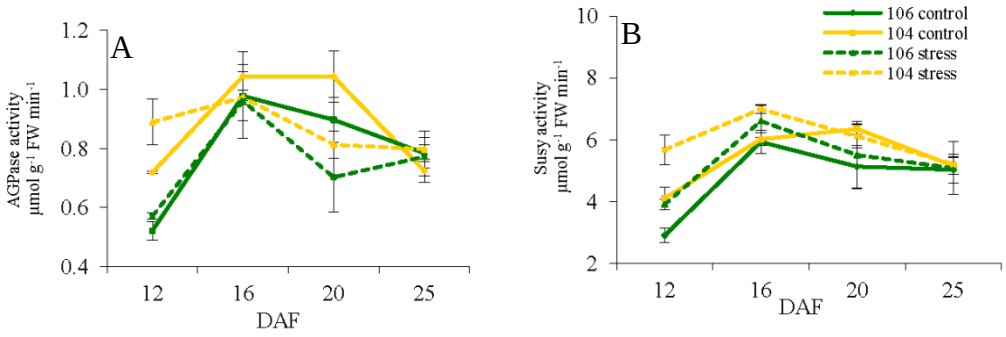


Figure 28. Higher activities of both AGPase (A) and sucrose synthase (B) of LP 104 compared to LP 106 under control and drought conditions. Values are mean ±S.D, n=4

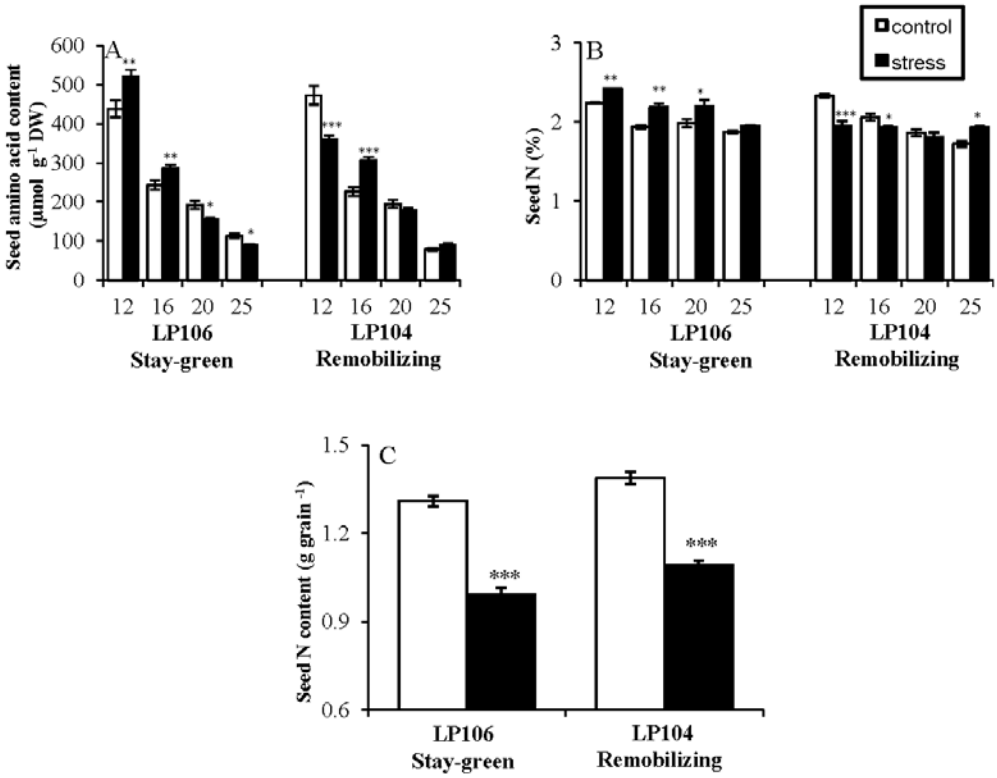


Figure 29. Altered seed nitrogen metabolism under drought.

A. **Total free amino acid content of the seed under drought.**Values are mean ±S.D, n=3 and expressed as umol per gram dry weight.

B. **Total seed N% of the seed under drought.** Values are mean ±S.D, n=4 and expressed as percentage.

C. **N content/seed was reduced under drought.** Values are mean ±S.D, n=6.

Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

(Figure 29A). There was little difference in the pattern of free amino acid content between the genotypes under drought except at 12 DAF, where it was increased in the senescing genotype over its control. However, total seed N% measured across different stages showed that drought significantly increased seed N% in the stay green during all stages except at 25 DAF; an opposite trend was observed in the senescing genotype (Figure 29B). Nitrogen content measured in matured seeds and expressed as N content per grain showed that seed nitrogen content was decreased in both genotypes under stress and that senescing genotype had higher N content per grain under both control and stress conditions compared to stay green (Figure 29C).

3.2.12 Metabolite pattern of developing seed in response to drought stress treatment

Through liquid chromatography combined with mass spectrometry (LC-MS), the contrasting genotypes were also analysed for various metabolite patterns of the developing seed under terminal drought. We measured the steady state metabolite levels at 12, 16, 20 and 25 DAF. The LC-MS approach detected ~90 metabolites of central metabolism including sugars, amino acids, nucleotides and intermediates of TCA cycle and glycolysis (Figure 30). With respect to the general stress response of caryopsis, it appears that the level of most free amino acids was elevated under stress, becoming most obvious for Asn and those derived from PEP/pyruvate. Several intermediates of the TCA cycle (malate and 2-oxoglutarate), of the glycolytic pathway (PEP and Fructose-1, 6-diP) and energy-rich nucleotides (ATP, UTP, CTP) dropped significantly under stress, indicating a shift (lower apparent rate) in energy metabolism. While sugars, especially sucrose and raffinose family of oligosaccharides are reported to increase under stress, there was no obvious trend in caryopsis. In addition, the Gaba shunt (bypass of TCA cycle) seems not to be induced under stress as concluded from the nearly constant levels of Gaba and SSA. Drought stress is known to elevate the ascorbate-gluthathione cycle metabolites which also became apparent here for both GSSG and GSH (oxidized and reduced form, respectively). This is coincident with the accumulation of their precursors (Gly and Cys), and indicates rising ROS (reactive oxygen species) levels upon stress treatment. Finally, stress treatment caused a significant drop in the level of trehalose-6-P, playing a role in the regulation of carbon partitioning (in particular cell wall metabolism and stress responses).

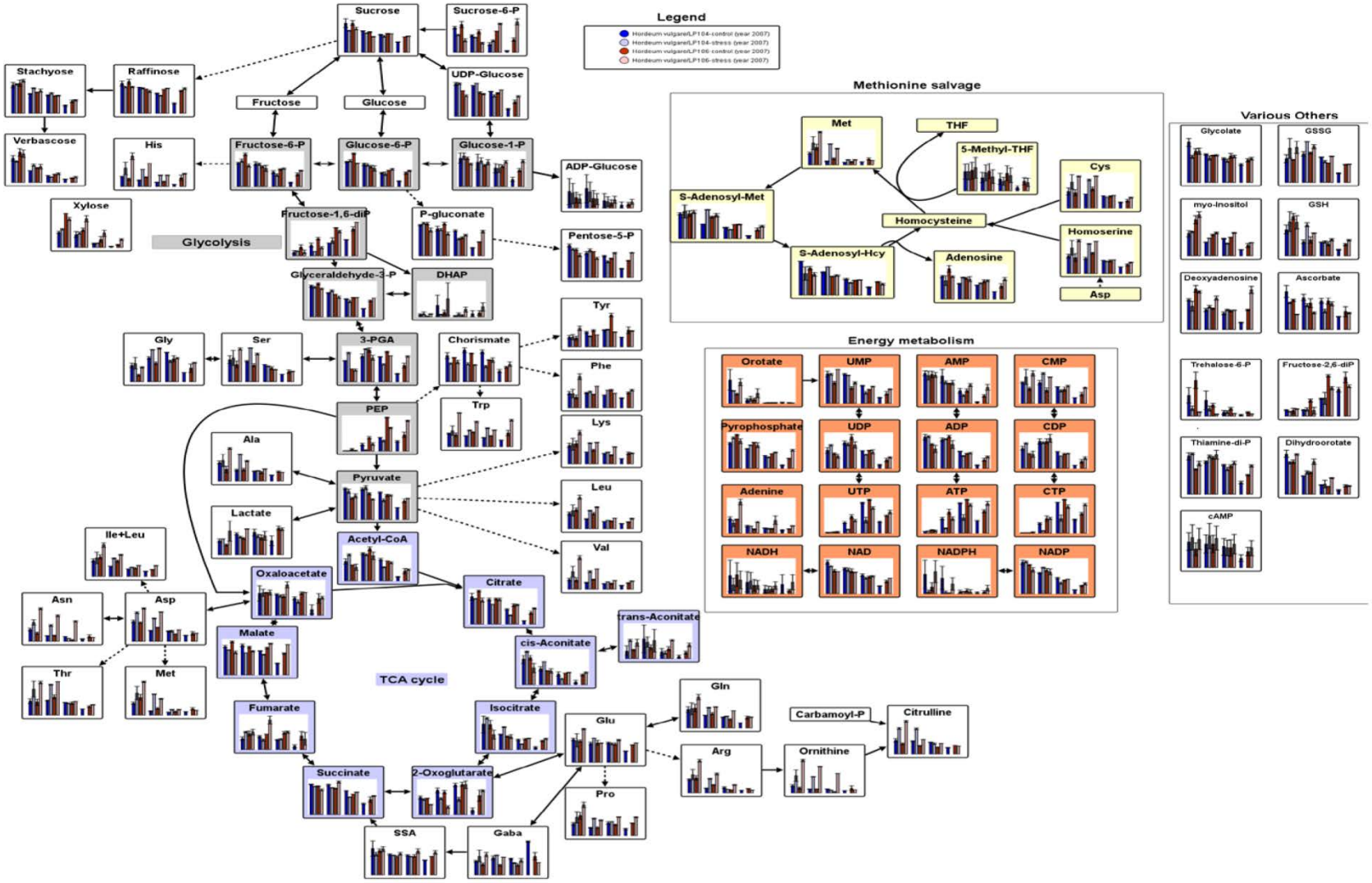


Figure 30. Metabolite pattern of developing seeds of the selected contrasting genotypes identified by LC-MS based method. The figure was made using VANTED software.

Comparison of the two genotypes revealed relatively minor differences in the stress response. Most prominent finding was that NADPH dropped upon stress in line LP104 but increased in LP106. Cofactors like NADPH are of central importance for metabolism and crop productivity. NADPH is primarily produced via OPPP and photosynthesis, and provides the reducing equivalents for various biosynthetic reactions, protects against the toxicity of ROS and allows the regeneration of GSH. Drought stress is also known to induce polyamines like putrescine which in turn can modulate plant senescence. Putrescine is synthesized from Arg with ornithine as an intermediate, the latter being also the precursor for citrulline (central metabolite in the urea cycle). Both Arg and ornithine were more drastically induced in LP106 versus LP104. While the level of ADP-glucose remained fairly unaffected by the stress treatment, there was a tendency towards higher levels in LP104 versus LP106, which coincided with the higher starch levels found in mature seeds of LP104 under stress. Taken together, metabolite profiles suggest that the caryopsis responds to stress treatment by adjustments in both nitrogen and carbon metabolism with prominent inductions in the metabolism of amino acids and antioxidants but reductions in energy metabolism. Both genotypes show largely overlapping stress responses, but there are also some indications for genotype-specific adaptations (NADPH, Arg, ornithine).

3.2.13 Parameters measured on the main stem

Stem Remobilization

Remobilisation of stem reserves is an important component of seed filling in cereals especially under terminal stress. A simple and effective method for determining stem remobilization is to measure the difference in stem weight few days after anthesis (when the maximum stem weight is observed) and at maturity (Ehadie et al., 2006). Figure 31A shows that stem weight in senescing genotype was significantly reduced from 8 DAF to maturity under both control and stress conditions. Although there was a reduced trend under stress compared to control at maturity, it was not statistically significant. On the other hand, an opposite trend was observed in the stay green genotype where the stem weight at maturity under both conditions was significantly increased compared to 8 DAF. Quantitation of stem WSC was in good agreement with the data obtained from the stem weight in these genotypes (Figure 31B). Drought significantly decreased stem WSC in the senescing genotype, whereas it was increased in the stay green genotype compared to their respective controls. At 20 DAF,

stem WSC was reduced to 30% of control in senescing genotype while in stay green it was increased to 45% under stress. In general, amount of WSC was always higher in the stay green genotype under both conditions and the peak of WSC content in both genotypes was observed at 12 DAF. Although the two genotypes did not differ significantly in stem weight between control and stress at maturity, there was a significant difference in WSC at this stage.

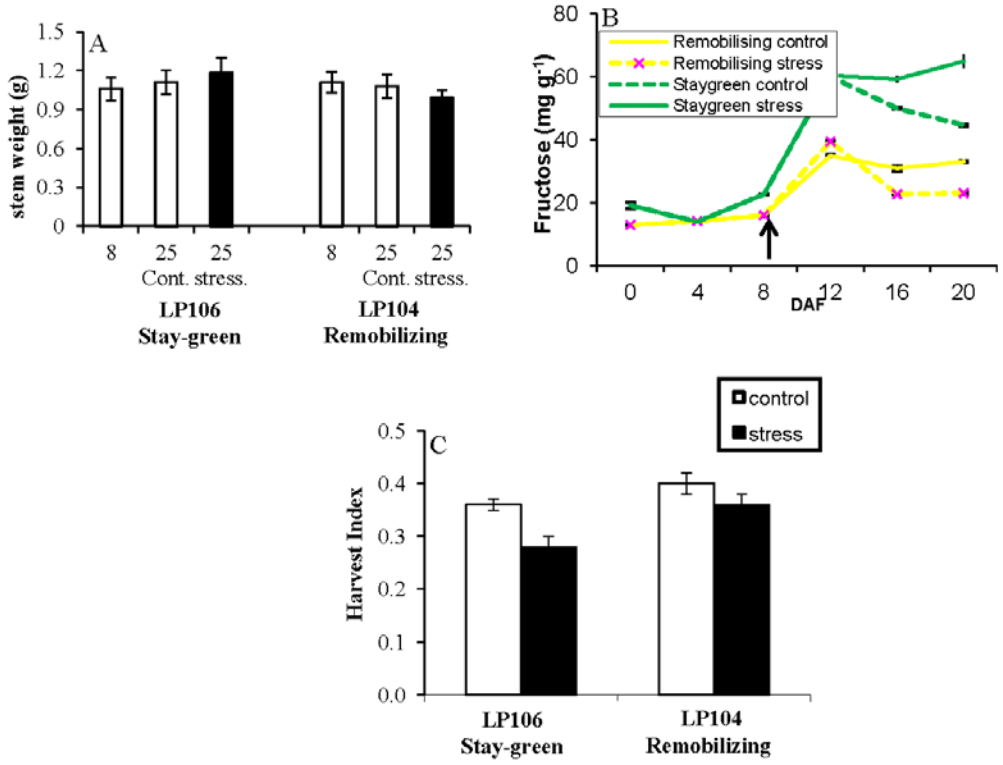


Figure 31. Drought induced higher remobilisation of stored stem resrreves in the senescing genotype. Stem weights at 8 DAF and maturity (A, n=12) and stem water soluble carbohydrate (B, n=4) and harvest index (C, n=10). Values are mean ±S.D. Significant difference between the treatments are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Harvest index (ratio of economic yield to total biological yield) is an important component of seed yield especially in small grained cereals like barley. The difference in stem remobilisation between the genotypes was also reflected in their harvest indices (Figure 31C). In general, HI was high in the senescing genotype compared to stay green under both conditions. The reduction in HI under drought was more severe in stay green compared to senescing over their controls. From the above results it could be summarized

that the senescing genotype was better in utilizing the stem reserves under terminal drought. Stable isotope labeling with $^{13}CO_2$ was carried out to study the remobilization capacities of LP 104 and LP 106 under terminal drought. At the beginning of drought imposition (10 DAF; 13 days after pulse labeling), both genotypes had similar percentage of total ^{13}C partitioned into their leaf (15-17%), grain (25%) with highest percentage (57-60%) in the stem (Supplemental Figure S5). After two weeks of stress (25 DAF, 30 days after labeling), both genotypes showed difference in the percentage distribution of ^{13}C present in their organs. In the remobilizing line, ^{13}C decreased from 69% under control to 62% under stress

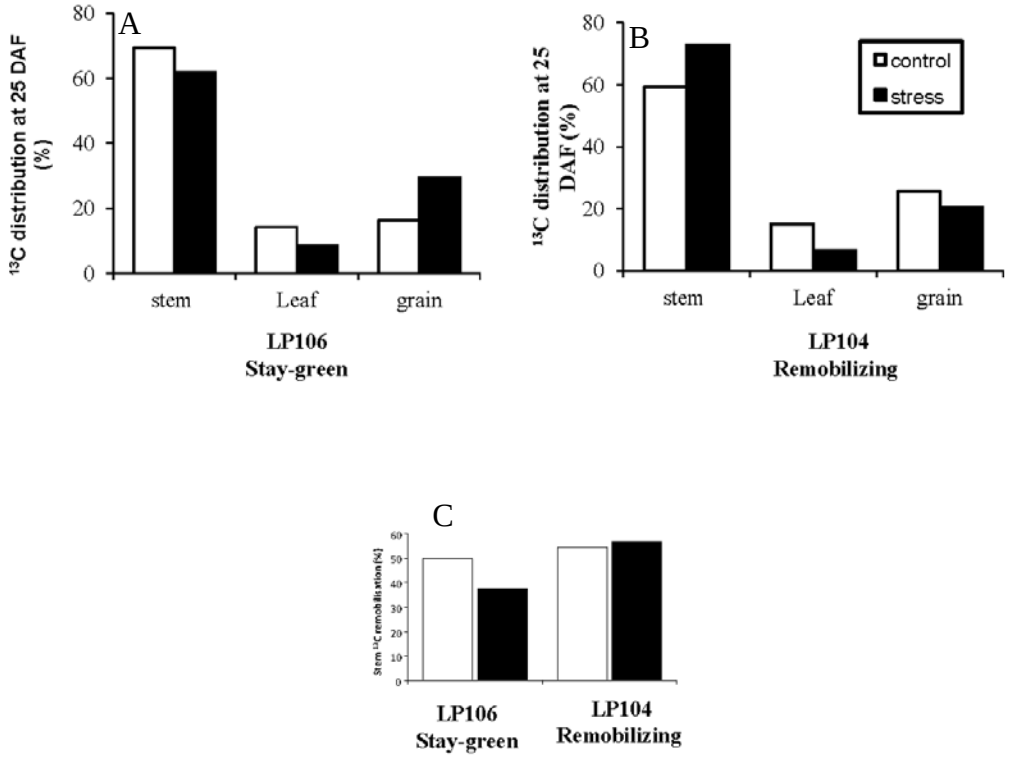


Figure 32. Percentage distibution and remobilisation of ^{13}C in stem, leaf and grains of contrasting genotypes at maturity. Distribution of ^{13}C in the stay green genotype (A), Distribution of ^{13}C in the senescing genotype (B) and Drought induced higher remobilisation of ^{13}C in the senescing genotype (C).

in the stem whereas in the grain it increased from 16% under control to 25% under stress. In the stay-green line, ^{13}C increased from 59% under control to 73% under stress in the stem whereas in the grain it decreased from 26% under control to 21% under stress (Figures 32A

and 32B). Calculation of ^{13}C remobilization from the stem showed that stress has inhibited stem remobilization in stay-green genotype where it was reduced from 50% under control to 37% under stress while in senescing genotype it remained almost unchanged between the treatments (54% under control and 57% under stress; Figures 32C). From the various experiments carried out in stem, it could be summarized that the senescing genotype was better in utilizing the stem reserves under terminal drought compared to stay green.

3.2.14 Yield trail

An yield trial with the contrasting genotypes along with two other check varieties, Scarlet and Martha, was carried out at three different locations in Germany, namely Granskevitz, Gudow and Böhnsausen by the breeding company Nordsaat. At all locations, the highest yield was obtained from the senescing genotype LP 104; although the stay green genotype LP 106 yielded more compared to the other two check varieties (Figure 33).

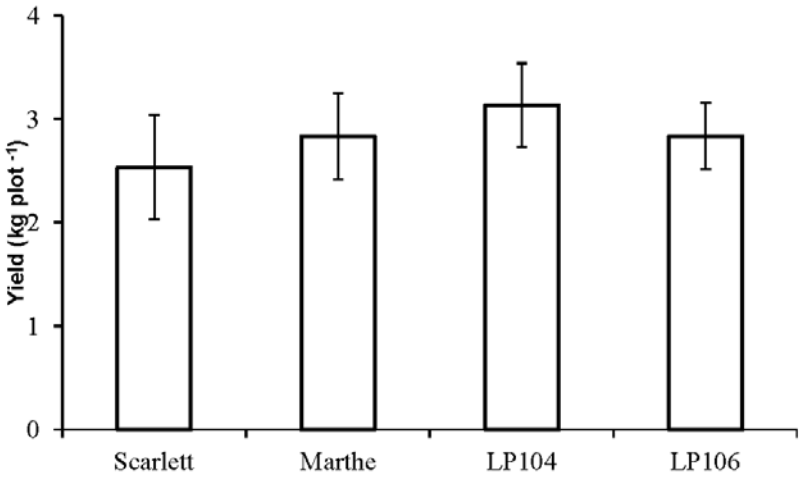


Figure 33. High yielding of senescing genotype under yield trail

An yield trail was conducted at 3 different locations in Germany under prevailing natural field conditions. Although LP 104 yield was not statistically different from LP 106 and Marthe, the relative ranking of LP 104 always high under all three environmental conditions compared to others. Each value is a mean of 3 independent field experiments and bars represent SD.

4 DISCUSSION

4.1 AB-QTL ANALYSIS TO IDENTIFY THE GENOMIC REGIONS INFLUENCING TERMINAL DROUGHT TOLERANCE IN BARLEY FOR SEED YIELD AND QUALITY

Wild barley is a repertoire of favourable alleles for variety of traits including terminal drought tolerance (Ellis et al., 2000; Talame et al., 2004; Pillen et al., 2004; Inostroza et al., 2009; Lakew et al., 2011, 2012). The present study was under taken to identify the exotic genomic regions influencing yield and seed quality traits under terminal drought using a BC_3-DH population developed by Li et al. (2006) between a German two row cultivar Brenda as the recurrent parent and the wild accession, Hs 584, as the donor parent. Significant differences between the parents in thousand grain weight (TGW) and seed quality parameters under terminal drought (Figures 6A and 6B) in addition to general difference in seed breadth and length (Figures 6D and 6E), the sub components of seed weight (Coventry et al., 2003), indicated the suitability of this population for QTL analysis under terminal drought. Stem reserves are an important component of seed filling under terminal drought, and barley varieties with taller stems contribute a higher percentage of pre anthesis reserves to grain filling under both control and drought conditions compared to dwarf ones (Austin et al. 1980a). Similarly, in wheat also it has been suggested that varieties with taller stems would have yield advantage under terminal drought compared to ones with shorter stems (Borrell et al., 1993). Though this character was not studied in the present investigation, significant difference in stem length between the parents (Figure 6F) in addition to other contrasting features mentioned above, makes it an apt population for QTL analysis for terminal drought tolerance.

In the present study, terminal drought under field condition was mimicked by spraying crop canopy with potassium iodide (KI), 0.4% at 10 DAF. Application of crop canopies with chemical desiccants such as KI has often been used in screening for terminal drought tolerance in variety of crops and the reduction in TGW obtained with chemical spray was generally well correlated with reduction observed under terminal drought (Nicolas and Turner, 1993; Blum, 1998; Sawhney and Singh, 2002). However, in the present study, TGW obtained with KI treatment was poorly correlated with TGW obtained from the drought imposed experiment in green house. A similar finding was reported by Gavuzzi et al. (1997),

who found that chemical desiccant treatment to be less reproducible than other laboratory-based tests and suggested that in wheat, extent of damage caused by chemical desiccant was greatly influenced by growing conditions. A possible reason for the above finding in the present study could be due to the spraying of KI to the whole plant including spikes which results in poor sink strength because of the bleaching agent (Nicolas and Turner, 1993). This could also be the reason for a poor correlation observed between yield and TGW in the field but not under green house under drought conditions.

Phenotypic evaluation of the mapping population under different environmental conditions showed that the two parents differed significantly in various traits scored (Supplemental Table S4 and S5), and transgressive segregation was evident in the progenies for most of the traits indicating the presence of both positive and negative alleles of a trait in both parents.

4.1.1 QTL analysis

QTL analysis of different traits (Table 1) showed that considering control and drought conditions together, a significant number of QTLs was contributed by wild accession, Hs 584 (42%). Similarly, the wild parent was found to contribute 34% and 52% of the favourable QTLs detected in two previous studies by Pillen et al. (2003) and Talame et al. (2004), respectively, using AB-QTL analysis.

One of the major hindrances to QTL analysis is the instability of QTLs as a result of large environmental effects. In this aspect, QTLs obtained in the present study could be relatively more stable as the plants were grown in diverse environment during different years and only common QTLs that appeared in at least two different environments were considered for analysis. Of the stable QTLs, 46% of the QTLs were contributed by wild parent; a notable feature of these QTLs was the less stability compared to Brenda especially for yield and TGW characters (Table 5). A probable reason for the above could be the selective breeding of the cultivated parent for its yield potential that would have less impact under diverse environments.

QTLs detected in a particular population relates to the genetic background of the population studied. Comparisons are made with similar studies that have used different populations either to reinforce the importance of common QTLs detected in these studies or to highlight a novel QTL controlling a particular trait in a specific population. In this context, QTLs detected in the present study (for yield and TGW) were compared to previous studies of AB-QTL analysis that were carried out mainly under Mediterranean conditions. Comparison to previous studies have been made whenever possible using the consensus markers present between the studies or comparing the markers present in the different studies to a barley consensus linkage map published by Varshney et al.(2007) using 765 SSR markers.

4.1.2 Yield

Yield is a complex trait governed by various internal and external factors. A number of QTL studies have been addressed in barley for yield and its contributing characters using wild barley as one of the parent (Pillen et al., 2003; Talame et al., 2004; Von Korf et al., 2006; Lakew et al., 2012). The QTL, qYLD1.1 corresponding to the marker Bmac0154 was found to match one reported by Talame et al. (2004); in both these studies, this particular QTL was derived from the cultivated parent. The qYLD2.1 could also be mapped to similar locations as reported by Talame et al. (2004) and Lakew et al. (2012); however, its precise location could not be verified due to the lack of common markers between the studies. However, this yield QTL (qYLD2.1) corresponding to the marker EBmac0684 was found to match a thousand grain weight QTL and a QTL for grain carbon isotope discrimination (Δ) detected by Teulat et al. (2001c) and Teulat et al. (2002), respectively, using the population developed from a cross between Tadmor and Er/Apm that was grown under Mediterranean conditions. Carbon isotope discrimination is a surrogate for water use efficiency and hence grain yield, especially under conditions of limited water availability (Farquhar and Richards, 1984). In particular, grain Δ has been found to be the most appropriate parameter for grain yield under Mediterranean conditions (Voltas et al., 1998; Merah et al., 2001). The collocation of yield (present study), TGW and grain Δ at the marker EBmac0684 indicate that this could be a potential region influencing the grain yield under terminal drought. Another interesting QTL that was derived from wild genotype is qYLD3.1, as this was not reported in earlier AB-QTL studies addressing yield under Mediterranean environments.

4.1.3 Thousand grain weight (TGW)

Thousand grain weight or seed weight in barley is an important trait determining yield and quality of the produce; and had been one of the main targets of selective breeding during domestication in a number of cereals (Doganlar et al., 2000). A significant correlation in TGW between different environments with a higher magnitude compared to yield (Table S8) is consistent with the fact that TGW is a more heritable trait than yield. Although it has a high heritability compared to other yield traits, grain weight is a more complex trait governed by a number of both genetic as well as environmental factors (Coventry et al., 2003). Yield reduction under post anthesis drought is primarily due to reduction in TGW and hence, is an important trait to be scored for terminal drought tolerance (Tanksley 1993).

In the present study, a total of 11 QTLs were detected for TGW, and two locations (qTGW3.1 and qTGW3.2), the increase in grain weight under control condition was contributed by Hs 584. Li et al. (2006) using the same population, found a total of 15 QTLs for TGW which were distributed to all 7 linkage groups, and at 8 places, the increased TGW was contributed by Hs584. Two QTLs, qTGW1.3 (GBS3269) and qTGW6.1 (GBMS222) derived from Brenda in the present study could be matched to the ones earlier detected by Li et al. (2006) indicating the stability of these QTLs over different environmental conditions. A significant reduction in the number of QTLs detected for yield and TGW in the present study compared to those of Li et al. (2006) could be due to the less number of progenies analysed in the present study (67 in the present study and 120 by Li et al., 2006) or due to the strict criteria of QTL assignment (LOD $\geq$3 with appearance in minimum 2 environments); although the effect of environment cannot be ignored.

Two QTLs, qTGW1.3 and qTGW2.1 corresponding to the markers Bmag0382 and Ebmac0684, respectively, were found to match to the ones detected by Lakew et al. (2012); however, the qTGW1.3 represented a yield QTL in the later study. Similarly, qTGW1.1 and 3.1 were also found to match to similar locations to Lakew et al. (2012), although its precise location could not be verified due to lack of common markers between the studies. A QTL near the marker Bmag 0225 (qTGW3.2) detected in the present study was also reported by Talame et al. (2004) and Teulat et al. (2002), indicating the influence of a particular gene/genes controlling TGW in different genetic back grounds and a potential target a future

study. Similarly, qTGW6.1 could be traced to similar location as found by Talame et al. (2004) and Teulat et al. (2002) after comparing the markers between the studies to the consensus linkage map developed by Varshney et al.(2007). Interestingly, no TGW QTLs were detected in the present study and by Talame et al. (2004) in the chromosome 4H.

The presence of both treatment specific and constitutive QTLs for yield and TGW (Table 5 and 6) indicate that crop yield under drought is determined by the concerted action of both constitutively and drought specific expression of genes. Large number of QTLs detected in Brenda for yield and TGW compared to Hs 584 could probably due to the selection pressure applied on the cultivated parent for yield during domestication.

4.1.4 Drought tolerance index of yield and TGW

All drought tolerance QTLs for yield (qDTI-Y) were co-localized with QTLs for yield except the exotic qDTI-Y1.3. Similarly, all drought tolerance QTLs for TGW (qDTI-TGW) was co-localized with QTLs for grain weight (Figure 9). The above observation possibly indicates that it is the genetic potential of the genotypes that imparts tolerance to yield under terminal drought.

4.1.5 QTLs for seed morphology

Grain morphological traits such as length, breadth, shape and density are important sub components contributing to seed weight as well as grain quality in cereals (Kovach et al., 2007). A number of studies have addressed these parameters in rice and wheat (Gegas et al., 2010; Li et al., 2011). Although grain size and shape parameters are found to be associated with flour quality such as protein content and hydrolytic enzymes which in turn determine the end quality of the seed, this has been largely overlooked in barley possibly because of its major use as feed.

One of the QTL study addressing seed length in barley was reported by Backes et al. (1995) using a DH population developed between Igri and Danilo using RFLP markers where 2 QTLs for seed length were detected on chromosome 4H and 7H. In the present study, a total of 5 QTLs were detected for seed length (Table 7) which were all derived from Hs 584, the parent superior in this trait value, but none of them was mapped to 4H and 7H. Hence, all novel QTLs identified in this study for seed length would be a source for studying seed

length character and breeding for yield in barley. Another notable feature of this trait was that, none of the ILs had higher seed length compared to Hs 584 (Supplementary Table S2). The above observation together with the identification of all seed length QTLs from Hs 584 indicates that Brenda did not possess any positive alleles for seed length. Detection of most of the seed length QTLs only under control except qSL2.1, that was expressed in both conditions indicates stability and constitutive nature of qSL2.1 in influencing seed length in this population.

With regard to seed breadth, to the best of our knowledge, no QTL reports were found in barley; although quite a number of studies have been carried out in other crops such as wheat, rice and tomato which also resulted in map based cloning of some of the QTLs identified (Frary et al., 2000; Fan et al., 2006). A total of 6 QTLs were detected for seed breadth which were all derived from Brenda, the parent having a higher trait value. At two locations, QTLs for seed breadth (qSB1.1 and qSB2.1) and seed length (qSL1.2 and qSL2.1) were co-localized. Although it cannot be resolved from this study whether such collocation is due to linkage drag or pleiotropic effects, similar studies in rice with QTL for seed weight (GS3 and GW2) was found to have pleiotropic effect on both grain width and length (Fan et al., 2006; Takano-Kai et al., 2006).

QTLs for seed length to breadth ratio which indicates seed shape had a total of 10 QTLs, which were all derived from Hs 584 except qSLB 5.1 and qSLB7.2. Although in most cases qSLB was co-localized with either seed breadth or length QTLs, there were many independent QTLs located on 5H (qSLB5.1, qSLB5.2 and qSLB5.3) and 7H (qSLB7.2), hinting at the genes specifically influencing seed shape in barley. In tomato, it was found that, fruit size and shape are largely controlled by independent genes (Frary et al., 2000; Van der Knaap and Tanksley, 2001). However, in a recent study for grain size and shape variation in wheat using 6 different mapping populations, Gegas et al. (2010), found that, many of the QTLs that affected grain size also had influence on grain shape. Another interesting observation was that, L/B ratio was negatively correlated to major Brenda contributed traits like TGW, seed breadth and seed starch content and positively correlated to Hs 584 contributed traits like seed nitrogen content and length (Table 3). Given the nature and

direction of correlation between the traits, it is urging to assume that, the major QTLs where these traits are co-localized is probably due to pleiotropic effects of the underlying genes; however, the possibility of linkage drag can only be excluded, if the segregating population at this region is further developed.

4.1.6 Seed quality traits

Seed starch and N content are the two most important parameters determining the end use of barley for malting. Generally a low protein concentration is desirable for malting as they interfere with starch degradation during malting and also due to the fact that, there is a strong negative correlation between yield and protein content in cereals (Briggs, 1978). All QTLs detected for seed N% in the present study were derived from Hs 584, which had for seed N%For seed nitrogen, all the QTLs detected under both conditions were obtained from the parent Hs 584, the parent that had higher value for this trait. Two QTLs, qSN2.1 and qSN7.1 were found to match the wild contributed QTLs reported by Li et al. (2005) using a BC_3-DH population developed between Brenda and wild accession, Hs213.

Similarly, qSN2.1 corresponding to the marker EBmac0684 was found to match the QTL for grain protein content reported by Von Korf et al. (2005) using AB-QTL analysis of a BC_2DH population developed between the spring barley cultivar Scarlett and the wild barley accession ISR42-8. Other QTLs present on 1H and 6H could not be matched to the above study because of lack of common markers. Similarly, qSN6.1 was also found to be located in a similar region for a major barley GPC locus as repoted by see et al. (2002). Interestingly, all the QTLs detected for grain N% were either specific to control or drought condition indicating that increased grain nitrogen observed under a variety of stresses in barley may not be the resultant of genes involved in general N metabolism, but rather could be the result of specific genes expressed under drought. However, absence of constitutive QTLs for grain N% is a bit intriguing as change in N metabolism under drought might also involve genes involved in general N metabolism under control conditions. Hence, as the QTL analysis for grain nitrogen was based on only one environment, it is possible that, we did not detect other QTLs involved in N metabolism which are constitutively expressed.

For seed starch content, a total of 5 and 3 QTLs were detected under control and drought conditions, respectively, which were all derived from Brenda. Interestingly, all these these QTLs were co-localized with QTLs for seed N% except qSS7.1 which was control specific. Co-localization of seed starch and N% and a strong negative correlation observed between these two traits (Table 3) possibly indicates that the universal negative correlation observed between yield and grain protein content in cereals is probably the result of pleiotropic effect of genes controlling these traits. In barley and other cereals, grain N% and yield are negatively correlated (Acreche and Slafer, 2009), as a result, the modern varieties with improved yield are often associated with reduced N% or grain protein content (Calderini et al., 1999). Using altered source-sink relations in wheat grown under Mediterranean conditions, Areche and Slafer (2009) found that lower N% of the modern day wheat varieties is a result of increased grain number and the negative correlation between yield and grain protein content could be broken if source strength is enhanced.

The detection of qSS7.1, which did not co-localize with seed starch content indicates that the relationship between yield and grain N% can also be altered if the genes underlying such QTLs are identified and altered specifically. This is evident in a recent study by Weichert et al. (2010), who reported that increasing the sink capacity of the wheat grains through over expression of a barley (*Hordeum vulgare*) sucrose transporter HvSUT1 (SUT) under the control of an endosperm-specific Hordein B1 promoter (HO) has resulted in increased grain protein content in addition to seed weight. Similarly, seed specific expression of a bacterial PEP carboxylase in V*icia narbonensis* was also found to increase seed protein content together with seed weight (Rolletchek et al., 2004). From the above discussion, it is clear that yield and protein relation in cereals could be altered by altering either source or sink strength. However, a more negative correlation observed between yield and grain protein content under terminal drought compared to optimal conditions in cereals like barley is attributed to the differential accumulation of starch and protein during grain filling in cereals (Morgan and Riggs, 1981; Brooks *et al.*, 1982).

4.1.7 Co-localization of traits

Since many of the agronomic characters are correlated, the phenomenon of different traits co-localizing to a QTL is observed in a variety of crops including barley and wheat (Zhu et al.,

1999; Quarrie et al., 2005). Such co-localization can be due to either tight linkage of genes underlying a particular QTL with nearby genes or due to pleiotropic effect of the gene/genes underlying a QTL. In tomato (*Solanum pennellii*), fine mapping of a QTL for fruit mass has resolved the QTL into three closely linked loci (Eshed and Zamir, 1995). On the other hand, grain weight and size QTL associated with sdw1 locus (semi dwarfing locus) in barley so far could not be separated indicating the pleiotropic effect of sdw1 locus on grain size (Barua et al., 1993). A similar observation is the association of *Vrs* 1 located on chromosome 2H with grain size in barley, with two row genotypes having heavier grains (Karakousis et al., 2003). However, it is difficult to distinguish the effect of pleiotropy from a linked QTL in an initial mapping population due to restriction in population size or recombination event between the linked loci. Hence, further fine mapping is needed to resolve the nature of such QTLs detected in the present study.

4.1.8 Candidate genes underlying the important QTLs

Some of the possible candidate genes underlying some QTLs detected in the present study could be identified as the linkage map developed by Worch et al. (2011) for this population also involved gene based SNP markers in addition to SSR markers. Several genes located in chromosomal regions involved in drought stress response are identified as transcription factors or to be transcriptionally regulated under water deficit (Nevo and Chen, 2010). We mapped drought responsive myb transcription factors to the regions on chromosome 1H (GBS3286) and 3H (GBS3145). Several other transcriptional regulators were localised on 2H (GBS3215, GBS3217, GBS3143, GBS3149, GBS3243 and GBS3224). The qYLD3.1 which harbours glutamate decarboxylase gene (GBS3192) was detected under control condition. Among the QTLs for biochemical traits, qSS2.1 is interesting as it contains two starch branching enzymes (GBS3257 and GBS3274) and a sucrose synthase gene (GBS3273). The qSN1.1 which also collocates with many other traits contains at least 4 amino acid metabolism genes (GBS3193, GBS3249, GBS3250 and GBS3251).

Among the candidate genes, an interesting one is, glutamate decarboxylase (GAD), the first enzyme of the GABA shunt pathway which is involved in the unidirectional conversion of glutamate to GABA by decarboxylation . GABA is a four-C nonprotein amino acid that has a primary role in central C-N metabolism which is particularly relevant under stress (Bown and

Shelp, 1997; Bouche and Fromm, 2004). In a recent study where the deregulated GAD was over expressed in Arabidopsis seeds under seed maturation-specific phaseolin promoter, the transgenic plants were characterized by an increased N metabolism resulting in higher C/N ratio (Fait et al., 2008). As yield is a complex trait and CN metabolism is an important aspect of various developmental stages, GAD might be involved in some aspects of plant development that influences yield components in barley.

Other interesting possible candidate genes are starch branching enzyme and sucrose synthase that underlies qSS2.1. Starch branching enzymes are the ones which introduce α-1,6 glycosidic bonds into α-polyglucans and hence, determine the amylopectin structure. In cereal species such as barley and wheat, three classes of starch branching enzymes (SBE I, SBE IIa, and SBE IIb) have been reported and was the primary target for altering the seed quality by altering amylase to amylopectin ratio (Boyer and Preiss, 1978; Wei et al., 2009). In addition, antisense repression of SBE IIa and IIb in barley has resulted in a significant reduction in TGW of the transgenic line compared to wild type (Regina et al., 2010). Sucrose synthase which catalyses the conversion of sucrose to fructose and UDP-glucose is considered to be the marker enzyme of sink strength in several crops including cereals (Sun *et al*., 1992; Wang *et al*., 1993; Kato, 1995; Jiang et al, 2011). Its activity was found to be the major determinant of seed filling duration in barley and wheat under both optimal and drought conditions (Chevalier and Lingle 1983; MacLeod and Duffus 1988). While the activity and expression of SBE genes in rice were generally suppressed under both drought and heat stress (Yamakawa et al., 2007; Peleg et al., 2011), sucrose synthase activity was suggested not to be a limiting factor under drought in a number of studies (Ober et al., 1991; Dorion et al., 1996; Sheoran and Saini 1996). The control specific expression of qSS2.1 indicates that genes underlying this QTL are negatively affected by drought and further fine mapping is needed to reveal the genes responsible in this region.

4.1.9 Selected contrasting lines and the importance of C/N ratio

Selected contrasting genotypes revealed that yield reduction in the inferior genotypes was primarily due to the integration of Hs segments at the major Brenda QTLs influencing yield and TGW. A significantly lower yield and TGW in ILHS 5 and 44 compared to other inferior lines indicate that qHS1.1 could be an important region determining both yield and TGW.

Similarly, qHS2.1 also could be an important QTL as it harbours two important candidate genes influencing grain weight, starch branching enzyme and sucrose synthase. Seed development in barley and other cereals is an important phase during which two main storage compounds, carbohydrates and proteins are accumulated. The process of carbohydrate and protein accumulation are differentially affected by drought which alters the seed C/N ratio and hence seed quality. It is generally believed that N accumulation is less sensitive to drought compared to starch accumulation resulting in higher N accumulation relative to starch under drought (Morgan and Riggs, 1981; Brooks *et al.,* 1982; Triboi et al., 2001). This was also evident in the selected inferior lines where N% was significantly increased under stress over control compared to superior lines. This has resulted in a significantly reduced C/N ratio in the inferior lines compared to superior lines under drought. Although seed N accumulation is also influenced by a number of environmental factors other than drought, the results from our study indicate that C/N ratio could be a potential parameter for screening drought tolerance, not just for seed quality but also for yield performance under terminal drought.

4.2 CHARACTERIZATION OF THE IMPORTANCE OF STAY GREEN/ SENESCENCE PHENOTYPES UNDER TERMINAL DROUGHT

Yield in cereal crops, especially under terminal stresses is largely dependent on the availability of assimilates for seed filling. In general, current photosynthesis and stored reserves are the two important sources of assimilates for seed filling (Schnyder, 1993; Yang et al., 2006). Under terminal drought, relative contribution of current photosynthesis and stored reserves to seed filling which are governed by stay green and senescence process, respectively, depends on the crop species and/or the severity of stress (Blum, 2005). The present investigation was undertaken to characterise the mechanism of terminal drought tolerance (stay green/senescence) in contrasting lines of barley that were selected based on an initial screening for drought tolerance under field conditions. Various physiological parameters combined with molecular and metabolite measurements made on flag leaf were used to characterise the extent of senescence variability and to study the effect of drought on CN metabolism in these genotypes. Finally, the contrasting genotypes were characterised in detail for stem reserve mobilization and seed filling under terminal drought.

4.2.1 Characterisation of the extent of senescence/stay green under terminal drought

Physiological parameters:

One of the initial responses of plants to drought stress is reduction in photosynthesis as a result of stomatal closure. Drought drastically reduced the stomatal conductance of all three genotypes with a concomitant reduction in assimilation rate that was more severe in the two senescing genotypes (LP 104 and LP 110) compared to stay green (LP 106). A strong positive correlation between stomatal conductance and assimilation rate under drought (Supplemental Figure S6) indicates that, among all limitations, stomatal limitation to photosynthesis is the major cause for drought induced reduction in photosynthesis (Chaves et al., 2009; McDowell, 2011). Down regulation of the transcripts involved in the light reaction and calvin cycle (RuBisCO small sub units) in the two senescing genotypes indicates that, in addition to stomata, the biochemistry of photosynthesis was also adversely affected by drought in these two genotypes. Further, a significantly increased Ci in the remobilizing line under drought over its control suggests that biochemistry of CO_2 assimilation is impaired in this particular genotype compared to other two. Indeed, the biochemistry involved in

photosynthetic CO_2 assimilation has been found to be rather stable under drought except during a very severe stress, where RuBisCO activity and RuBP regeneration were found to be negatively affected (Galmes et al., 2011; Aranjuelo, 2011). Hence, among the genotypes, the remobilizing genotype is more susceptible to drought with an impaired biochemistry of CO_2 assimilation, in addition to stomatal inhibition.

Reduced photosynthesis and degradation of chlorophyll are the conspicuous events of drought induced senescence in plants (Hensel et al. 1993). Significantly reduced chlorophyll pigments (both a and b) under drought at 16DAF in both the senescing genotypes indicate that they are more susceptible to drought compared to stay green. Down regulation of the transcripts involved in tetrapyrrole biosynthesis in both the senescing genotypes are in good agreement with the chlorophyll measurements obtained from these genotypes.

Tetrapyrroles and its various intermediates are the potential phytotoxic compounds especially under stress as they have the ability to form toxic compounds when they combine with various reactive oxygen species, (ROS) such as singlet oxygen (Tanaka and Tanaka, 2007; Tanaka et al., 2011). Hence, it is imperative for the plants experiencing drought to limit their production so as to avoid the harmful effects of their accumulation. In this context, a large reduction in the transcripts of tetrapyrrole biosynthesis in both the senescing genotypes compared to stay green indicates that the senescing genotypes are experiencing more stress compared to stay green. The two senescing genotypes had little difference in chlorophyll or related transcripts. Further, RWC of leaf, which in general indicates health of a plant especially under drought is consistent with other physiological data showing higher susceptibility of both the senescing genotypes among which, LP 104 was more susceptible. In short, various physiological parameters clearly indicate that the two senescing genotypes were more susceptible to drought compared to stay green and among the senescing genotypes, LP 104 was characterised by an early/faster initiation of drought induced senescence compared to LP 110.

4.2.2 Drought induced flag leaf carbohydrate metabolism and senescence induction.

Accumulation of various osmolytes, including sugars under drought, has long been proposed to be an adaptive mechanism in plants contributing to tolerance through different mechanisms such as osmotic adjustment, protection of membranes and proteins, scavenging of ROS etc. (Morgan 1984; Chen and Murata, 2011). However, a number of studies have show no advantage of osmotic adjustment to crop yield, or sometimes showing a negative impact of osmotic adjustment on crop yield under drought (Grumet *et al*., 1987; Sanchez et al., 2011b). In the present study, both the senescing genotypes were characterized by a marked increase in osmolality under drought which did not correlate with their leaf water status compared to stay green genotype. Hence, osmolite accumulation may not contribute to drought tolerance in the genotypes studied.

Proline, a universal osmolite which gets accumulated under stress was significantly increased under drought in all genotypes and was more pronounced in the senescing genotype LP 110. The role of Proline as a universal osmolite contributing to drought tolerance is a controversary. While some authors claim a positive effect of proline accumulation under drought, others regard accumulation of proline as a mere passive response of plants to drought, which confers no tolerance or yield advantage under drought (Ibarra-caballero et al., 1987). In the present study also, higher accumulation of proline under drought in the senescing genotype, LP 110 was not correlated with its leaf water status. A similar observation was also noticed in other studies in barley, where the sensitive genotype was found to accumulate more proline, glycine betaine and other compatible solutes than the tolerant cultivar (Chen *et al*. 2007; Sanchez et al., 2012).

A Significantly increased content of various water soluble sugars in both the senescing genotypes especially during initial stages of drought compared to stay green indicates that, difference in osmolality observed between the genotypes under stress could primarily be due to the accumulation of water soluble carbohydrates, although we have not measured other potential osmolytes in barley.

A general increase in soluble sugar content under drought across the genotypes in the present study is consistent with the earlier observations that drought induces accumulation of

reducing sugars as well as sucrose, where they are implicated to function in turgor maintenance (Geigenberger *et al*. 1997; Villadsen et al., 2005; Rogiers et al., 2011). Accumulation of soluble sugars in spite of reduced photosynthesis under drought has been attributed to various reasons such as conversion of starch into hexoses, inhibition of growth, excess carbon availability resulting from decreased amino acid synthesis and reduced export of sugars because of sieve tube occlusion (Jongebloed et al. 2004; Chaves and Oliveira, 2004). Reduction in leaf starch content together with the up regulation of starch degrading enzymes such as α and β-amylases under drought in all genotypes hints that conversion of starch to soluble sugars could be a general mechanism in barley for soluble sugar accumulation under drought; although other mechanisms could also be operating.

Further, upregulation of the transcripts of invertases together with increase in hexose (glucose and fructose) content found in all genotypes under drought is consistent with other studies that drought causes the accumulation of hexose content through the activation of invertases (Andersen, 2002; Xue et al., 2008a). Although there was a significant increase in sucrose content under drought, the transcripts involved in the sucrose synthesis were not differentially regulated in the present study. Hence, increased sucrose content observed under drought in the present study could probably be due to the inhibition of sucrose export from leaf which has also been found in other studies (Jongebloed et al. 2004; Farooq, 2009).

A high carbohydrate level, “carbon feast”, is known to induce senescence in plants, and the role of sugars in senescence induction has been widely discussed (Pourtau et al., 2006; Wingler and Roitsch, 2008; Van Doorn, 2008; Wingler et al., 2012). Senescing leaves in Arabidopsis are characterized by a higher accumulation of glucose and fructose content (Parrot et al. 2007, Pourtau et al. 2006). Role of sugars in senescence induction was also evident from stem girdling experiment in barley, where accumulation of sugars as a result of inhibited transport was found to induce senescence (Fro¨hlich and Feller 1991; Parrott et al. 2005). Similarly, a higher differential accumulation of all three soluble sugars especially during the initial stages (12 and 16DAF) in the senescing genotypes compared to stay green under drought could be related to the senescing nature of these genotypes. Further, early initiation of drought induced senescence in LP 104 compared to LP 110 could be related to the higher accumulation of sugars observed at 12 DAF in LP 104 compared to LP 110.

In addition to the role of sugars in senescence induction, they have also been shown to be important in various signaling functions in plants (Rolland et al., 2002; Bolouri-Moghaddam et al., 2012). Elevated levels of carbohydrates are known to inhibit photosynthesis (Neals and Incoll, 1968). Accumulation of hexoses, resulting from sucrose hydrolysis was found to inhibit photosynthesis that is mediated by hexokinase signaling system (Goldschmidt and Huber,1992; Eom et al., 2011). This was evident from the transgenic potato overexpressing yeast invertase which had reduced photosynthesis resulting from higher accumulation of hexoses (Bussis et al., 1997). The hexose mediated photosynthesis inhibition is thought to be the resultant of Pi sequestration in the cytosol and hence, depriving chloroplasts of Pi required for ATP generation through light reaction (Foyer, 1988; Paul and Foyer, 2001). In the present study, both the senescing genotypes were characterised by a significantly increased hexose to sucrose ratio especially during the early stage (12 DAF) compared to stay green, which could be related to the reduction in photosynthesis as well as extent of senescence observed in these genotypes under drought.

Another aspect of sugar signaling is the interaction of sugars with metabolites including hormones, in mediating various plant developmental processes under different environmental conditions (Rolland et al. 2006; Cho et al., 2009). Co-expression of some of the sugar metabolizing and ABI (ABA insensitive) genes found from the meta analysis of large number of metabolites and gene expression studies under stress possibly indicates the interaction of sugars and ABA in mediating various co-regulated functions in plants under stress (Pinheiro and Chaves 2011). Given the role of ABA in drought induced senescence (Nooden et al., 1997; Weaver et al., 1998), higher accumulation of both sugars and leaf ABA in both the senescing genotypes compared to stay green especially during the initial stages of drought could be related to the interaction between sugars and ABA in signaling the senescence induction; however, this has to be explored in greater details.

Although various other parameters could clearly differentiate the early senescing nature of LP 104 genotype from LP 110 genotype, accumulation of ABA at 12 DAF did not differ much in these genotypes (Figure 22). This could probably be due to the degradation of already accumulated ABA in the LP 104 genotype where senescence was induced early

compared to LP 110. Although ABA degradation products have not been quantified in these genotypes, in a similar study involving a senescing and a stay green genotype, at 16DAF, the senescing genotype was characterized by a significantly higher accumulation of ABA degradation products such as PA and DPA compared to stay green However, the two genotypes did not differ much in differential accumulation of ABA under drought (data unpublished; Supplemental Figure S7). In short, various aspects of sugar metabolism observed in the present study reveal that accumulation of soluble sugars could more be associated to senescence induction rather than functioning as osmolytes.

4.2.3 C/N and Gly/Ser ratio as markers to characterize senescence variability

Another important parameter that distinguished the senescence genotype from stay green is the carbon to nitrogen ratio. CN ratio is an important aspect of plant development and a relatively high carbon to nitrogen ratio is often implicated in the process of senescence induction (Zheng, 2009; Mangelsen et al., 2010). Further, importance of carbon to nitrogen ratio in senescence induction was revealed in the studies, where senescence specific genes such as SAG 12 were specifically induced in Arabidopsis grown on a glucose/low nitrogen medium compared to glucose/high nitrogen medium (Schmid et al. 2005; Pourtau et al. 2006). In the present study, accumulation of soluble sugars accompanied by an increased remobilisation of leaf nitrogen under stress resulted in an increased leaf carbon to nitrogen ratio in the remobilizing genotype compared to the other two. A significantly increased CN ratio especially during the early stages of drought treatment in the remobilizing genotype combined with a relatively higher expression of the various SAG 12 transcripts (Supplemental Figure S2) indicates an early induction of senescence in this particular genotype compared to other two. Interestingly, although the genotype LP 110 has shown several signs of senescence under drought with various physiological parameters, C/N ratio was very similar to the stay green as leaf nitrogen status was maintained even under drought condition.

We have also used glycine to serine ratio to characterise the extent of senescence in these genotypes. Glycine and serine are the two intermediates of photorespiration and higher Gly/Ser ratio is used as an indicator of both increased photorespiration as well as induction of

senescence process (Diaz et al., 2005). Diaz et al. (2005) in a study to determine the markers and to characterise the extent of senescence using five RILs of the Bay-0 and Shahdara population of Arabidopsis having differences in senescence phenotype showed that early senescing genotypes were characterised by a higher accumulation of Gly/Ser ratio compared to the late senescing ones. Similarly, in the present study also, the two senescing genotypes were characterised by a higher Gly/Ser ratio compared to stay green; among the senescing genotypes, Gly/Ser ratio was higher in the remobilising genotype compared to LP 110 especially at 12 DAF which could be related to the early induction of senescence in this genotype compared to LP 110. The reduction in Gly/Ser under stress at 16 DAF in both the senescing genotypes however could not be explained.

4.2.4 Senescence induced remobilisation of leaf nitrogen under terminal drought

In monocarpic crops like barley, majority of the nitrogen required for plant development is acquired before anthesis and stored in vegetative tissues mainly in the leaf. About 80-90% of the grain nitrogen in these crops is acquired through the remobilisation from the vegetative tissue which is closely associated to the process of plant senescence (Peoples & Dalling, 1988, Uauy *et al*., 2006a, b; Wingler et al., 2010). One of the reasons for higher nitrogen remobilisation observed in crops grown under Mediterranean condition is attributed to the whole plant senescence which enhances the nitrogen remobilisation from the vegetative tissues when the crops face drought during grain filling period (Palta et al., 1986). A higher nitrogen remobilisation observed in all three genotypes under drought confirms our results with the earlier mentioned finding that water stress at grain filling period enhances nitrogen remobilisation.

Among the genotypes, a higher nitrogen remobilisation observed under stress in the remobilizing genotype could be attributed to the drought induced early senescence in this genotype. In addition, this genotype was also characterised by a higher nitrogen remobilisation under control condition that could be explained by the early flowering of this genotype by 2-3 days compared to other two (data not shown). The importance of flowering time in senescence induced remobilisation of leaf nitrogen is evident from the studies in barley, where the low GPC germplasms were characterized by delayed senescence as result

of late flowering compared to high GPC germplasm which flowered earlier by 3-4 days under control conditions (Jukanti and Fischer, 2008a, b; Parrot et al., 2012). Another notable feature with respect to senescence induced nitrogen remobilisation in the present study is that, although the genotype LP 110 also exhibited several senescing features under drought, enhanced nitrogen remobilisation was not evident in this genotype and it behaved similar to stay green. Hence, in addition to senescence there could be other factors influencing nitrogen remobilisation in barley or it could be genotype specific.

Chloroplast represents the major site of reduced leaf nitrogen, which is predominantly constituted by RuBisco followed by various pigment protein complexes (Peoples and Dalling 1988, Hörtensteiner and Feller, 2002). Hence, remobilization of nitrogen essentially includes the degradation of these chloroplast proteins to various transportable forms of nitrogen (Krupinska and Humbeck 2004; Feller et al., 2008). Different classes of proteases activated during senescence ensure that various leaf proteins are degraded into amino acids which are eventually transported to the deveopling grains. The carbon skeletons required for the synthesis of various amino acids are derived from metabolic intermediates of respiration including both glycolysis and TCA cycle (Huppe and Turpin, 1994; Scheible et al., 1997). Hence, a general increase in the transcripts involved in glycolysis and TCA cycle especially in the senescing line could be seen as the source of carbon skeleton required for amino acid synthesis. Further, channeling of sugars primarily to respiration is evident from the down-regulation F2KP (Fructose 2- kinase phosphatase) and Fructose 1, 6-BPase which are the major regulators of sugar metabolism in plants leading to the synthesis of sucrose or the channeling of sugars to glycolysis (Huber, 1986; Stitt, 1990; Nielsen et al., 2004).

A significant reduction in total free amino acid content at 12 DAF under stress in LP 104 compared to other two could be an indication of drought induced early/faster mobilization of amino acids to the developing grain as a result of early senescence induction of senescence in this genotype. A similar observation was also made by Jukanti et al. (2008) a where a reduction in leaf free amino acid content in the senescing line compared to stay green was observed as a resultant of mobilization of leaf nitrogen to the developing grain. The major transported amino acids in barley and wheat are glutamate, followed by varying levels of aspartate, glutamine, threonine and serine (Winter et al. 1992; Caputo et al. 2001; Kichey et

al. 2006). A similar trend as that of total leaf amino acid content was evident in all these major transported amino acids across the genotypes.

Up-regulation of the various senescence induced protease family genes and transcripts of amino acid transporters preferentially in the remobilizing genotype compared to other two (Supplemental Figure S2) might support the idea that early induction of senescence in the remobilizing genotype has resulted in the faster remobilisation of leaf nitrogen in this genotype under drought. Characterization of Gpc (Grain protein concentration) locus in wheat and a study with NAM-B1 antisense lines in wheat showing delayed senescence with reduced grain mineral (N, Fe, Zn) content in the transgenic plants (Uauy et al., 2006a, b; Waters et al., 2009) elucidates the importance of senescence induced remobilization of not alone nitrogen but also other nutrients. From these perspectives, it is clear that the genotype LP 104 has the advantage of early induction of senescence compared to other two genotypes under both control and terminal drought.

4.2.5 Senescence induced remobilisation of stem reserves under terminal drought

In cereal crops, carbon assimilated during vegetative and early reproductive stages is stored in the stem (also leaf sheath) as carbohydrate reserves, which are remobilized during grain filling. At peak of their content, which is usually a few days after anthesis, these stored reserves, mainly composed of fructans in barley, constitute a significant portion of the stem weight (Slafer and Savin, 1994). Hence, stem weight at anthesis has often been considered as an indirect measure for screening genotypes tolerant to terminal drought (Pierre et al., 2010). Further, a significant negative correlation observed between the stem weight or WSC at maturity and percentage reduction in grain weight in a number of studies under terminal drought reinstates the importance of stem reserves for grain filling under terminal drought (Pierre et al., 2010).

In the present study, a significant reduction in stem weight from the time of anthesis (8DAF) to maturity in the remobilizing genotype under drought indicates the efficient utilization of stored stem assimilates to grain filling. Whereas in the stay green plants, stem weight at maturity under stress was significantly increased compared to 8DAF indicating hindered stem remobilisation and/or a continued deposition of assimilates to stem as a result of reduced sink strength. Further, even under control condition, stem weight at maturity had a

decreasing trend in the senescing genotype as opposed to stay green, when compared to 8DAF. This could probably be due to the early flowering nature (2-3 days, data not shown) of the remobilising genotype which was also evident in leaf nitrogen remobilisation and seed filling (discussed later) characters of this genotype under control condition.

As water soluble carbohydrates constitute a significant portion of the stem weight, results from the WSC measurement was in good agreement with the observed differences in stem weight at maturity between the genotypes indicating that stem remobilisation was enhanced by drought in the remobilsing genotype. Both genotypes had their peak of WSC at 12 DAF which might also coincide with stem weight; hence, for future studies in barley with respect to stem remobilisation, 12 DAF could be considered for the maximum stem weight rather than 8 DAF used in the present study. However, this will not affect the conclusion of the present study that under terminal drought, the genotype, LP 104 was better able to remobilize the stem reserves for grain filling compared to stay green which is evident from the results of isotope labeling and harvest index (discussed in a later section). An important observation from the present study was the poor performance of stay green genotype in spite of higher amount of water soluble carbohydrates in stem compared to the senescing one under terminal drought.

This is in agreement with some of the previous studies showing inconsistent relationship between yield and stem WSC at flowering under terminal drought (Evans & Wardlaw, 1996; Ehdaie *et al*., 2006; Ruuska *et al*., 2006). This was also evident in a study by Zhang et al (2009) correlating the expression of 1-FEH and stem remobilisation within the two wheat varieties Kauz and westonia under terminal drought. The genotype Kauz, having a higher WSC content and concentration with accelerated senescence compared to the Westonia was more susceptible to terminal drought with a higher reduction in TGW compared to later reinforcing the observation that amount of stem WSC at anthesis alone cannot be a good indicator of terminal drought tolerance. In agreement with the above results that the senescing genotype was better able to utilize the stem reserves for seed filling, HI (harvest index) was better in the senescing genotype both under control and stress conditions. Similarly, stable isotope labeling using $^{13}CO_2$ for studying stem remobilisation showed that drought enhanced the ^{13}C remobilization from the stem over its control in the remobilizing genotype whereas in the stay green, it was inhibited by drought.

Ear length is an important secondary trait often implicated in tolerance to terminal drought. Greater heat tolerance of awn to photosynthesis, higher tissue water potential, and greater availability of ear assimilates to grain compared to leaf are some of the characters that make ear assimilation an important component of grain yield especially under drought (Blum, 1980; Morgan 1980). Hence, a significantly higher ear length in the senescing genotype compared to stay green could have contributed to its better performance under terminal drought, although its contribution to seed filling was not ascertained in the present study.

4.2.6 Senescence induced seed filling under terminal drought

Grain size, a major determinant of cereal yield is determined by rate of dry matter accumulation and grain filling duration. Reduction in grain yield observed under terminal drought is mainly due to the reduction in TGW that results from both reduced source and sink activities and reduced grain filling duration. Rapidly decreasing seed moisture content and higher starch accumulation in the senescing genotype under drought indicate that drought has significantly enhanced seed maturation and seed filling in the senescing genotype compared to stay green. TGW obtained from the matured plants after physiological maturity indicated that the senescing genotype was better able to maintain the grain weight and starch content under drought in spite of reduced grain filling duration compared to stay green. In accordance with the TGW data, AGPase and SuSY activities were always high in the senescing genotype under both control and stress conditions compared to stay green.

Activities of both AGPase and SuSy were enhanced by drought especially during the initial stages (12 DAF), which could be correlated to the starch accumulation pattern in these genotypes. However, during the subsequent stages, a significantly reduced AGPase acivity compared to the relatively non responsiveness of SuSy activity indicates that AGPase could be the major limiting factor influencing sink activity and hence seed weight under stress. This is in accordance with other studies, where SuSy activity was little affected by drought while AGPase was more susceptible leading to a reduced seed weight under drought (Caley *et al.,* 1990; Dorion *et al.,* 1996; Sheoran and Saini 1996; Geigenberger *et al.,* 1997). In short, results from the seed filling characters from the present study revealed that the senescing genotype was better able to maintain the seed weight in spite of reduced photosynthesis under drought. A significantly enhanced remobilisation of stem reserves in

the senescing genotype coupled with better seed filling under drought clearly signifies the importance of stem reserves for seed filling under terminal drought and the importance of senescence induction in mediating these processes (Brocklehurst, 1977; Nocolas and Turner, 1992; Yang et al, 2003b).

4.2.7 Senescence induced nitrogen remobilisation and grain nitrogen content

Grain filling in barley essentially means the accumulation of starch and protein which are the major components of matured barley grain. In general, about 70 to 90% of the grain nitrogen at maturity is obtained through the process of remobilization from the vegetative tissues which are acquired before anthesis. On the other hand, starch accumulation is primarily determined by the post anthesis assimilation of CO_2. Hence, under situations like post anthesis drought or heat stress, starch accumulation is more sensitive compared to nitrogen accumulation (Palta et al., 1994; Triboi et al., 2001). Therefore, the observed increase in grain protein concentration under post anthesis drought is primarily due to the reduction in starch content although grain protein content per se may not increase (Morgan and Riggs, 1981; Brooks et al., 1982).

In the present study, two genotypes exhibited contrasting response in terms of nitrogen accumulation under post anthesis drought. Except at 25 DAF, grain N% under drought was significantly increased in the stay green, while it was reduced in the senescing genotype over control. However, the nitrogen content per seed was reduced in both genotypes and was more severe in the stay green. The differences in grain N% accumulation between the genotypes are in good agreement with the results obtained with starch accumulation and stem remobilization in these two genotypes. A significantly enhanced N% in the stay green under stress could be due to a corresponding decrease in starch accumulation resulting from a reduced photosynthesis.

On the other hand, decrease in photosynthesis in the senescing genotype was offset by an increased remobilisation of stem reserves contributing to higher accumulation of starch and possibly diluting the N% resulting in reduced N% under stress compared to control. Importance of senescence induced remobilisation for maintaining grain protein content is evident from the identification of GPC locus in wheat and barley that codes for a NAC transcription factor, NAM-B1. The stay green genotype with low GPC is characterized by

presence of a non-functional allele at the GPC locus as opposed to a functional form in the senescing genotypes with high protein content (Uauy et al., 2006). Although in the present study, expression analysis of NAM genes have not been carried out, a significantly enhanced leaf nitrogen remobilisation coupled with a higher grain nitrogen content in the remobilising genotype compared to stay green indicates the importance of senescence induced remobilisation of nitrogen to the developing grains in barley. Transgenic wheat with antisense NAM in wheat (RNAi-NAM) further revealed that, senescence induced remobilisation is important not just for nitrogen, but also for other important minerals such as Fe and Zn (Uauy et al., 2006b; Waters et al., 2009

CONCLUSION FIGURE

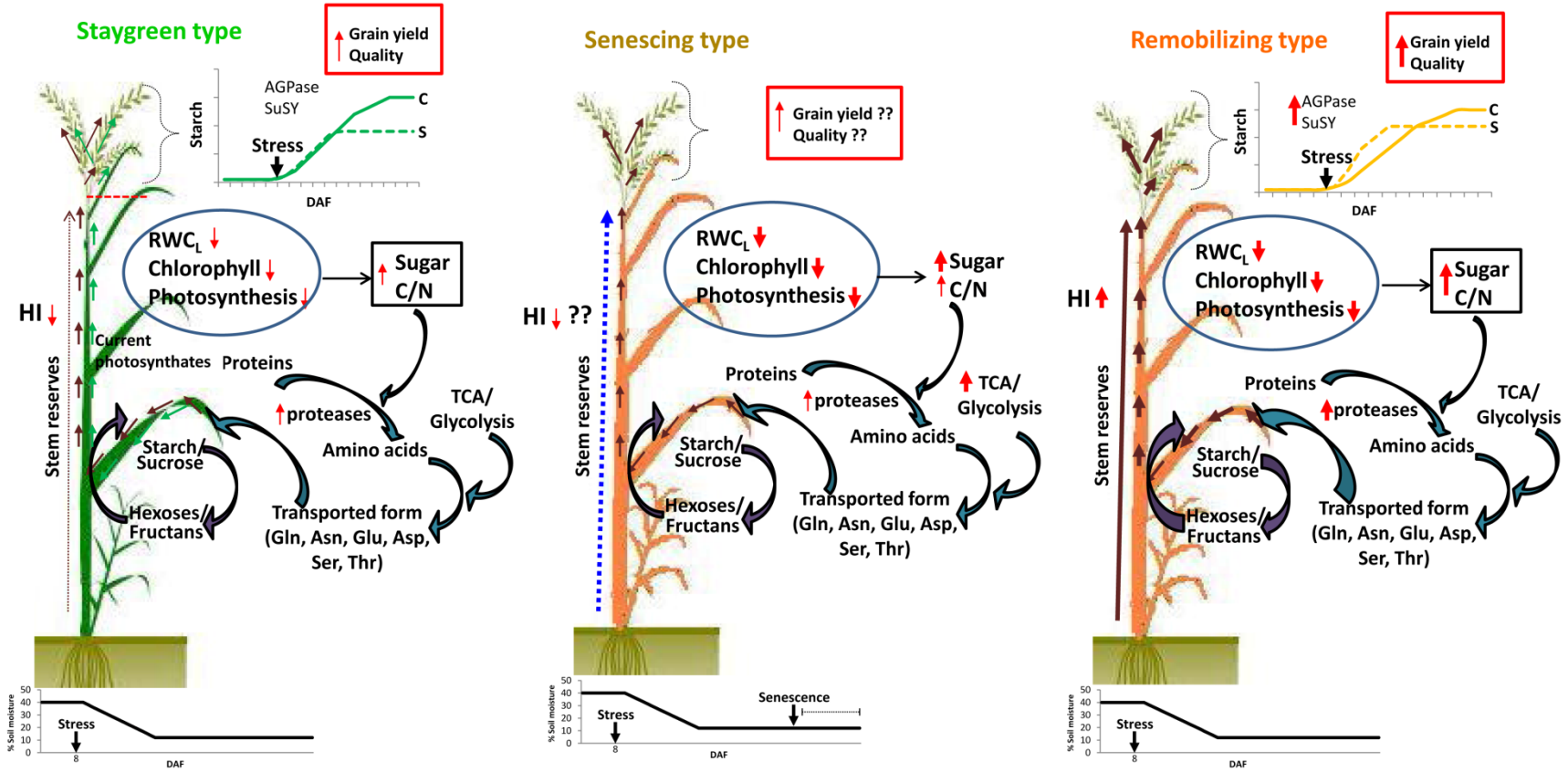


Figure depicts the responses of three selected genotypes to terminal drought where in the remobilizing genotype has outperformed both staygreen and senescing genotype in terms of seed yield and quality. Intensity of a process is indicated by thickness of an arrow. Drought induced early senescence in the remobilizing genotype which is evident from a severe reduction in physiological parameters, a higher accumulation of sugar and a high C/N ratio. This resulted in better mobilization of leaf nitrogen and stem reserves to developing seed which had higher sink strength. Finally, seed yield and quality were better in the remobilizing genotype compared to other two. Major difference between remobilizing and senescing genotype was in terms of C/N ratio and proteases induction which indicates that senescence might be initiated late in this genotype compared to remobilizing. Red dotted line in staygreen genotype indicates that current assimilate was not efficiently used in seed filling as sink strength was found to be low which also resulted in increased stem weight and hence low harvest index. Stem reserves in senescing genotype is indicated in dotted blue and was not ascertained in the present study. However, it is assumed that senescing genotype might perform inferior to remobilizing as late senescence may not be advantageous under severe terminal drought.

5 SUMMARY

Terminal drought is one of the major constraints for barley production that affects both seed yield and quality. The major objective of the present investigation was to understand the mechanism of terminal drought tolerance using two complementary approaches. Firstly, an AB-QTL was employed to identify exotic regions influencing terminal drought tolerance using an introgression line population (BC_3-DH) developed between the cultivated parent Brenda and a wild accession, Hs 584. Secondly, an integrated omics approach was used to understand the importance of stay green/senescence mechanisms of terminal drought tolerance in barley using elite breeding lines. The following results were achieved.

- Wild barley is a valuable source for various agronomic traits including seed quality and resistance to both biotic and abiotic factors. Identification of drought specific yield QTL (qYLD3.1) and control specific TGW QTLs (qTGW3.1 and qTGW3.2) contributed by the wild accession Hs 584, illustrates the potential of wild barley for varietal improvement in breeding for improved varieties for optimal and drought conditions. Although in general, introgression of the exotic segments into the cultivated background caused yield reduction. Interestingly, QTLs such as qTGW3.2 from the wild parent were also identified in two other studies (Talame et al., 2004; Teulat et al., 2001) using different populations grown under Mediterranean conditions. In addition, the present study highlights the importance of the QTLs, qYLD2.1 and qTGW3.2 which are also found to correlate to yield in other studies using different populations.
- Collocation of QTLs for drought tolerance index of yield and TGW (qDTI-Y and qDTI-TGW) respectively with yield and/or TGW under control condition suggests that yield of barley under drought is primarily determined by the genetic potential of the crop and less by the environment. All QTLs for malting qualities such as seed starch and grain N% were derived from the cultivated parent and wild accession, respectively. Among various QTLs for seed N%, qSN2.1 is particularly interesting as it was reported in two previous studies of AB-QTL analysis using different population (Li et al., 2005; von Korff et al., 2008). This emphasizes the importance of this particular wild species contributed QTL in determining seed quality and its

potential for incorporation into various breeding programmes for the increase in quality of barley especially when used as feed. In addition, the present study also focused on the identification of QTLs for seed morphological characters such as length, breadth and shape which was mostly neglected in previous studies in barley.

- A major observation from the present QTL analysis is the collocation of a large number of traits at various regions in the genome. Further development of the present population would help to resolve the nature of these QTLs (linkage or pleiotropic) and to verify some of the possible candidate genes identified in the present study. In addition, this could also lead to the development of NILs, which are better adapted to terminal drought in terms of seed yield and quality.

- With regard to stay green and senescence components of terminal drought, the present study revealed that senescence induced remobilization of stem reserves is an important component of seed filling under terminal drought. The better performing remobilizing genotype, LP 104 was characterized by an early drought induced senescence induction which was evident from the down regulation of various transcripts related to photosynthesis and accumulation of various sugars. Upregulation of various transcripts related to invertases together with the induction of various senescence specific genes such as SAG 12 in the remobilizing genotype under drought indicated the role of sugar accumulation in senescence induction especially under conditions of low plant nitrogen status. Senescence induction in the remobilizing genotype seems to have triggered an early/higher remobilisation of nitrogen and stem reserves resulting in better maintenance of the seed yield and quality compared to the stay green genotype. In addition to increased nutrient remobilisation, senescence also seems to posseess positive effect on the sink strength, which is evident from the higher activities of both AGPase and Susy in the remobilizing genotype compared to stay green. However, the role of senescence in altering the sink strength has to be explored in detail to understand the components of senescence involved in altering the sink strength.

- The present study also revealed that osmolite accumulation has little advantages in maintaining plant water status under drought. This was evident in senescing

genotypes which accumulated more proline that did, however, not correlate with its leaf water status. Another important observation from the present study was the poor yield of the stay green genotype, in spite of having higher amounts of stem WSC at anthesis. These results suggest that, osmolite accumulation such as proline and stem WSC at anthesis cannot alone be used as secondary trait for screening for drought tolerance in barley.

- Importance of sink metabolism to terminal drought tolerance is evident from the identification of the starch branching enzyme and sucrose synthase as possible candidates underlying a major seed starch content QTL (qSS2.1 or qHS2.1) by AB-QTL analysis.
- The present study also highlights the importance of genetic potential for yield in determining the terminal drought tolerance in barley. The better performance of the senescing genotype, LP 104, in different yield trials conducted under natural conditions and collocation of various drought tolerance QTLs for yield and TGW (DTI-Y and DTI-TGW) with corresponding traits (yield and TGW), highlights the importance of genetic potential of a crop in determining its drought tolerance. Further, identification of gene/genes underlying the major TGW QTL obtained from the wild barley (qTGW3.2) could be an interesting option to further improve the genetic potential of the breeding line LP 104 for its yield performance.
- Although the present investigation reveals the importance of senescence induced remobilization as a major component of terminal drought tolerance, the advantage of stay green mechanism would be evident when drought occurs during pre-anthesis, where crop yield components are mainly determined by the availability of current assimilates.

7 CONCLUSION

- Under severe terminal drought, stored stem reserves is crucial to seed filling in barley that can outweigh the loss of current photosynthesis.

- The better yield performance of the remobilizing genotype was also characterised by a higher sink activity, indicating that apart from assimilate availability, sink activity is important in crop yield under terminal drought.

- In addition to yield, the better seed quality of the remobilizing genotype in terms of seed N content indicates that senescence induced remobilization is important for both yield and seed quality under terminal drought.

- Contribution of QTLs for yield, thousand grain weight and other traits by the wild genotype under both control and drought conditions further strengthens the importance of wild barley in crop improvement.

- A significantly increased seed N% relative to carbon in the inferior genotypes of introgression line population and the drought susceptible stay green genotype compared to better performing genotypes indicates that C/N ratio could be a useful indicator to screen for seed yield and quality under terminal drought along with other parameters.

- Finally, the present study highlights the importance of yield potential of a crop in determining yield under terminal drought. Hence, future breeding programmes for terminal drought could be targeted towards the general improvement in yield potential of a genotype.

8 Zusammenfassung

Zusammenfassung

Trockenheit ist eine der Hauptbeschränkungen der Produktivität bei Gerste und beeinträchtigt Samenertrag und –qualität. Das Hauptziel der vorliegenden Arbeit war die Mechanismen der terminalen Trockentoleranz unter Verwendung zweier komplementärer Ansätze zu verstehen. Zunächst wurde ein AB-QTL-Ansatz angewendet, um eingebrachte Regionen einer Wildgerste zu identifizieren, die die terminale Trockentoleranz beeinflussen. Dazu wurde eine Population von Introgressionslinien (BC3-DH) verwendet, die aus der Kreuzung zwischen dem Gerstenkultivar Brenda und einer Gersten-Wildform (Hs 584) entwickelt wurden. Zweitens wurde eine integrierter omics-Ansatz verwendet, um die Wichtigkeit von „Stay-green-“ / Seneszenzmechanismen in Gerste unter terminaler Trockenheit zu verstehen. In diesem Ansatz wurden Elite-Züchtungslinien untersucht. Folgende Ergebnisse wurden erzielt:

- Wildgerste ist eine wertvolle Quelle für verschiedene agronomische Merkmale, wie z.B. Samenqualität und Resistenz gegenüber biotischen und abiotischen Stressfaktoren, darunter Trockenheit. Die Identifizierung von Ertrags-QTLs spezifisch für Trockenheit (qYLD3.1) und spezifisch für Kontrollbedingungen (qTGW3.1 und qTGW3.2), die durch die Wildgerste Hs 584 beigesteuert wurden, illustrieren das Potenzial der Wildgerste in der Züchtung, um verbesserte Sorten bei optimalen sowie Trockenstress-Bedingungen zu erhalten. Obwohl im Allgemeinen die Einkreuzung von Segmenten einer Wildform in den genetischen Hintergrund einer kultivierten Sorte zu Ertragsverlusten führte. Interessanterweise, wurden QTLs wie z.B. qTGW3.2 aus der Wildform auch in zwei anderen Studien identifiziert (Talame et al., 2004; Teulat et al., 2001), bei denen verschiedene Populationen untersucht wurden, die unter mediterranen Bedingungen angezogen wurden. Zusätzlich hebt die vorliegende Studie die Wichtigkeit der QTLs qYLD2.1 und qTGW3.2 hervor, die auch in anderen Arbeiten mit unterschiedlichen Populationen in Bezug auf ihre Korrelation zum Ertrag identifiziert wurden.
- Die Kollokation von QTLs für Trockentoleranzindex von Ertrag bzw. TGW (qDTI-Y bzw. qDTI-TGW) mit solchen für Ertrag und / oder TGW unter Kontrollbedingungen deutet darauf hin, dass der Ertrag von Gerste unter Trockenstress vorrangig durch das

genetische Potenzial des Getreides und weniger durch die Umwelt bestimmt wird. Alle QTLs der Brauqualität wie z.B. Stärke- bzw. Stickstoffgehalt der Samen stammten von der Kultur- bzw. die Wildform als Elternteil. Unter verschiedenen QTLs für Samen-N-Gehalt, ist qSN2.1 besonders interessant, da dieser QTL bereits in zwei vorangegangenen AB-QTL-Studien mit einer anderen Population beschrieben wurde (Li et al., 2005; Korff et al., 2008). Dies unterstreicht die Wichtigkeit des durch diese spezielle Wildgerste eingebrachten QTLs in der Beeinflussung der Samenqualität. Weiterhin hat dieser QTL das Potenzial in verschiedene Züchtungsprogramme zur Erhöhung der Qualität der Gerste integriert zu werden, insbesondere wenn diese als Futterpflanze verwendet wird. Zusätzlich konzentrierte sich die vorliegende Arbeit auf die Identifizierung von QTLs für Samenmorphologie wie z.B. Länge, Breite und Form der Körner, was in bisherigen Studien mit Gerste meist vernachlässigt wurde.

- Eine Hauptbeobachtung der vorliegenden QTL-Analyse ist die Kollokation einer großen Anzahl an Merkmalen in verschieden Bereichen im Genom. Eine Weiterentwicklung der jetzigen Population in fortgeschrittene Generationen würde helfen, die Natur dieser QTLs (Kopplung oder pleiotropisch) zu klären und einige der möglichen Kandidatengene zu verifizieren, die in dieser Arbeit identifiziert wurden. Zusätzlich könnte dies auch zur Entwicklung von NILs führen, die in Bezug auf Samenertrag und –qualität besser an Trockenstress angepasst sind.
- In Bezug auf „stay-green“ und Seneszenzkomponenten der terminalen Trockenheit wurde mit dieser Arbeit dargelegt, dass seneszenz-induzierte Remobilisierung von Stängelreserven ein wichtiger Bestandteil der Samenfüllung unter Trockenheit ist. Der effizientere remobilisierende Genotyps LP 104 war durch eine vorzeitig durch Trockenheit induzierte Seneszenz charakterisiert, was aufgrund der Herabregulierung verschiedener Transkripte der Photosynthese und Zuckerakkumulation offenkundig wird. Die Hochregulierung von Transkripten der Invertase-Familie zusammen mit der Induktion von seneszenz-spezifischen Genen wie z.B. *SAG12* im remobilisierenden Genotyp unter Trockenstress deutet auf die Rolle der Zuckerakkumulation bei der Seneszenzinduktion hin, besonders unter Bedingungen mit geringem Stickstoffgehalt in den Pflanzen. Die Seneszenzinduktion scheint im remobilisierenden Genotyp eine

frühe/verstärkte Remobilisierung von Stickstoff und anderen Stängelreserven ausgelöst zu haben, was zu einem stabilen Ertrag und einer Erhaltung der Samenqualität verglichen mit dem „stay-green“ Genotyp führt. Zusätzlich zur erhöhten Nährstoffremobilisierung hat die Seneszenz möglicherweise auch einen positiven Effekt auf die Samen-sink-Stärke, was aus den höheren Aktivitäten von AGPase und Susy im remobilisierenden Genotyp verglichen mit „stay-green“ deutlich wird. Die Rolle der Seneszenz bei der Änderung der sink-Stärke muss jedoch detailliert untersucht werden, um die Komponenten der Seneszenz, die an der Veränderung der sink-Stärke beteiligt sind, besser zu verstehen.

- Die aktuelle Studie zeigte auch, dass die Osmolytakkumulation nur wenige Vorteile zur Aufrechterhaltung des Wasserstatus der Pflanze mit sich bringt. Dies wurde im seneszierenden Genotyp klar, der zwar mehr Prolin akkumulierte, was jedoch nicht mit seinem Wasserstatus korreliert werden konnte. Eine weitere wichtige Erkenntnis der vorliegenden Arbeit war der geringe Ertrag des „stay-green“ Genotyps, trotz größerer Mengen an Stängel-WSC zum Zeitpunkt der Blüte. Das deutet darauf hin, dass die Osmolytakkumulation wie z.B. Prolin und Stängel-WSC bei der Blüte nicht allein als sekundäres Merkmal für das Sreening nach Trockentoleranz bei Gerste herangezogen werden können.
- Die Bedeutung des sink-Stoffwechsels hinsichtlich terminaler Trockentoleranz wird deutlich aus der Identifizierung des Stärkeverzweigungsenzyms (starch branching enzyme) und der Saccharose-Synthase als mögliche Kandidaten, die einem Haupt-QTL des Samenstärkegehalts unterliegen (qSS2.1 oder qHS2.1), welcher innerhalb der AB-QTL-Analyse identifiziert wurde.
- Die vorliegende Arbeit hebt auch die Wichtigkeit des genetischen Potenzials für den Ertrag hervor, wodurch die terminale Trockentoleranz bei Gerste bestimmt wird. Die bessere Leistung des seneszierenden Genotyps LP 104 in verschiedenen Ertragsversuchen unter natürlichen Bedingungen und die Kollokation verschiedener Trockentoleranz-QTLs für Ertrag und TGW (DTI-Y und DTI-TGW) mit den entsprechenden Merkmalen (Ertrag und TGW) hebt die Wichtigkeit des genetischen Potenzials eines Getreides bei der Bestimmung seiner Trockentoleranz hervor. Weiterhin wäre die Identifizierung von Genen, die dem Haupt-QTL TGW

(qTGW3.2, aus der Wildgerste erhalten) unterliegen, ein interessante Möglichkeit, das genetische Potenzial der Züchtungslinie LP 104 hinsichtlich ihres Ertrags weiter zu verbessern.

- Obwohl die vorliegende Untersuchung die Bedeutung der seneszenz-induzierten Remobilisierung als wichtigen Teil der terminalen Trockentoleranz hervorhebt, würde sich der Vorteil des „stay-green“ Mechanismus eher auswirken, wenn Trockenstress vor der Blüte stattfindet. Zu diesem Zeitpunkt werden Ertragskomponenten von Getreiden hauptsächlich durch die Verfügbarkeit von momentanen Assimilaten bestimmt.

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10 SUPPLEMENTARY FIGURES

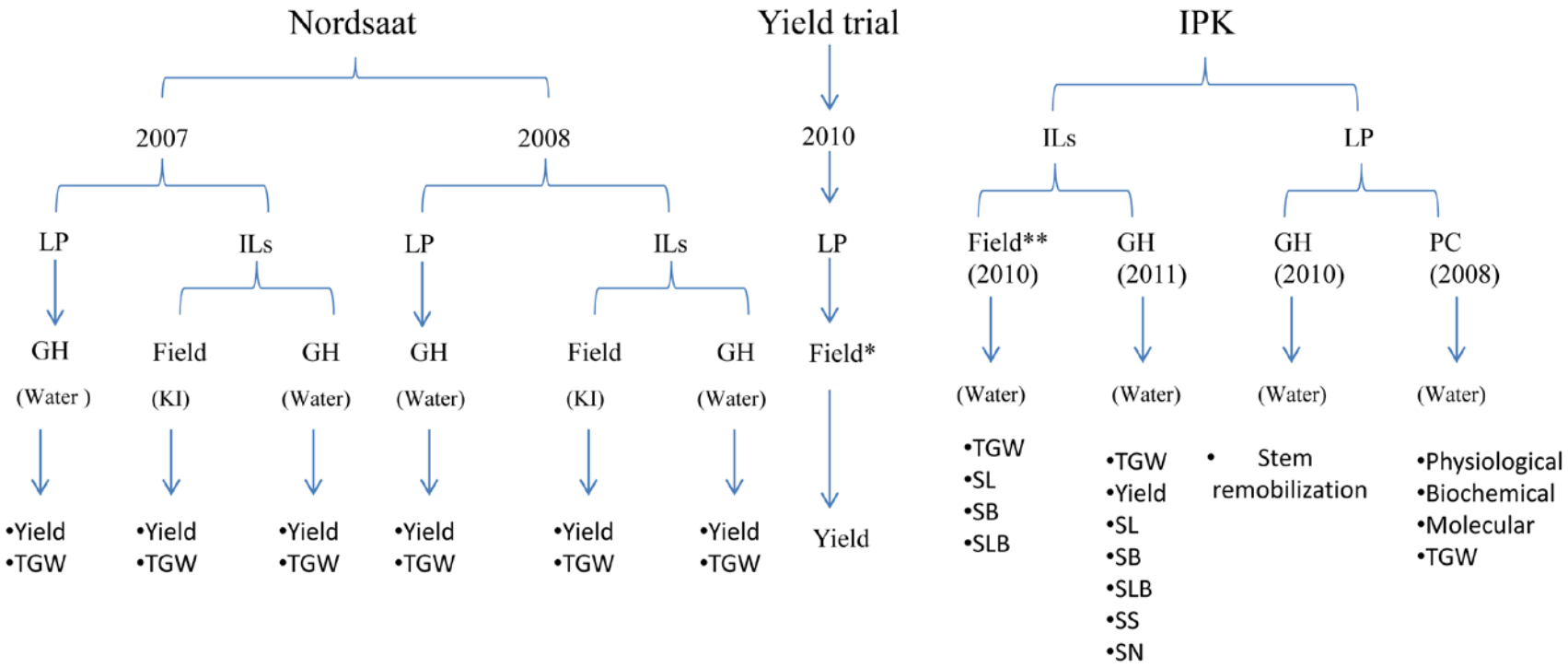

Figure S1. Details of the various growing carried out during the study.
Experiments were carried out mainly at two locations, Nordsaat and IPK in Germany during different years. Terminal drought was imposed by either withholding water or spraying with 0.4% potassium iodide at 10 DAF, and is indicated in brackets below each environment in Nordsaat experiments. At IPK, all drought experiments were carried out by withholding water. Various traits scored in each environment are given under the respective environments. Abbreviations- LP- Lochow-Petkus genotypes, IL- introgression lines,GH- green house, PC- phytochamber, KI- potassium iodide, TGW- thousand grain weight, SL- seed length, SB- seed breadth, SLB- seedlength to breadth ratio, S
S- seed starch content, SN- seed nitrogen content.
** this experiment was carried out under control condition only.
*this experiment was carried out using selected contrasting LP genotypes under natural conditions at three locations in Germany, namely, Granskevitz, Gudow and Bohnsausen.

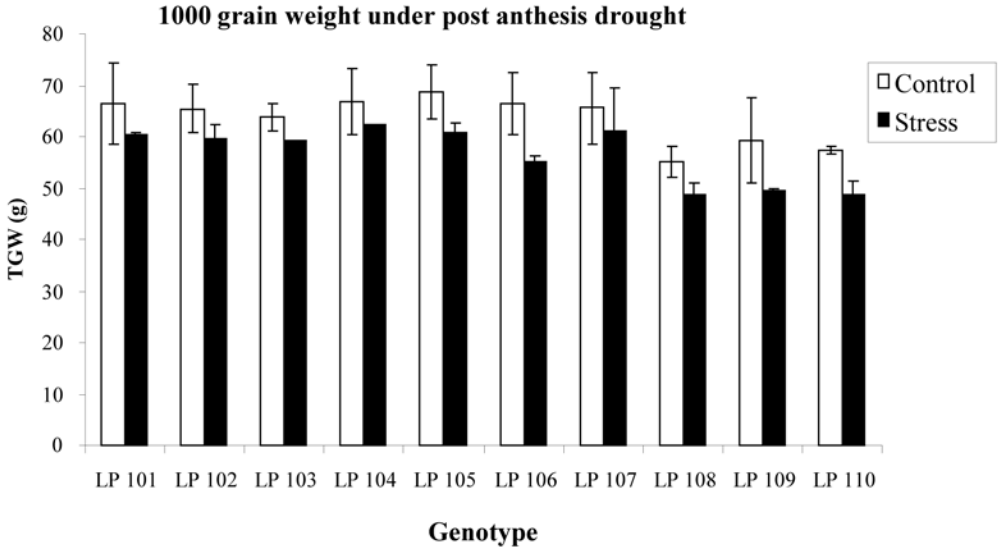

Figure S2. Reduction in 1000 grain weight under post anthesis drought.
Plants were grown under field conditions at Nordstadt, Germany and drought stress was imposed 8 DAF. Each value is an average of two independent experiments carried out in 2007 and 2008 and error bars represent ± S.D. TGW, Thousand grain weight.

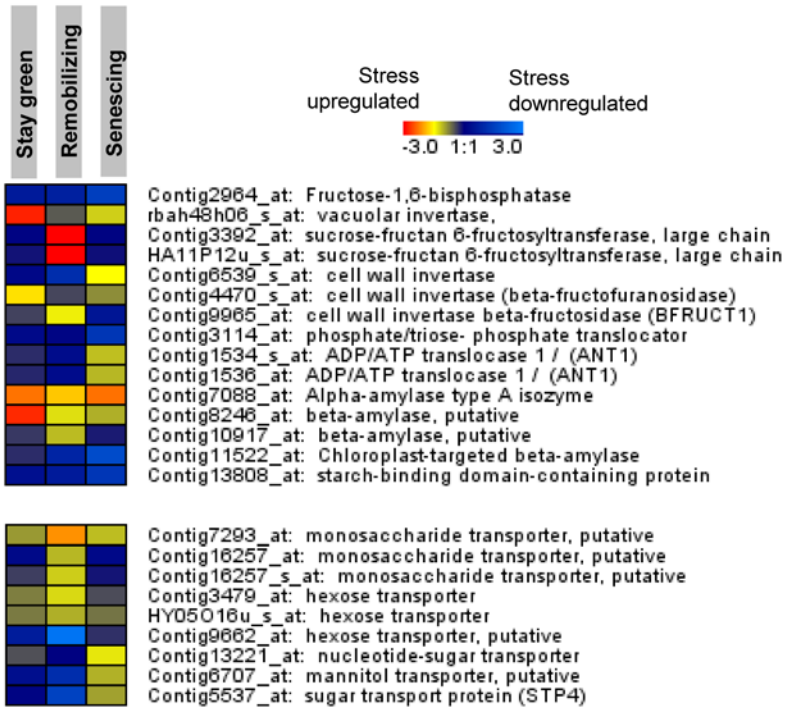

Figure S4. Drought induced differential expression of transcripts involved in starch degradation, fructan synthesis and sugar transporters in the selected contrasting genotypes.

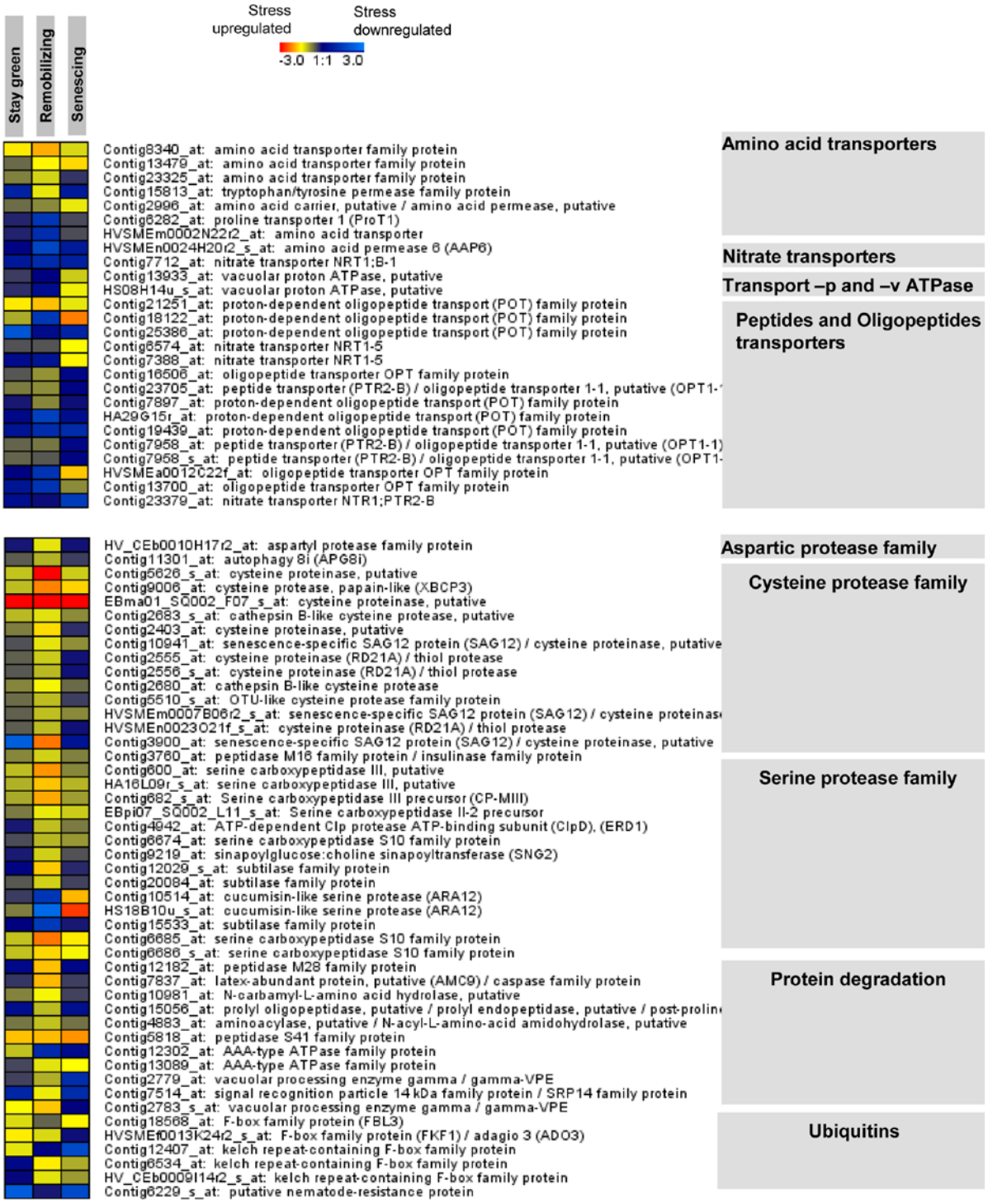

Figure S2. Differential expression of transcripts involved in nitrogen transport and various protease family under drought.

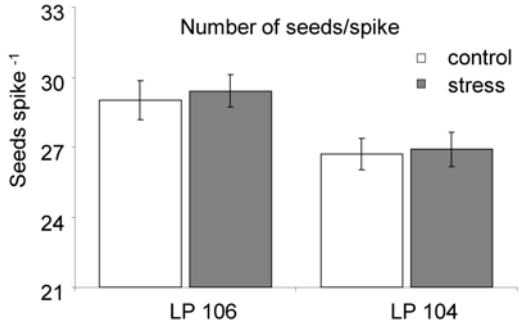

Figure S5. Seed number was little affected by terminal drought in both genotypes

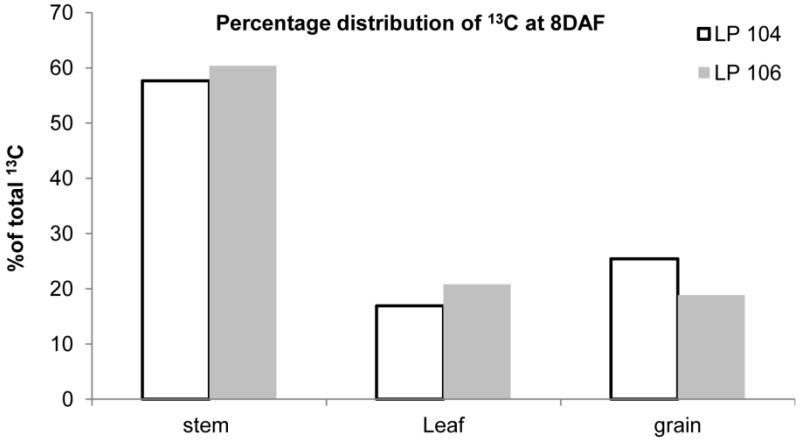

Figure S6: Percentage distribution of 13C at 8DAFin the contrasting genotypes. After isotope labeling at 5 days before anthesis, samples were collected 8 DAF from the main stem and the percentage of total stem ^{13}C present in different plant organs were calculated.

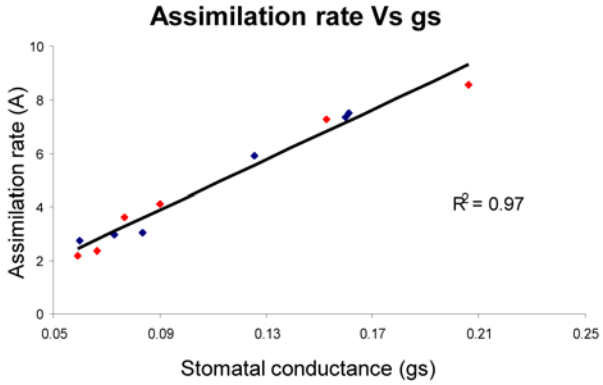

Figure S7: Stomatal control of photosynthesis under drought.
Correlation of stomatal conductance and photsynthesis at 12 and 16 DAF under drought in different barley genotypes. In addition to the selected contrasting genotypes of the present Study (marked in red), the figure also contains the data from 3 additional lines (Brenda, Hs 584 and LP 103, blue points).Each point is an average of 5 replications measured under drought and the correlation is significant at *** ($p<0.001$).

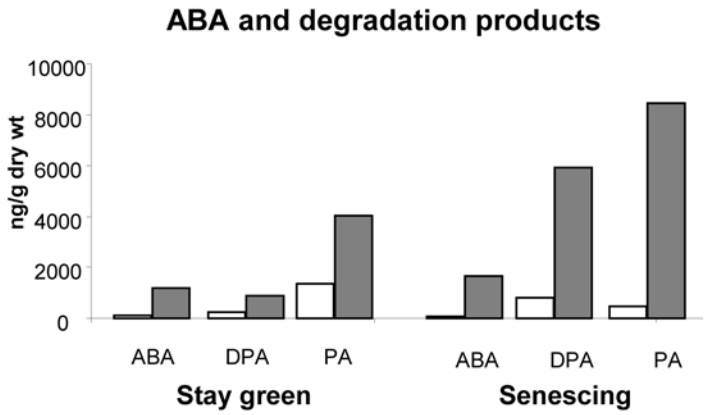

Figure S7: Higher accumulation of ABA degradation products in the senescing genotype. Drought induced accumulation of DPA and PA at 16 DAF were greatly enhanced in the senescing genotype over its control compared to stay green; although difference in ABA accumulation between the treatment was not that high between the genotypes compared to degradation products.

11 SUPPLEMENTARY TABLES

Table S1: Parameters for mass spectrometric analysis of metabolic intermediates coupled to ion chromatography. Filtered Masses and potentials of MS/MS transitions in negative mode. DP Declustering Potential, EP Entrance Potential, CE Collision Energy, CXP Collision Cell Exit Potential,
*Not the optimum potential to reduce sensitivity due to the high concentrations in seeds.

Compound	RT	Q1 mass	Q3 mass	DP	EP	CE	CXP
Lactate	2.5	89	43	-45	-10	-18	-1
Glycolate	2.5	75	47	-35	-10	-12	-7
Succinate semialdehyde	2.6	101	57	-45	-10	-14	-1
Pyruvate	2.9	87	87	-45	-10	-12	-5
Trehalose-6-phosphate	7.7	421	79	-85	-10	-60	-5
Glucose-1-phosphate	7.9	259	79	-60	-10	-42	-5
Succinate	12.8	117	73	-45	-10	-16	-1
Malate*	13.0	133	71	-80	-10	-22	-3
Glucose-6-phosphate	17.7	259	97	-50	-10	-22	-5
Fructose-6-phosphate	18.7	259	79	-55	-10	-60	-5
2-Oxoglutarate	18.2	145	101	-55	-10	-12	-17
Fumerate	19.2	115	71	-40	-10	-12	-11
AMP	20.6	346	79	-75	-10	-66	-5
Oxaloacetate	18.5	131	87	-20	-10	-10	-5
NADH	27.2	664	79	-100	-10	-116	-3
Citrate*	30.5	191	87	-70	-10	-24	-5
Isocitrate	31.7	191	73	-20	-10	-30	-5
UDP-Glucose	32.6	282	111	-35	-10	-16	-7
cis-Aconitate	31.8	173	85	-35	-10	-18	-15
Phosphoenolpyruvate	32.6	167	79	-35	-10	-14	-5
trans-Aconitate	32.6	173	85	-35	-10	-18	-15
ADP	33.4	426	79	-75	-10	-90	-5
Fructose-1,6-biphosphate	33.5	339	241	-50	-10	-20	-15
NADPH	34.7	371	79	-45	-10	-68	-13
UDP	34.4	403	79	-60	-10	-100	-5
ATP	34.6	506	159	-80	-10	-40	-29
UTP	35.2	483	79	-80	-10	-40	-9

Table S2: Parameters for mass spectrometric analysis of metabolic intermediates coupled to hydrophilic interaction chromatography (negative mode). Filtered Masses and potentials of MS/MS transitions. DP Declustering Potential, EP Entrance Potential, CE Collision Energy, CXP Collision Cell Exit Potential

Compound	RT	Q1 mass	Q3 mass	DP	EP	CE	CXP
cAMP	17.2	328	134	-70	-10	-35	-11
Glucose	11.6	179	89	-50	-10	-12	-10
Fructose	12.0	179	89	-55	-10	-12	-8
Stachyose	14.2	665	383	-120	-10	-46	-25
Sucrose	12.9	341	179	-180	-10	-18	-15
Raffinose	13.6	503	179	-100	-10	-30	-8
Citrulline	14.0	174	131	-35	-10	-16	-9
Verbascose	14.4	827	89	-135	-10	-100	-7
Asn	14.5	131	113	-50	-10	-14	-10
NAD	17.1	662	540	-35	-10	-24	-19
Ascorbate	18.0	175	115	-25	-10	-14	-7
Thiamine-di-P	22.4	423	302	-50	-10	-20	-7
UMP	22.6	323	79	-65	-10	-62	-5
Pentose-5-P	22.8	229	97	-50	-10	-16	-7
NADP	23.4	742	620	-50	-10	-24	-19
5-Methyl-THF	23.6	458	329	-75	-10	-34	-9
CDP	24.0	402	79	-55	-10	-92	-13
ADP-Glucose	25.2	588	79	-65	-10	-10	-7
CTP	27.4	482	158	-80	-10	-36	-9

Table S3: Parameters for mass spectrometric analysis of metabolic intermediates coupled to hydrophilic interaction chromatography (positive mode). Filtered Masses and potentials of MS/MS transitions. DP Declustering Potential, EP Entrance Potential, CE Collision Energy, CXP Collision Cell Exit Potential

Compound	RT	Q1 mass	Q3 mass	DP	EP	CE	CXP
Deoxyadenosine	6.5	252	136	66	10	25	10
S-Adenosyl-Met	13.4	399	250	66	10	23	18
S-Adeonosyl-Hcy	14.4	385	136	61	10	29	10
Ornithine	14.0	133	70	36	10	25	12
CMP	22.7	324	112	66	10	20	8
5-Methyl-THF	23.6	460	313	71	10	27	22
Homocysteine	13.5	269	105	66	10	21	10
Acetyl-CoA	26.8	810	303	111	10	39	24
Adenosine	9.3	268	136	50	10	25	10
GSSG	23.8	613	355	60	10	33	14
Trp	12.4	205	188	41	10	13	12
GSH	19.8	308	179	50	10	17	12
Asn	13.9	133	87	31	10	17	16
Arg	14.2	175	70	40	10	35	14
Lys	14,9	147	84	36	10	25	16
Ser	13.9	106	60	40	10	17	10
Thr	13.5	120	74	36	10	17	14
Phe	11.9	166	120	46	10	19	22
Ile	11.5	132	86	40	10	17	12
Met	12.3	150	133	30	10	13	10
Cys	13.5	122	76	35	10	20	10
Tyr	13.3	182	165	41	10	15	12
Val	12.1	118	72	41	10	15	12
Pro	12.6	116	70	46	10	20	6
Leu	11.1	132	86	36	10	15	10
Gln	13.5	147	84	36	10	25	10
Gly	13.7	76	76	41	10	10	10
His	14.0	156	110	41	10	21	12
Glu	17.5	148	84	41	10	23	14
Ala	12.9	90	44	41	10	25	8
GABA	13.8	104	87	31	10	15	14

Table S4: Descriptive statistics for the various traits scored under different environments.

		NS-2007				NS-2008				IPK		
		Field		Green House		Field		Green House		GH		Field
Traits	Statistics	control	stress	control	stress	control	stress	control	stress	control	stress	control
Yield	Brenda	36.8	26.2	50.3	41.1	787.5	360	97	76	33.9	19.4	-
	Hs 584	-	1.1	-	-	-	-	-	-	-	-	-
	DH mean	44.6	35.3	51.6	44.9	612.8	299	62.6	73	29.2	26.7	-
	SD	19.5	15.9	14.9	13.4	193	137	25	25.5	17.1	19	-
	CV%	43.8	45	28.9	29.8	31.5	45.8	39.9	34.9	58.6	71.2	-
	Minimum	8.2	9.7	5	13.2	58	22	2.7	5	1.7	1.5	-
	Maximum	105.5	75.4	86.5	77.5	956	666	118	118	71.6	71	-
DTI-Y	Brenda	0.5		0.8		0.8		1.8		0.8		-
	Hs 584	-		-		-		-		-		-
	DH mean	0.9		0.9		0.5		1.3		0.9		-
	SD	0.7		0.4		0.3		0.7		1		-
	CV%	79.5		44.2		60.5		56.1		104.3		-
	Minimum	0.2		0.2		0.1		0		0.1		-
	Maximum	3.4		2.2		1.1		3		3.5		-
TGW	Brenda	48.6	48.3	52.3	50	44.1	41	58.9	47.8	39	37.4	44.7
	Hs 584	-	-	-	-	-	-	-	-	-	-	-
	DH mean	47.4	43.5	53.1	48.2	44.8	43.7	58.4	48.2	33.9	33.7	45.3
	SD	4.8	5.2	4.3	4.1	3.1	4.2	7.7	6.2	5.4	7.5	10.2
	CV%	10.1	12	8.1	8.5	6.9	9.6	13.2	12.9	15.9	22.3	22.6
	Minimum	30.6	29.6	37.6	30.2	35.6	33.4	37.7	25.6	17.7	14.2	37.7
	Maximum	56.3	54.8	59.4	56.8	50.4	57.1	74.8	60.1	48.4	53	51.3
DTI-TGW	Brenda	1		0.9		0.9		0.8		1.3		-
	Hs 584	0.9		-		0.3		0.4		-		-
	DH mean	0.9		0.9		1		0.8		1		-
	SD	0.2		0.1		0.1		0.2		0.3		-
	CV%	18.1		12		14.7		21.9		30.9		-
	Minimum	0.6		0.6		0.7		0.3		0.4		-
	Maximum	1.3		1.1		1.4		1.2		2		-

At IPK field condition, only control experiment was carried out. In most cases, Hs584 could not be scored because of seed shattering.

Table S5: Descriptive statistics for seed morphological and quality traits from the experiments carried out at IPK.

		IPK		
		Green house		Field
Traits	Statistics	control	stress	control
Seed starch	Brenda	23.3	22.1	-
	Hs 584			-
	DH mean	22.8	20.7	-
	SD	5.6	6.3	-
	CV%	24.6	30.4	-
	Minimum	7.9	8.2	-
	Maximum	33.5	33.4	-
Seed nitrogen	Brenda	-	-	-
	Hs 584	-	-	-
	DH mean	1.7	2.3	-
	SD	0.3	0.5	-
	CV%	17.6	21.7	-
	Minimum	1.3	1.5	-
	Maximum	3	3.6	-
Seed breadth	Brenda	3.8	3.6	4.3
	Hs 584	-	-	3.3
	DH mean	3.6	3.5	4.1
	SD	0.1	0.2	0.1
	CV%	2.8	5.7	2.4
	Minimum	3.3	3.1	3.7
	Maximum	3.9	4.1	4.4
Seed length	Brenda	8.7	8.6	9.9
	Hs 584	-	-	12.1
	DH mean	8.9	9	10.4
	SD	0.5	0.5	0.4
	CV%	5.6	5.6	3.7
	Minimum	7.4	8	9.8
	Maximum	9.7	10.1	11.4
Seed L/B ratio	Brenda	2.5	2.4	2.3
	Hs 584	-	-	3.7
	DH mean	2.5	2.6	2.5
	SD	0.1	0.2	0.1
	CV%	4	7.7	5.5
	Minimum	2.1	2.1	2.3
	Maximum	2.5	2.9	3

In IPK field condition, only control experiment was carried out.

Table S6. Trait correlation for IPK_M_2008 (control condition only)

1_TGW_C_M_IPK				
2_SB_C_M_IPK	0.641***			
3_SL_C_M_IPK	-0.041	-0.226		
4_L/B_C_M_IPK	-0.43***	-0.76***	0.803***	

Abbreviations are TGW- Thousand grain weight, SB-Seed Breadth, SL- Seed Length, L/B- Length to Breadth ratio, C- control. Karl Pearson correlation significance at $p<0.05$, $p<0.01$ and $p<0.001$ are represented by *, ** and *** respectively.

Table S7. Correlation between yields from different environments

Environments	1	2	3	4	5	6	7	8	9	10
1_Y_C_F_07_NS										
2_Y_S_F_07_NS	0.642***									
3_Y_C_GH_07_NS	0.182	0.24*								
4_Y_S_GH_07_NS	0.257*	0.27*	0.488***							
5_Y_C_GH_IPK	-0.097	-0.203	0.25*	0.227						
6_Y_S_GH_IPK	-0.088	0.044	-0.19	-0.071	0.068					
7_Y_C_F_08_NS	0.189	0.349**	0.394**	0.317*	0.158	0.268*				
8_Y_S_F_08_NS	0.083	0.327**	0.318*	0.175	0.187	0.167	0.664***			
9_Y_C_GH_08_NS	0.074	0.296*	0.551***	0.264*	0.063	0.063	0.673***	0.671***		
10_Y_S_GH_08_NS	0.111	0.28*	0.594***	0.425***	0.199	0.064	0.673***	0.585***	0.811***	

Table S8. Correlation between thousand grain weight from different environments

Environments	1	2	3	4	5	6	7	8	9	10	11
1_TGW_C_F_07_NS											
2_TGW_S_F_07_NS	0.345**										
3_TGW_C_GH_07_NS	0.559***	0.379**									
4_TGW_S_GH_07_NS	0.567***	0.178	0.698***								
5_TGW_C_GH_IPK	0.256*	0.207	0.181	0.135							
6_TGW_S_GH_IPK	0.277*	0.097	0.294*	0.491***	0.204						
7_TGW_C_F_08_NS	0.391**	0.185	0.453***	0.372**	0.31*	0.456***					
8_TGW_S_F_08_NS	0.344**	0.211	0.323**	0.342**	0.354**	0.403**	0.569***				
9_TGW_C_GH_08_NS	0.379**	0.397**	0.572***	0.587***	0.137	0.32*	0.18	0.134			
10_TGW_S_GH_08_NS	0.461***	0.198	0.438***	0.613***	0.019	0.232	0.043	0.015	0.599***		
11_TGW_M_C	0.558***	0.095	0.333***	0.547***	0.187	0.317*	0.365**	0.232	0.356**	0.482***	

Table S9. Correlation between drought tolerance index for yield from different environments

Environments	1	2	3	4	5
1_DTI_Y_F_08_NS					
2_DTI_Y_F_07_NS	0.206				
3_DTI_Y_GH_08_NS	0.696***	0.139			
4_DTI_Y_GH_07_NS	0.297*	0.213	0.454***		
5_DTI_Y_GH_IPK	0.208	-0.199	0.068	-0.029	

Table S10. Correlation between drought tolerance index for TGW from different environments

Environments	1	2	3	4	5
1_DTItgw_F_08_NS					
2_DTItgw_GH_08_NS	0.115				
3_DTItgw_F_07_NS	0.372**	0.459***			
4_DTItgw_GH_07_NS	0.257*	0.458***	0.333**		
5_DTItgw_GH_IPK	0.529***	0.281*	0.296*	0.262*	

Table S11. Correlation between seed breadth from different environments

Environments	1	2	3
1_SB_C_M_IPK			
2_SB_C_GH_IPK	0.179		
3_SB_S_GH_IPK	0.16	0.196	

Table S12. Correlation between seed length from different environments

Environments	1	2	3
1_SL_C_Marian			
2_SL_C_GH_IPK	0.624***		
3_SL_S_GH_IPK	0.453***	0.433***	

Table S13. **Different drought screening indices used for selection of contrasting genotypes for terminal drought tolerance.**

Genotype	TGW (g)		Drought screening index		
	control	stress	DSI	DTI	KI (%)
LP 101	66.5	60.5	0.8	1.0	9.0
LP 102	65.4	59.8	0.8	1.0	8.5
LP 103	63.9	59.1	0.7	0.9	7.5
LP 104	**66.9**	**62.2**	**0.6**	**1.0**	**7.0**
LP 105	68.7	60.7	1.1	1.0	11.7
LP 106	**66.3**	**55.0**	**1.5**	**0.9**	**17.0**
LP 107	65.6	61.3	0.6	1.0	6.5
LP 108	55.2	48.7	1.1	0.7	11.8
LP 109	59.3	49.3	1.5	0.7	16.9
LP 110	57.3	48.8	1.4	0.7	14.9
Average	**63.5**	**56.5**			

Thousand grain weight from two independent experiments, 2007 and 2008 (details of the experiment as in Fig.1) were combined and different drought screening indices were calculated based on TGW obtained under control and drought condition. DTI-drought tolerance index, DSI-drought susceptibilty index, KI- Kernal injury. The selected contrasting genotypes are highlighted in bold.

12 Appendix

Chemicals and Reagents

Chemicals and reagents used while performing the experiments are listed below along with their company names.

ABA	Sigma
ABA-D6	ICON
Acetic acid	Roth
Acetone	Roth
Acetonitrile	J.T. Baker
Amino acid standard	Sigma
Amyloglucosidase(3500U/457 mg Lyo. powder)	Roche
AQC kit	Waters
Asparagine	Sigma
ATP	Roche
Boric acid	Sigma
Calcium disodium EDTA	Sigma
DMSO	Sigma
Ethanol	Roche
Ethyl acetate	Sigma
Fluridone	Sigma
Fructose	Sigma
Fructosidase (750000U/2.07 g Lyo.powder	Roche
G6PDH (1000U/mL	Roche
Glucose	Sigma
Glutamine	Sigma
HCl	Roth
Hexokinase (1600U/mL)	Roche
Imidazole	Calbiochem
Invertase	Roche
KOH	Sigma
Methanol	Fluka

MgCl2	Duchefa
NAD	Roche
NaOH 50%	J.T. Baker
PGI (3500U/mL)	Roche
Sodium acetate anhydrous	Fluka
Starch	Boehringer Mannheim
Sucrose	Sigma
Tri sodiumCitrate dehydrate	
Triethanolamine	Aldrich

Instruments

Centrifuge (5415 R)	Eppendorf
Data logger (Irrigation system)	Detla T Devices
Elemental analyzer (Vario EL III)	Elementar
HPLC (Summit)	Dionex
Ion Chromatography system	Dionex
Marvin digital seed analyzer	Hoopman Eqpmt and Eng.
Mass Spectrometer (1200L)	Varian
Portable photosynthetic system, IRGA (LcPro+)	ADC Biosystem
Soil moisture meter (HH2 meter)	Detla T Devices
Soil moisture sensor (SM 200)	Detla T Devices
Spectrophotometer (Uvikon)	Goebel
Thermomixer (comfort)	Eppendorf
Centrifuge Vaccum evaporator (Univapo 100H)	Uniequip
Vapour pressure osmometer (5500)	Wescor

Materials

Columns

AccQ Taq column (3.9 x 150 mm)	Waters
Carbopac PA20 (3 x 150 mm)	Dionex
Trap column, Ion Pac (4 x 35 mm)	Dionex
Guard column for AccQ. Taq (3.9 x 20 mm)	Waters
Guard column for Carbopac PA20 (3 x 30 mm)	Dionex

Others

Alumimium cups	Elementar
CO2 catridges	Liss
Copper granules	Elementar
Disposable cuvettes	Ratiolab
HPLC vial for Summit (1.1-STVG)	Chromacol
HPLC vial for IC	Zinsser analytic
Membrane filter (Multiscreen, 96 well)	Millipore
Vivaclear, 0.8 um PES	Sartorius stedim
Genesis C18 column ((4µm, 100 mm)	Vydac

Buffers and Enzyme solutions

All chemicals were dissolved in double distilled water unless if it is specifically mentioned.

80% aqueous buffered acetone

To 100 ml of acetone (100%), 667 uL of 1mM KOH was added and pH adjusted to 8.6

Eluent A for Amino acid analysis (pH-5.8)

Triethanolamine HCl (MW-185.5) - 7mM

Sodium acetate anhydrous (MW-57.42) - 140mM

pH adjusted to 5.8 with acetic acid

Borate buffer for amino acid derivatization (pH-8.8)

Boric acid (MW- 61.83) - 0.2M

calcium disodium EDTA (MW-374.27) - 5 m*M*

Weigh 1.24 g of boric acid and add 100 mL of water, add 187 mg of calcium disodium EDTA. Adjusted the pH to 8.8 with sodium hydroxide (a solution made fresh from pellets).

Imidazole buffer components

Imidazole (MW-68.08)	-100mM	pH adjusted to 6.9 and stored at 4C
MgCl2*6H2O (MW-203.3)	-5mM	
ATP (MW-605.2)	-1mM	
NAD (MW-717.4)	-2mM	

ATP and NAD stocks were made separately and stored at -20C. Fresh buffer was made each time by mixing different components to the desired concentration.

Citrate buffer

-Tri sodium citrate dehydrate (MW-294.1).

pH adjusted to 4.6

Enzyme solutions for starch estimation

Amyloglucosidase

-Amyloglucosidase (3500U/457 mg lyophillised powder)

Made by dissolving 10.29 mg of lyophillised powder in 1 mL of 100mM citrate buffer pH-4.6 (79U/mL).

Hexokinase

-Hexokinase(1600U/mL)

Made by dissolving the enzyme solution and water in 1:1 ratio (800U/mL)

Curriculum vitae

Personal Data

Full Name: Kalladan Rajesh
Gender: Male
Date of birth: 02-02-1978
Place of birth: Paloor, mahe, India
Marital status: Married
Nationality: Indian
Email ID: rajesh@ipk-gatersleben.de, rajdani@gmail.com

Education and employment

August 2007 to present:	PhD student at stress genomics group, molecular genetic department, IPK, Gatersleben, Germany.
March 2007 to June 2007:	Senior research fellow at International centre for genetic engineering and biotechnology, New Delhi, India.
2004 to August 2007:	Senior research fellow, Department of Microbiology, CFTRI, Mysore, India.
2001 to 2004:	Master student in the Department of crop physiology, GKVK, Bangalore, India.
1996 to 2000:	Bachelors student at Pandit Jawaharlal Nehru College of agriculture and research institute, karaikal, India.

Publication

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.

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Rajesh Kalladan,
Stress Genomics,
Department of Molecular genetics,
IPK, Correnstrasse 3,
06466 Gatersleben.

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 ..... _____