Institut für Nutzpflanzenwissenschaften und Ressourcenschutz

Genetic dissection of shoot traits and proline content under control and drought conditions in barley

Inaugural-Dissertation

Zur Erlangung des Grades

Doktorin der Agrarwissenschaften
(Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn 2018

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Tag der mündlichen Prüfung	12.03.2018

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

ACKNOWLEDGEMENT

This thesis represents not only my work at the keyboard; it is a milestone in more than one decade of work at INRES and specifically within the Plant Breeding group. Foremost, I would like to express my sincere gratitude to my advisor **Prof. Dr. Jens Léon** for the continuous support of my PhD study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my PhD study. Besides my advisor, I would like to thank the rest of my group leader **PD Dr. Ali Ahmed Naz** for his encouragement, insightful comments, and hard questions.

I thank my fellow lab mates, and technical staff, for the stimulating discussions, helps and for all the fun we have had during my entire PhD period. My research would not have been possible without their helps. My thanks are due to **Dr. Benedict Chijioke Oyiga**, **Said Dadshani,** and **Mirza Majid Baig** for their continuous support throughout my PhD. I am grateful and deeply indebted to Frau **Karin Woitol, Karola Müller, Anne Reinders** and **Alexa Brox** for their generous encouragement and kind help during my stay in the institute. I am also very grateful to Higher Education Commission (HEC), Pakistan for funding my research.

Last but not the least; I would like to thank my family: especially my parents **Muzammil Ishtiaq**, **Shagufta Parveen** and my elder sister **Naila Haseeb**. They were always supporting me and encouraging me with their best wishes and at the bottom of me spiritually throughout my life.

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List of abbreviations

ABA	:	Abscisic acid
ABF	:	ABRE-binding factors
ABREs	:	ABA-responsive elements
BBCH	:	Bundesanstalt, Bundessortenamt und CHemische Industrie (German scale to assess the development stage of plants)
BC2DH	:	Backcross (second generation)-doubled haploid
BOPA	:	Barley oligo pool assay
CC	:	Chlorophyll Content
cDNA	:	Complementary Deoxyribonucleic acid
cM	:	centiMorgan
cm	:	centimetre
CORR	:	Correlation
DAS	:	Days after stress
DH	:	Double haploid
DNA	:	Deoxyribonucleic acid
et al.	:	et aleri
F2	:	Second filial generation
FRS	:	Resonant frequency shift
GLM	:	General linear model
h2	:	Heritability
HE	:	Heading
Hsp	:	*Hordeum vulgare* ssp. *spontaneun*
Hv	:	*Hordeum vulgare* ssp. *vulgare*
ILs	:	Introgression lines
IQS	:	Inverse quality factor shift
NILs	:	Near isogenic lines

NL	:	Number of leaves
NS	:	Number of spikes
P5CS	:	pyrroline-5- carboxylate synthetase
PCR	:	polymerase chain reaction
PH	:	Plant height
Ppd	:	Gene associated with photoperiod (heading)
PROC	:	Procedure
QTL	:	Quantitative trait loci
R2	:	Genetic variance
REML	:	Restricted maximum likelihood method
RIL	:	Recombinant inbred line
RNA	:	Ribonucleic acid
RP	:	Relative performance
SAS	:	Statistical Analysis System software
S42	:	Scarlett × ISR42-8 population of barley
SFW	:	Shoot fresh weight
SDW	:	Shoot dry weight
SNPs	:	Single nucleotide polymorphisms
SS	:	Sum of squares
ssp.	:	Subspecies
TFBS	:	Transcription factor binding sites
VAR	:	Variance
VMC	:	Volumetric moisture content
°C	:	Degrees Celsius
WS	:	Wilting score

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Abstract

Drought is the most severe threat to world crop cultivation and production especially in water shortage areas**.** Wild barley diversity contains notable variation in phenotype that is essential for its adaptation to abiotic stress like drought. In the current study, we performed QTL mapping for shoot traits and proline content accumulation under control and drought conditions. A library of 73 (BC3S4:S10) S42ILs derived from German cultivar Scarlett and wild accession from Israel (ISR42-8) was used in this experimental study and genotyped for shoot traits with a 1,536-SNP Illumina BOPA1 set.

Plants were analyzed and phenotypic data was collected for eight shoot traits and physiological trait i.e; proline content. All studied traits showed high significant differences between both treatments. Genetic mapping reveals total twenty QTLs for shoot traits and five QTLs for drought inducible proline accumulation all over the barley genome and had main effects on improving or reducing the traits under control and drought stress conditions. The most important QTL which have been obtained in the current study is for proline content on 1H chromosome. Further mapping and validation in a high resolution population revealed that Qpro.S42-1H underlie a previously unknown HvP5CS1 allele originated from wild barley. The functional mutations were found in the promoter motifs for DNA binding transcription factor i.e; ABRE-binding factors (ABF1, ABF2), where the number and arrangements of ABFs binding motifs in the wild P5CS1 allele in ISR42-8 appeared to imply transcriptional up regulation and excessive proline accumulation under extreme drought conditions. Higher proline accumulation in QTL allele bearing ILs S42IL-143 and S42IL-141 conferred improved physiological activity and photosynthetic yield, thus confirming functionality of an exotic P5CS1 allele in the cultivated barley. The present findings brought up a first insight on the molecular and evolutionary regulation of an essential drought physiological traits in crop plant. These resources offer opportunity to understand adaptive biology of crop plants and can serve as direct target for trait improvement in barley and related species.

Abstract (in Deutsch)

Die Kultivierung und der Ertrag von Feldfrüchten wird hauptsächlich durch Trockenheit insbesondere in Anbaugebieten mit zunehmender Wasserverknappung gefährdet. Die Wildformen unserer Kulturgerste bieten ein hohes Maß an Variation des Phänotyps und somit an Anpassungsmöglichkeiten an diverse abiotische Stressszenarien wie etwa Trockenheit. Für die vorliegende Arbeit wurde eine Kartierung quantitativer Merkmale für Sprossparameter, sowie des Gehalts von Prolin, einem Pflanzenhormon, unter Kontroll- und Stressbedingungen durchgeführt. Eine Population bestehend aus 73 (BC3S4:S10) S42-Introgressionslinien abstammend von der deutschen Kultursorte Scarlett und der exotischen Linie ISR42-8 aus Israel wurde im Hinblick auf Sprossmerkmale mithilfe eines Illumina BOPA1 Sets anhand von 1536 SNPs genotypisiert. Die Versuchspflanzen wurden im Hinblick auf acht phänotypische Sprossmerkmale, sowie physiologische Merkmale, wie z.B. Prolingehalt analysiert. Alle untersuchten Parameter wiesen hochsignifikante Unterschiede zwischen den beiden Behandlungen auf. Die genetische Kartierung ergab insgesamt 20 QTLs für Sprossmerkmale und fünf QTLs für trockeninduzierte Prolinanreicherung, verteilt auf das gesamte Gerstengenom und hatte wichtige Effekte in Bezug auf die Ausprägung der entsprechenden Merkmale unter Kontroll-bzw. Stressbedingungen. Für Prolingehalt konnte in der aktuellen Studie ein wichtiges QTL auf Chromosom 1H lokalisiert werden. Die weitere Kartierung und Validierung in einer höher auflösenden Population ergab, dass der Genort Qpro.S42-1H einem bislang unbekannten HvP5CS1 Allel aus Wildgerste entstammt. Funktionelle Mutationen wurden in der Promotorregion für DNA-bindende Transkriptionsfaktoren wie z.B. ABRE-Bindungsfaktoren (ABF1, ABF2) entdeckt, wobei deren Anzahl und Anordnung im exotischen P5CS1 Allel in ISR42-8 eine deutliche Prolinanreicherung unter extremen Trockenstressbedingungen reguliert. Die Introgressionslinien S42IL-143 und S42IL-14, welche das entsprechende QTL-Allel für eine Prolinanhäufung tragen, zeigten eine verbesserte physiologische Aktivität und Photosyntheserate und bestätigten damit die Funktionalität des P5CS1-Allels in der Kulturgerste. Die vorliegenden Ergebnisse geben einen ersten Einblick in die Regulierung eines entscheidenden physiologischen Merkmals auf molekularer Ebene in Pflanzen. Dadurch werden Möglichkeiten zum Verständnis der Anpassung von Pflanzen an Trockenstress und die Nutzung dieser wertvollen Ressourcen als Quelle zur Leistungsverbesserung von Gerste und verwandten Spezies eröffnet.

1.INTRODUCTION

1.1 Barley

Barley (*Hordeum vulgare* L.) is one of the first and abundant cultivated cereal crops from grass family. The genus *Hordeum* composed of 45 *taxa* and 32 species that consist of diploid (2*n* = 2*x* = 14), tetraploid (2*n* = 4*x* = 28) and hexaploid (2*n* = 6*x* = 42). Most of the species belonging to Hordeum are perennials and are reproductively different from each other including the cultivated barley (*H. vulgare ssp*. *vulgare* L.) and its wild progenitor (*H. vulgare ssp. spontaneum C. Koch*.).

Barley adapts well to a wide variety of climates and is grown as a summer crop in temperate areas and as a winter crop in tropical climates. It is considered to be an early maturing crop and germinates within one to three days after sowing. The world barley production in 2016/2017 (Figure 1) was approximately 145.2 million metric tons (MMT) produced in 54.13 million hectares (MH) of arable lands. Europe had the largest growing area of barley, producing 59.74 MMT, followed by Russia with 17.55 MMT in 2016/2017 (FAO; 2017).

Barley use as food in the European Community was even less (0.3%) than in the United States. The largest use for barley as a food was in Morocco (61%), Ethiopia (79%), China (62%), and India (73%) (Kent and Evers 1994). It is also used as animal feed and has many health benefits and is largely used in malting. Barley is a rich source of nutrients like protein, B vitamins, dietary minerals, and dietary fiber. The grain is a particularly good source of manganese and phosphorus. Raw barley is 78% carbohydrate, 10% protein, 10% water, and 1% fat. Dehulled barley is used to prepare a number of food items like flour, flakes, grits, etc.

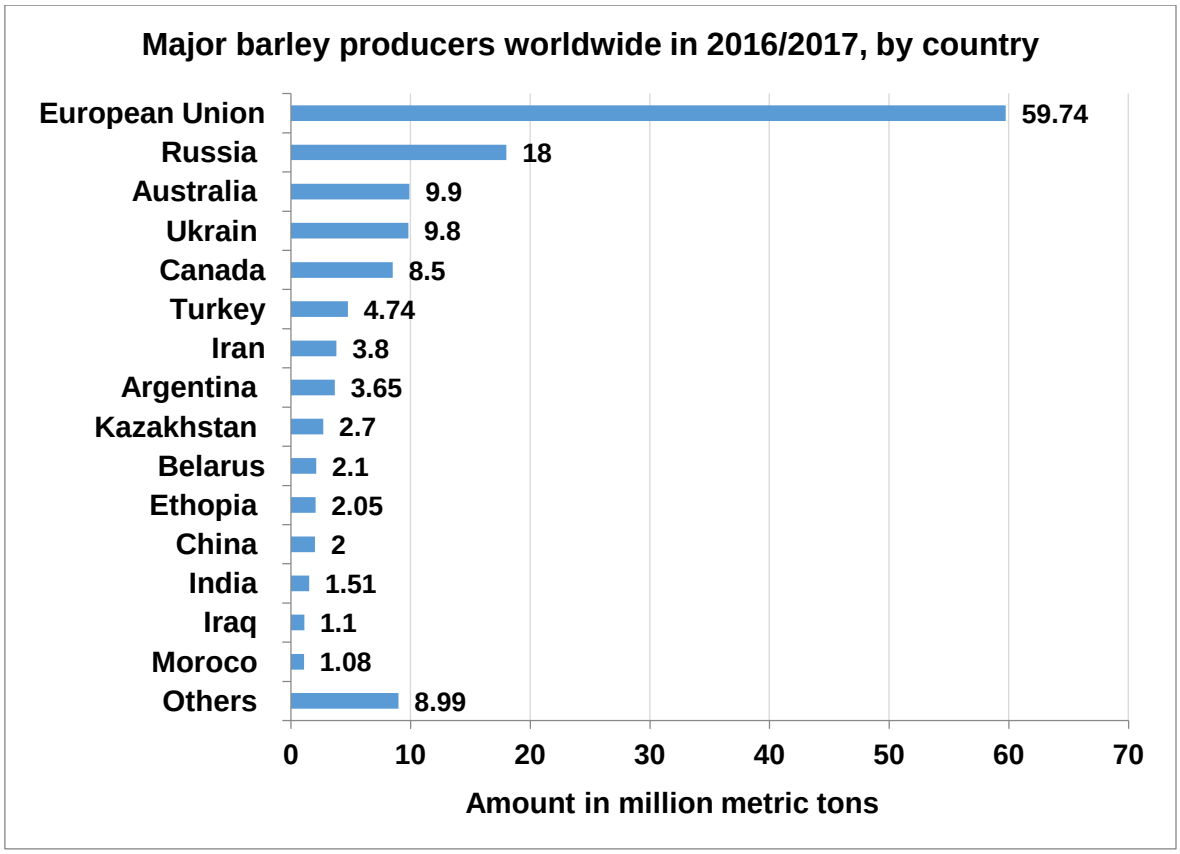


Figure 1: Major barley producing countries in 2016/2017.

1.2 Drought stresses

Plants are frequently subjected to adverse climatic conditions – abiotic stresses, playing key role in crop production along with the species to be exposed to a particular environment (Boyer, 1982, Chaves *et al*., 2003). Among the abiotic streses, drought is considered to be the most important factors limiting crop production by causing a significant reduction of crop growth and productivity (Bagci et al., 2007; Passioura, 2007). A recent study analyzed the data of studies published from 1980 to 2015 to report up to 21 and 40% yield reductions in wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.), respectively due to drought on a global scale (Daryanto *et al.,* 2016). Report has shown that about 25% of global agricultural land is affected by drought stress (Jajarmi, 2009). Drought is considered the single most devastating environmental stress, which decreases crop productivity more than any other environmental stress (Lambers et al., 2008). Drought stress significantly reduced cereal production by 10% on average between 1964 and 2007 and this percentage was found to increase annually due

to the rising drought severity (Lesk *et al*., 2016). Moreover, grain yield reduction of up to 85% due to drought stress has been observed in barley (Ouda *et al*., 2016).

Consequently, increasing desertification and looming water shortages lead to more and longer drought periods, which affect the crop productivity especially in tropical, semi-arid and arid regions worldwide during grain-filling phase and results in yield losses dramatically (Samarah, 2005; Pennisi, 2008). Keeping in view all the environmental changes occurring, Figure 2 depicts that most of the global arable lands would be under drought condition by 2070(Source: Eurowasser study, University of Kassel).

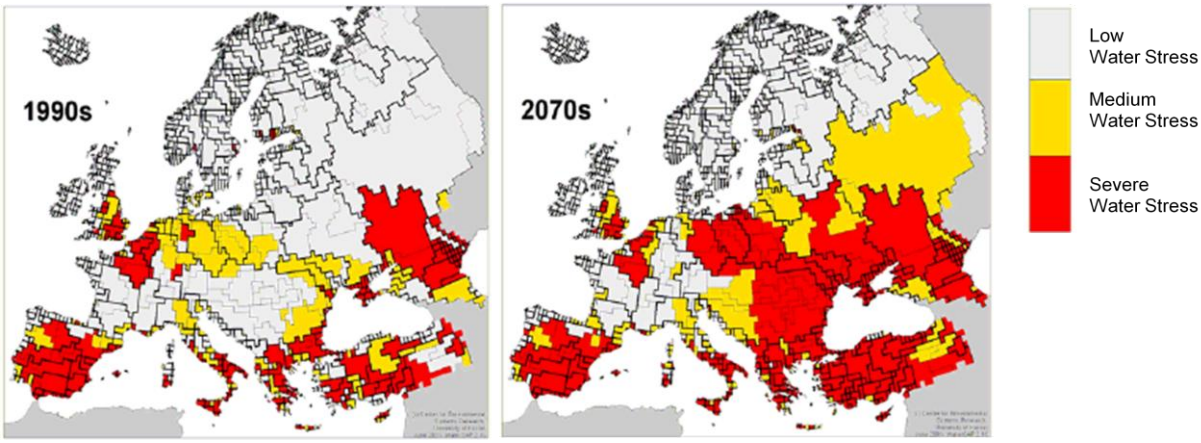


Figure 2: Depiction of areas under drought stress in 2070

Drought is a major risk with its extensive impacts on economic losses to livelihood. Water deficit soil cause low water potential that is the major natural problem hindered the cultivation and end productivity of natural as well as agricultural ecosystems generates large economic losses in many regions of the world. Artificial irrigation has been a key for this problem, but due to high societal demands water supplies became at an increasingly high financial and environmental cost (Wu and Cosgrove, 2000). Thus, the cultivation of drought tolerant genotypes in drought prone agro-ecologies appears to be the best strategies to tackle the increasing aridity of the arable land.

1.3 Effect of drought stress on plants

Drought stress reduces germination and seedling vigor (Harris et al., 2002, Kaya et al., 2006), resulting in poor plant growth and development. In pea, drought has been reported to cause drastic effect on seedling growth (Okcu et al., 2005). Similarly, in alfa alfa drought reduced germination, hypocotyls length, root and shoot fresh as well as dry weights (Zeid and Shedeed, 2006). Plant growth and development depends on cell division, cell enlargement and differentiation, morphological, physiological, genetic and ecological processes and their interactions (Figure 3).

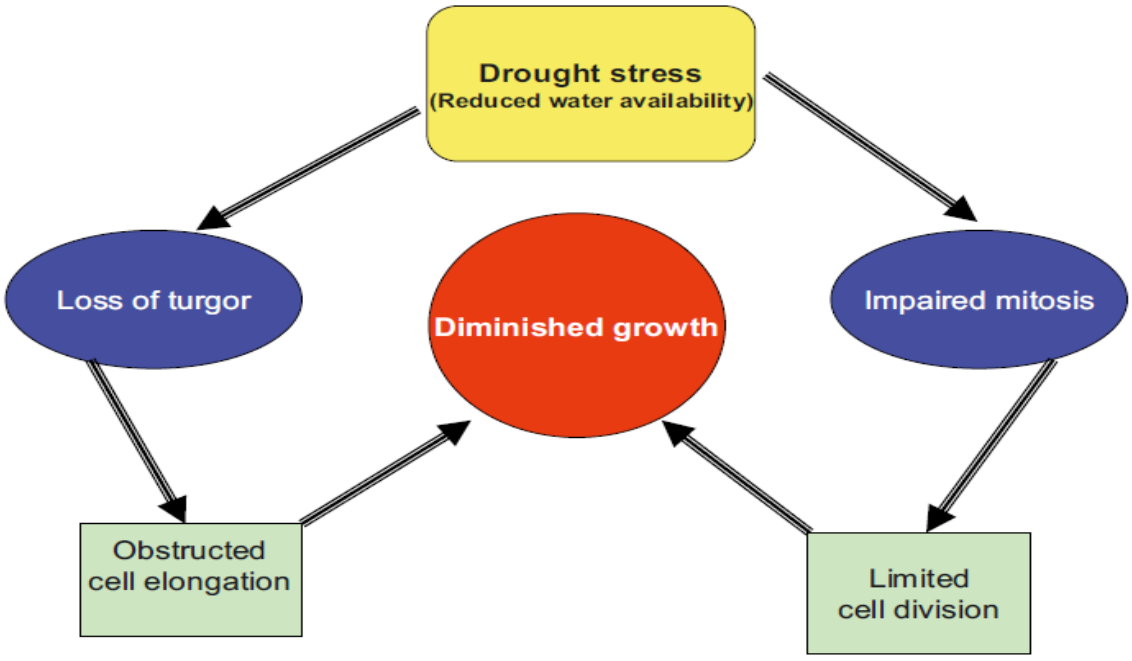


Figure 3: Description of possible mechanisms of growth reduction under drought stress.

Water deficiency induced reduction in yield in crops, because water stress can shortened grain filling duration (Estrada-Campuzano et al., 2008. Samarah, 2005). The reduction is associated with the negative impact of drought stress on the yield related traits including number of tiller, number of spike, grain size, grain weight and number of grains per plant has been reported in barley. Reduction in grain yield due to water stress has been reported in many crop species (Cattivelli et al.,

2008, Frederick et al., 2001, Pettigrew 2004, Ahmadi and Baker, 2001, Taiz and Zeiger, 2006). Water deficiency during reproduction period cause kernal abortion in maize and shortened grain size and kernal growth in wheat crop (Morgan, 1990; Ober *et al.,* 1991). While, water scarcity during flowering caused different plant response and resulted into yield stagnation by 40-55% (Nam et al., 2001). On the other hand, water stress during the grain filling stage boosted up the mobilization of carbon reserves to grain thus increased the gain filling (Yang et al., 2001). Another major effect of drought is reduction in photosynthesis due to decline in leaf expansion. Drought stress not only altered photosynthesis process by changing photosynthetic pigments but also damaged photosynthetic apparatus that ultimately inhibits the growth (Anjum et al., 2003, Fu J. and Huang, 2001).

1.4 Drought Tolerance

Drought tolerance is defined as the ability to grow, flower and display economic yield under suboptimal water supply (Farooq et al. 2009). The plant reactions to drought stress are tissue and organ dependent (Kranner et al., 2010). Moreover, duration and level of stress, cause particular impact and make the responses more complex (Taiz et al., 1991, Larcher et al., 2003). Plants respond to drought stress by the induction of several such as morphological, biochemical and physiological mechanisms

Morphological mechanism

Drought escape and drought avoidance are most common morphological responses of plants under severe drought conditions. Drought escape is the ability to complete the life cycle during wet season before serious soil and plant water deficits develop. This form of adaptation needs an extremely short life cycle, where seeds are produced during short rainy seasons (Levitt, 1980). Early flowering is an important trait related to drought escape (Araus et al., 2002). Developing early flowering verities has been an effective strategy to avoid the period of drought stress and less yield loss (Kumar and Abbo 2001).

Drought escape is only possible when phonological development occurs exactly when soil moisture is available. But, to maximize the water uptake during this particular period, plants need to produce more root biomass. The ability of a genotype to regulate its root growth according to prevailing circumstances is

termed as root plasticity (Kano et al., 2011). Drought stress has negative effect on plant root growth even in tolerant plants, but effect is more drastic on susceptible genotypes. More root biomass and root length in resistant genotypes resulted in more yield compared to genotypes with less root and short length (Jongrungklang et al., 2013).

The ability of plants to maintain relatively high tissue water potential by reducing water loss from plants, due to stomatal control of transpiration loses is drought avoidance. The root characters such as biomass, length, density and depth are the main drought avoidance traits that contribute to final yield under terminal drought environments (Subbarao et al. 1995; Turner et al. 2001). Furthermore, an enhanced stomatal resistance, less small stomata, reduced leaf area and a change in leaf orientation are other important drought avoidance traits to minimize water loss due to transpiration under drought stress conditions (Aroca, 2012).

Biochemical mechanism

At molecular levels, plants affected by drought developed many adaptive processes to modulate water stress. The stream of molecular responses to drought starts from stress perception, through signal transduction to cytoplasm and nucleus, to gene expression and resulting to metabolic changes (Ahmad and Prasad, 2012). Plants perceive the external and internal signals upon stress, via different independent or interlinked pathways to regulate different responses for its better development (Ciarmiello et al., 2011). Up-regulation of many genes as well as the accumulation of stress proteins has been reported to help the plant to withstand the stress conditions which leads to plant adaptation (Tuteja 2009, Kavar et al., 2008).

Plant responses to stress are complex integrated circuits within which multiple pathways are involved. Transcription factors are among the category of genes which are induced early within minutes of stress. Transcriptional activation of some of these early genes has been well studied. In 2002, Chen and Murata identified a group of genes including transcription factors of drought-responsive element / C-repeat (DRE/CRT) binding factor family as well as MYB proteins, bZIP/HD-ZIPs and AP2/EREBP domain proteins which were up-regulated under drought stress. Stress related transcription factors like MYB, dehydration-responsive element binding factor (DREB), WRKY and bZIP confer tolerance by

induction of genes by maintaining the osmotic equilibrium of the cell (Seki et al., 2002)

In addition to transcription factors, the expression of stress proteins like aquaporins increases the drought tolerance in plants. Aquaporins are integral membrane proteins which regulate the movement of water in and out of the cell, across plant vacuolar and plasma membranes; they are associated with plant tolerance to abiotic stresses (Li et al., 2015). Plant aquaporins can transport various physiological substrates in addition to water. With an increasing number of plant genome sequences available, aquaporin genes have now been fully described in several plants like *Arabidopsis thaliana*, maize, rice, soybean, tomato, and cotton (Reuscher et al., 2013, Park et al., 2010, Gupta et al., 2009, Chaumont et al., 2001, Johanson et al., 2001, Quigley et al., 2001, Sakurai et al., 2005) Several studies have shown that the over-expression of aquaporins increases the abiotic stress tolerance in plants (Ayadi et al., 2011; Hu et al., 2012; Liu et al., 2013).

Physiological mechanism

Plant water conservation, plant growth regulator and over production of the compatible solutes are physiological mechanism which plant adopt during stress. For water conservation, the osmotic adjustment may confer tolerance against drought, by accumulation of organic and inorganic solutes under water deficiency stress to create a high water status (Turner et al., 2001). With increased accumulation of solutes, the water potential of the cell is lowered, which help the cell to maintain its turgor pressure (Serraj and Sinclair, 2002).

Plant growth regulators like proline, auxins, gibberellins, cytokinins, ethylene and abscisic acid (ABA) are substances which help plant in development and play vital roles in drought tolerance of plants (Morgan 1990). Abscisic acid (ABA) is known as an important regulator for plant growth and adaptation to drought. It has been proposed that he increased synthesis of ABA leads to many changes in development, physiology and growth. ABA production also alters the growth rate of various parts of plant like leaf development, shoot and root dry weight and deeper roots as well. So, it activates physiological short-term adaptations to

drought like stomata closure as well as long term adaptation like root growth (Verma et al., 2016).

Role of proline under drought:

Plants affected by drought developed much adaptive physiological adaptation to modulate water balance. One of the most common stress tolerance strategies in plants is the overproduction of different types of active compatible organic solutes (Serraj and Sinclair 2002). These are osmoregulators and are of low molecular weight and high soluble compounds. Osmoregulators are confined mainly to the cytosol, chloroplasts, and other cytoplasmic compartments and protect cellular components from dehydration injury during osmotic stress. They include amino acids such as proline, glycine betaine, mannitol, and sugars that confer stress tolerance. In higher plants, proline is a candidate biochemical solute, which is involved in protection of cells against stress damage (Hare and Cress 1997) Reports have shown that proline is a plant defence response to water-deficit stress, including signal transduction, osmoregulation and antioxidant systems (Hare and Cress, 1997; Kishor et al., 2005; Szabados and Savouré, 2009). Moreover, application of different osmoregulators such as proline had a significant role on plant growth promotion and seed yield under normal or stress conditions as observed in some crops e.g., maize (Yang and Lu, 2006; Kaya et al.,2013; Reddy et al.,2013),canola (Dawood and Sadak, 2014), rice (Mohammed and Tarpley, 2011), wheat (Raza et al., 2014), chickpea (Kaushal et al.,2011)and faba bean (Taie et al., 2013; Dawood et al., 2014).

Proline accumulation is well known in plants during the adaptation to various types of environmental stress including drought (Öncel *et al*., 2000; Ruiz *et al*., 2002). Proline contents were increased under drought stress in pea cultivars (Alexieva *et al.* 2001). Drought-tolerant petunia (*Petunia hybrida*) varieties were reported to accumulate free proline under drought that acted as an osmoprotectant and induced drought tolerance (Yamada *et al.* 2005). The principal role of proline probably is not to reduce the osmotic potential, but to protect enzymes against dehydration (Thomas, 1991). Despite proline role under stress, many physiological roles have been assigned to free proline including a positive role of proline synthesis in flowering, stabilization of macromolecules,cell elongation, bolting and

many developmental process (Zhu 2002, Mattioli *et al*., 2008, 2009; Samach *et al.*, 2000). Thus, it is necessary to perform thorough investigation of the regulatory mechanism of proline metabolism in higher plants (Kishor *et al*., 2005).

Proline can be synthesized through two pathways; one from glutamate and the other one through ornithine. The glutamate pathway is normally located in the cytosol and chloroplasts (Armengaud *et al*., 2004). Glutamate-semialdehyde (GSA) by Δ1-pyrroline-5- carboxylate synthetase (P5CS) is produced in the result of glutamate reduction, and is converted to Δ1-pyrroline-5-carboxylate (P5C) and then P5C reduced to proline. In an alternative pathway, proline can be synthesised from ornithine, which occurs in mitochondria. Ornithine-δ-aminotransferase (δOAT) converts ornithine to GSA and P5C, which is then transported to the cytosol and converted to proline by P5CR. Proline is oxidised via the sequential action of proline dehydrogenase (PDH) producing P5C and Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH), which converts P5C to glutamate (Lehmann *et al.,* 2010; Szabados and Savouré, 2010). P5CS and PDH are regarded as key enzymes in proline synthesis and catabolism, respectively. Plant genomes usually contain two homologous genes encoding P5CS, as in *A. thaliana* (Funck *et al*., 2010; Strizhov *et al.,* 1997), *N. tabacum* (Ribarits *et al.,* 2007) and *M. truncatula* (Armengaud *et al.,* 2004). Early studies of proline metabolism established a “standard model” whereby increased synthesis and reduced degradation led to the accumulation of proline (Chaitanya *et al*., 2009; Miller *et al.,* 2005, 2009; Parida *et al*., 2008; Ribarits *et al*., 2007; Sharma *et al*., 2011). Based on this model, genetic manipulation to progress plant stress tolerance by over expressing the P5CS gene has achieved initial success (Verbruggen and Hermans, 2008; Mizoi and Yamaguchi-Shinozaki, 2013). Through further investigations, researchers came to realise that the dynamic transport and turnover of proline between different organs, rather than static cell-autonomous accumulation, are fundamental to the protective role of proline (Sharma et al., 2011). At present, proline transporter (ProT), which belongs to the amino acid transporter family has been shown to be localized at the plasma membrane and is involved in the intercellular transport of proline (Rentsch et al., 2007). Isolation of P5CS genes and comprehensively analyses of their expression patterns under drought stress conditions would serve as a guide towards gaining an in-depth understanding of the key function on the mechanism of proline metabolism in barley.

1.5 QTL mapping

Quantitative traits have been a major part of genetics study from almost a century. In order to begin with QTL mapping, two or more strains of organisms are needed that differ genetically with regard to particular trait of interest. Second, genetic markers are also required that distinguish between these parental lines. Several types of markers are used, including single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs, or microsatellites), restriction fragment length polymorphisms (RFLPs) and transposable element positions (Henry, 2006, Gupta & Rustgi, 2004; Vignal *et al.*, 2002). In all eukaryotes especially in crop plants, these markers provide a common feature of variation. Molecular markers are preferred for genotyping, as these markers are unlikely to affect the trait of interest. Afterwards, to carry out the QTL analysis, the parental strains are crossed, resulting in heterozygous (F1) individuals, and these individuals are then crossed using one of a number of different schemes (Darvasi, 1998). Finally, the phenotypes and genotypes of the derived (F2) population are scored. Markers that are genetically linked to a QTL influencing the trait of interest will segregate more frequently with trait values, whereas unlinked markers will not show significant association with phenotype. Sax in 1923 described the isolation of effect of single locus by the continuation of the crosses resulting in genetic background randomization regarding to all genes that are not linked to the genetic markers. Sax worked with bean and used morphological seed markers and found significant effect with some markers associated with seed weight.

During 1930-80s, only few QTL was detected and some of them were repeated because of the deficit of available adequate polymorphic markers. During 1980s the advancement was made and the discovery of the easily visualized variability at DNA level was discovered that could be used as markers. However, most of the markers are in non-coding regions of the genome and not affecting the trait of interest but, a few of these markers might be linked to QTLs and directly influence the trait of interest. Thus, it is assumed that QTL and the marker locus will co-segregate. Partitioning of the mapping population into genotypic classed and then application of correlation statistic is useful to understand whether a QTL is linked to a marker or not. Advancement in statistical packages also helped in analyzing the marker data. For last few decades, quantitative traits have been studied using

statistical tools based on means, variances and co-variances of relatives. These studies provide a base to understand the partitioning of the phenotypic variation into genetic and environmental variances in term of additive, dominance and epistatic effects. From this information, it became possible to estimate the heritability and ultimately the response of a specific trait to selection as well as number of genes that controlled that trait of special interest. However, little was known about what these genes were, where they are located, and how they controlled the trait. Apart from the fact, for any given trait, there were significant genes distributed randomly in a mendelian fashion in any specific population, mostly with additive effect (Kearsey and Pooni, 1996) and were called 'polygenes' by Mather (1949).

Normally QTL analysis is initiated in segregated mapping population like F2 population, recombinant inbred lines (RILs), near isogenic lines (NILs), backcross population and doubled haploid lines (DHs) populations. In the present research, a library of barley introgression line was used which was developed by a cross between cultivated and wild accession and generated by back crossing, various round of selfing and finally with the help of marker assisted selection. The lines were already been used for verification of QTLs for field experiments in order to highlight the applicability of the spring barley ILs.

Zamir (2001) described the numerous advantages of ILs and explained precisely about the advantage of ILs. It is assumed that once the homozygous IL set is developed, each IL can be used for breeding, because it is reliable and more stable source (Obando *et al.,* 2008, Eduardo *et al*., 2007, Fernandez-Trujillo *et al.,* 2007; Rousseaux *et al.,* 2005). Many researchers used ILs under stress for drought stress (Zhang *et al.,* 2006; Zhou *et al*., 2006; Siangliw *et al*., 2007) to detect putative QTLs respectively. IL sets for tomato (Finkers *et al*., 2007, Canady *et al*., 2005, Mon-forte and Tanksley 2000), *A. thaliana* (Keurentjes *et al*., 2007), rice wild species (Tan *et al*., 2007, Tian *et al*., 2006a, Li *et al*., 2005), as well as the D-genome of wheat (Pestsova *et al*., 2006), maize (Szalma *et al*., 2007), and melon (Eduardo *et al.,* 2005) were developed in recent years for advanced study on different crops.

1.6 Research Hypothesis

Taking into consideration the facts about barley and drought, sufficient literatures have discussed the genetic analysis of shoot traits under drought stress conditions. On other hand, the physiological characteristics are known to be important in improving drought tolerance in barley. In addition, there is limited knowledge on the inheritance of these traits like proline content, in particular studying the effect of QTLs by treatments I attempt to answer the following central research hypotheses:

1. Wild barley contains genetic diversity for drought tolerance and adaptation mechanism for better use in breeding system.
2. The introgression lines are useful source of QTL alleles of wild origin for improved shoot traits and proline content accumulation.
3. Proline accumulation is induced by drought stress and is regulated by a stress inducible gene P5CS1 in Barley.

1.7 Objectives

The main goal of this research was to identify and develop barley with improved adaptation to drought, and to find out markers in natural population for key traits linked with drought stress tolerance. The overall objectives of the proposed study were:

1. To conduct a genome wide analyses of QTL associated to shoot and physiological trait traits using 73 S42ILs lines of a cross between cultivar Scarlett and wild barley accession ISR42-8 under control and drought conditions.
2. To validate QTL effects of the exotic alleles in a set of ILs carrying ISR42-8 introgressions in the Scarlett background.
3. To assess variations in shoot traits and proline content of barley introgression library under control and drought stress conditions.
4. To identify and characterize the QTLs for shoot traits and proline content to improve drought tolerance.

2. MATERIAL AND METHODS

2.1 Plant material for shoot traits under drought

A library of 73 barley introgression lines (ILs) was used for this research. This set of library was developed as a result of cross between Scarlett (*Hordeum vulgare* L.) and ISR42-8 (*H. vulgare ssp*. *spontaneous*) which are German cultivar and a wild accession from Israel respectively, Hence named as S42ILs after their parents. Thereafter back crossing and ten round of selfing (BC3S4:S10) was carried out. ISR42-8 being a wild parent was utilized as the donor, while the Scarlett which is a cultivar was used as the recurrent parent for subsequent advanced backcrossing. Schmalenbach *et al.,* (2008) described the detail of the S42ILs development.

The shoot traits including plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC), wilting score (WS) were evaluated for the 73 S42ILs population across two years 2012 and 2013 under the plastic tunnels at the Institute of Crop Science and Resource Conservation, University of Bonn, Germany. List of evaluated traits and their methods of measurement is presented in Table 1.

2.2 The experiment

For the traits evaluation, the experiments were performed in plastic tunnels during the summer seasons of 2012 and 2013, at the Poppelsdorf experimental station, Faculty of Agriculture, Rheinische Friedrich-Wilhelms-University Bonn. The plants were sown in a split-plot design with two treatments drought and control. Each treatment had four replications of individual IL, where the S42ILs lines were assigned randomly.

The plants were kept under control condition with continuous irrigation for 30 days and then the drought stress treatment was applied as suggested by Lancashire *et al.,* 1991 at BBCH 29-31 that are plant development stages, by completely

carrying out the water supply. Each treatment had four replications. The drought stress was continued for 26 days until the Volumetric Moisture Content (VMC) was at the maximum drought stress threshold level (VMC near to 0%), but the control block was under the regular supply of irrigation. Environmental conditions during experimental period across 2012 and 2013 are presented in Table 2.

Table 1 List of investigated shoot traits and their methods of measurement in S42IL population.

Abbreviation	Trait	Method of measurement	Unit
PH	Plant height	Plant height was measured from the stem base to the shoot tip of each individual plant with a ruler	cm
NL	Number of leaves	Before harvesting total number of leaves of the main tiller were counted for each plant	Numbers
HE	Heading	After sowing heading was measured by counting number of days from sowing to first heading of each plant	Numbers of days
NS	Number of spikes	Before harvesting total number of spikes were counted for each plant	Numbers
SFW	Shoot fresh weight	Plants were cut and weighed individually	g
SDW	Shoot dry weight	Plants were dried in the oven at 50º C for seven days and weighed	g
CC	Chlorophyll content	Chlorophyll content was measured using SPAD meter	ug-cm^2
WS	Wilting scores	Measured using 'Standard evaluation system' (SES) for rice (IRRI, 1980).	Score 1-9

Table 2 Average temperature and relative humidity across years 2012 and 2013 at Poppelsdorf field station Bonn, Germany.

Months	2012		2013	
	T[a](^{o}C)	RH[b](%)	T (^{o}C)	RH(%)
April	14.4	50.1	11.4	52.9
May	20.9	63.3	14.	60.9
June	19.3	65.2	16.9	78.5
Average	18.2	59.5	14.2	64.1

[a] Temperature
[b] Relative humidity

The aim of water management in the control treatment was to hold the soil moisture near to field capacity (plant available water content AWC 100%). After 21 days of stress treatment a gradual reduction of water supply was observed in the stress block. Volumetric moisture content (VMC) was measured by the DL2e Data Logger soil moisture sensor.

Figure 4 showed and described the soil moisture content under control and drought stress condition during the days of stress period (A) the experimental design showing the clear difference between control and drought (A and B) position of plants in the pot and irrigation system supplied to make sure that all four plants get equal amount of water (C and D).

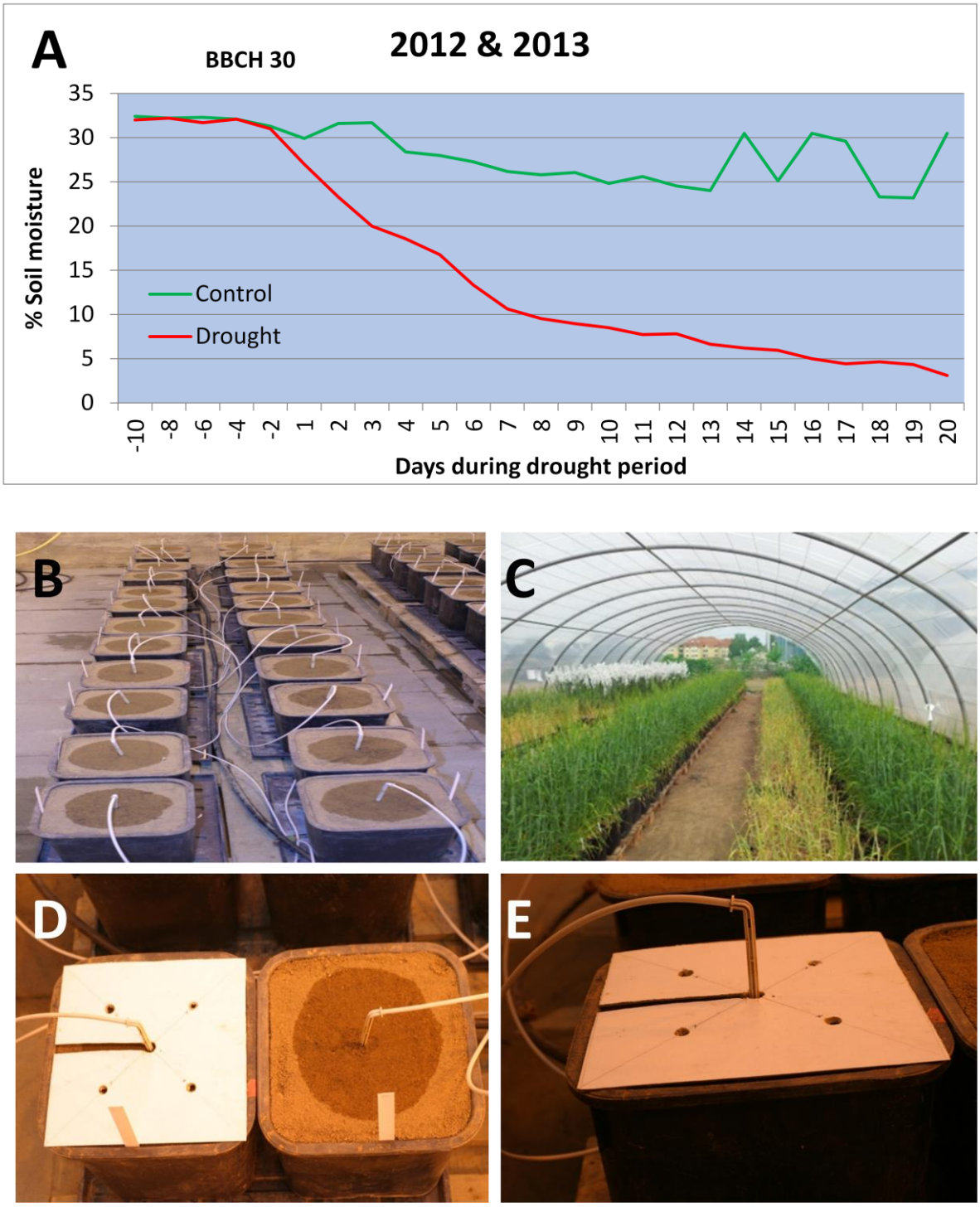


Figure 4: Soil moisture of the pots under control and drought conditions (A) The experiments were arranged in split plot design and conducted in plastic tunnels at INRES institute (B and C). Arrangement of plants in one plot and supply of water (D and E).

2.3 Growth conditions

The size of pots used was 22 x 22 x 26 cm for four plants per pot, containing a mixture of top soil, sand, silica, peat dust and milled lava (Terrasoil®, Cordel & Sohn, Salm, Germany). A drip irrigation system was used for water supply according to Netafilm, Adelaide, Australia, by giving the water to plants three times a day. VMC was determined digitally with Echo2 sensors (Decagon Dev., Pullman WA, USA) with the frequency domain technique. Plants were given fertilizer with 250 ml of NPK liquid fertilizer containing 7 % N, 6% K2O and 3% P2O5 three times per season. As per recommendation for barley cultivation the plants were sprayed against fungicides and insecticides.

2.4 Phenotypic data measurements

Seven shoot and physiological traits related to drought tolerance were investigated in this study.

1) **Heading (HE):** Heading was documented in the number of days since initial planting to the first heading.

2) **Wilting Score (WS):** Visual rating (from 0 up to 9), was enumerated at the end of the drought period, where 0 with no symptoms of stress effect and 9 with all plants most likely dried. (de Datta *et al.,* 1988).

3) **Plant height (PH):** was measured at maturity stage before harvesting the plants in centimeter from soil surface to the top of the spike excluding the awns.

4) **Numbers of leaves (NL):** Numbers of leaves (NL) were counted and recorded the every visible leaf on each plant.

5) **Chlorophyll content (CC):** Chlorophyll content (CC) of flag leaf of each plant was measured using SPAD 502 plus chlorophyll meter.

6) **Shoot fresh weight (SFW):** Shoot fresh weight (SFW) was measured in grams (g) by removing the whole above ground whole plant material and then packed them in airy bags to let them dry.

7) **Shoot dry weight (SDW):** For shoot dry weight (SDW), after calculating fresh weight put airy bags in drying chambers at 70 c for 5 days and fully dried shoots were weighed in grams (g).

2.5 Statistical Analysis

Microsoft Excel 2003 (Microsoft Corp., Redmond, WA, USA) was used for data evaluation. Statistical analysis was performed using the software package SAS Enterprise 9.2 (SAS Institute, 2008). Significance of genotypic differences between S42ILs was calculated with Dunnett test using Scarlett as a recurrent parent. Genetic correlation coefficients (r) between traits were estimated using least square means (Lsmeans) of 72 S42ILs with CORR procedure in SAS. Lsmeans were calculated with GLM procedure considering all replications and years separately for both control and drought conditions.

Some significant lines which carry overlapping introgressions in same chromosome and in same directions were identified as a putative QTL. The relative performance (RP) of a particular S42IL was calculated using the following formula:

$$\mathrm{RP(S42IL)} = \frac{[\mathrm{Lsmeans(S42IL)} - \mathrm{Lsmeans(Scarlett)}]}{\mathrm{Lsmeans(Scarlett)}} \times 100$$

Where, Lsmeans were calculated for each trait across all replications and treatments.

2.6 Analysis of variance of phenotypic data

The differences and variation among S42ILs population under both treatments over years were detected, performing ANOVA with the Statistical Analysis System SAS (SAS Institute, ver. 9.2 2008), PROC GLM procedure, as follow:

$$Y_{ijkl}=\mu+Gi+Tj+Yk+ Gi \times Tj+T_j(Yk)+ R_l +\varepsilon_{ijkl}$$

Where,

μ – the general mean
Gi – the fixed effect of *i*th genotype
Tj – the fixed effect of *j*th treatment
Yk – the random effect of *k*th year
R_l– the fixed effect of *j*th replication
Gi × *Tj* – the fixed interaction effect of the *i*th genotype with *j*th treatment
$T_j(Yk)$ – treatment effect with the year *k*

Each genotype was tested for significance with a post-hoc Dunnett (1955) test between S42ILs and Scarlett as recurrent parent. After a particular S42IL was tested which is significantly (P<0.05, P<0.01 and P<0.001) different from Scarlett for a particular trait across both treatments, then presence of a QTL was assumed. Significant lines which carry overlapping or flanking introgressions in same chromosome or chromosomal region for the same trait values were identified as putative lines for QTL of that particular trait. Variance components were estimated with VARCOMP in SAS program.

Coefficients of broad sense heritability (h^2) were performed for all studied traits across both treatments as:

$$h^2 = \frac{V_G}{V_G + \frac{V_{G\times T}}{t} + \frac{V_{G\times Y}}{y} + \frac{V_E}{tyr}} \times 100$$

Where,

VG – variance components of genotype
VG×T – variance components of genotype by treatment
VG×Y – variance components of genotype by year
VE – experimental error
t – number of treatment
y – number of years
r – number of replication

2.7 Phenotypic correlation of investigated traits

The phenotypic correlations between trait performances were calculated using the correlation procedure (PROC CORR), 73 S42ILs lines across years and separately for each treatment were used for the evaluation of the Pearson

correlation coefficients (r). Whereas, the Lsmeans were calculated considering all replications and years separately for both control and drought conditions with GLM procedure.

2.8 Genotypic data

This population was genotyped using an Illumina 1,536 SNP-array and genotyping by sequencing approaches according to Schmalenbach *et al.,* (2011) and Honsdorf *et al.,* (2014). This SNP map was associated with phenotypic data to find QTL region controlling to drought inducible proline accumulation. For this, each individual IL was compared with recurrent parent Scarlett under control and drought stress conditions using Dunnett-test according to Dunnett (1955). Later, the chromosomal introgression were compared among the ILs according to Naz *et al.,* (2014) showing significant difference of proline accumulation under drought stress conditions.

2.9 QTLs detection

For QTL detection, only QTLs for traits with heritability greater than 0 were considered. The post-hoc Dunnett test was performed for QTL discovery, to see the significant differences between the recurrent parent Scarlett and individual introgression of the S42IL lines either in control or drought stress treatment. If the particular IL was significantly different with Scarlett, it was assumed that this IL must have an introgression of wild parent, ISR42-8 carrying a putative QTL for particular trait. By comparing the common overlapping of wild introgressions among the ILs showing significant differences with Scarlett, he putative QTL regions were refined. The quantification of QTL effects was calculated by the relative performance (RP) of particular S42IL introgression line bearing the QTL in comparison to recurrent parent Scarlett.

2.10 Calculation of relative performance (RP [*Hsp*])

To evaluate the performance of the homozygous exotic genotype under drought conditions, the relative performance (RP) of a particular S42IL was calculated using the following formula:

$$\mathrm{RP(S42IL)} = \frac{[\mathrm{Lsmeans(S42IL)} - \mathrm{Lsmeans(Scarlett)}]}{\mathrm{Lsmeans(Scarlett)}} \times 100$$

Where, Lsmeans were calculated for each trait across all replications and treatments.

According to the relative performance of the exotic genotype (ISR 42-8), if it helps to improve the trait under drought conditions as well as matching with the breeding goals of drought tolerance, it was characterized as favorable QTL.

2.11 Proline accumulation under drought stress condition

Initial genetic mapping for proline accumulation under drought stress was carried out in a same barley introgression lines (ILs) population (BC3S4:S10) which was used for proline content (PC) in this research. For the phenotypic evaluation for proline content the S42IL population was planted in a split-plot design with four replicates of individual barley IL in a tunnel. The treatments (control and drought) were assigned to the sub-plots, within which the lines were assigned randomly as described above in the section 2.1.3 and 2.1.4. The first fully expanded leaf was harvested in liquid nitrogen for drought and control conditions and stored at -80°C before proline determination. Proline content was measured using a colorimetric procedure according to Bates *et al.,* (1973). The proline accumulation was quantified in µg/g of the harvested fresh leaf material.

2.12 Proline content (PC) measurement

Solutions: All the solutions are stored at -20°C.

Extract: 20 to 50 times diluted fresh weight (w/v), typically in a 70:30 ethanol:water mixture (v/v) (Hummel *et al.,* 2009).

Standards: proline solutions ranging from 1 ppm (parts per million) to 20 ppm (parts per million), in the same medium as the one used for the extraction.

Reaction mix: ninhydrin 1% (w/v) in acetic acid 60% (v/v), ethanol 20% (v/v). Protect from light.

Procedure:

1. Weigh the plant material before storing at -70 °C or homogenized.

2. Homogenized the frozen plant material in 3% aqueous sulphosalicylic acid (0.01g/ 0.5 ml) and the residue is removed by centrifugation at 12 000 g for 10 min.

3. Take 1 ml of the homogenized tissue reacts with 1 ml acid-ninhydrin and 1 ml of glacial acetic acid in a test tube for 1 hour at 100°C and the reaction is terminated in an ice bath.

4. Acid-ninhydrin is prepared by warming 1.25 g of ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid, with agitation, until dissolved. Kept cool (stored at 4°C), the reagent remains stable only for 24 hours.

5. The reaction mixture is extracted with 2 ml toluene, mixed vigorously and left at room temperature for 30 min until separation of the two phases.

6. The 1 ml upper phase containing toluene is measured at 520 nm using toluene as a blank.

7. The proline concentration is determined from a standard curve using D Proline.

$$\text{Prolin} = \frac{\mu g \frac{\text{proline}}{\text{ml}} \text{ x F x } 3\text{ml}}{\text{weight (g)}}$$

The factor F is calculated F = 3 ml / a ml supernatant

2.13 QTL validation in IL S42IL-143 and derived BC4S2 population

To validate the QTL effect in IL S42IL-143 and to test the segregation of QTL alleles, we performed a pilot experiment in a derived population (BC4S2) from QTL bearing IL S42IL-143 in which S42IL-143 was used as control parent together with Scarlett. For this, seeds of HR S42IL-143 population and control genotypes were sown in climate chamber under control conditions. The pots were randomized after sowing single seed per pot (10 × 10 × 12 cm). Drought stress treatment was executed 10 days after germination by eliminating the water supply

completely. The treatment pots were kept under stress and first fully expanded leaf was harvested for each drought and control levels for proline measurement. Leaf material was harvested at same time from 09 to 10 hours under light inside the growth chamber and frozen in liquid nitrogen immediately. Proline content was measured nine days after drought treatment by colorimetric procedure according to Bates *et al.,* (1973). The growth chamber was supplied 12 hours artificial light at day temperature 22°C and 18°C night temperature at 50% to 60% relative humidity.

To see the segregation of QTL alleles with the low and high proline phenotypes under control and drought stress conditions, we developed a diagnostic polymorphic SSLP marker from a putative candidate gene from the QTL region. This marker was developed across the 3`UTR of the putative candidate gene P5cs1 that reveals 44 bp deletion in ISR42-8 allele as compared to Scarlett allele. Around 237 BC4S2 segregating progenies were genotyped using this diagnostic SSLP-marker and phenotyped for proline variation under drought stress conditions. The allelic polymorphism of Scarlett and ISR42-8 alleles was visualized on 2.5% standard agarose gel.

2.14 Positional cloning of QTL QPro.S42-1H

Positional cloning of major QTL on chromosome 1H were performed using a high resolution population derived from allele of QTL bearing IL S42IL-143 through backcrossing with recurrent parent Scarlett followed by two successive self-pollination to reach the generation BC4S2. In the first step, we sow around 3300 BC4S2 seeds along with control parental genotypes S42IL-143 and Scarlett and ISR42-8 in ten replications each. In the next step, DNA was extracted using the CTAB extraction method according to protocol by Virginia Tech Small Grains Breeding (Blacksburg, Virginia, USA) from fully expanded leaves from one week old seedlings. For genotyping we established two SNP derived KASP markers at the left (KASP-L) and right (KASP-R) border of the QTL region. KASP-L was 1383233 bp away from QTL region while, KASP-R was 531024 bp away from QTL region. The position was confirmed on physical map with the help of ensembl genome browser. The KASP genotyping was outsource at TraitGenetics®,

Gatersleben, Germany. After KASP genotyping, informative recombinants were selected among the 3300 BC4S2 progenies that showed recombination between KASP-L and KASP-R markers. These informative recombinants were then subjected to drought stress for 9 days and proline accumulation was measured according to Bates *et al.,* (1973) as mentioned earlier. Later, we incorporated two additional polymorphic markers, M1-L and M2-R to refine the QTL region. These markers enable us to refine the QTL region to single candidate gene of which a gene-specific marker was established to confirm the co-segregation of Scarlett and ISR42-8 alleles with low and high proline accumulation, respectively among the recombinants under drought stress conditions. A list of marker utilized in this analysis and their corresponding primers sequence information given in Appendix, Table S1.

2.15 Promoter analysis

Promoter analysis between Scarlett and ISR42-8 was performed using MAFFT alignment tools (Katoh *et al.,* 2002). DNA binding motifs across the promoter were identified MULAN analysis according to (Ovcharenko et al., 2005). MULAN performs local multiple DNA sequence alignments of finished and draft-quality sequences. The draft approaches employs a combination of BLASTZ and refine programs (Schwartz et al. 2003b). Pair wise alignments between each secondary sequence and the reference sequence are done initially by BLASTZ. Effectively, this allows each reference sequence nucleotide to be covered by either one or no alignment block from one of the secondary sequence contigs in each set of pair wise alignments. Alignment post processing is carried out by the *refine* program, which collects all the pair wise alignments into a single FASTA-formatted gapped alignment file that is available for the user to download from the results Web page. It identifies transcription factor binding sites evolutionarily conserved across multiple species. All the sequences can have gene annotation and any of the sequences can be represented as a base sequence. Identification of transcription factor binding sites conserved across multiple species could be performed with the use of interconnected multi transcription factor tools.

2.16 RNA Isolation and cDNA Synthesis

RNA extraction, purification, and quantification were performed to assess the expression of proline-related genes using the TRIZOL RNA Isolation Protocol. The Thermo Fisher RT-PCR kit (Thermo Fisher, Rochester, USA) was used for cDNA synthesis following the manufacturer's instructions.

2.17 Expression analysis of P5cs1 mRNA

To determine the expression of the P5cs1 gene in Scarlett and S42IL-143 plants, RNA extracted from the leaves was analyzed using semi-quantitative RT-PCR. Prior to the extraction, 10 days old seedling were exposed to drought stress for 3, 6 and 9 days. Semi-quantitative PCR reactions were performed in 30 µl volumes containing 3 µl reverse transcription reaction products as templates. PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis. Three biological replicates were used for the analysis. Primer sequences used for expression analysis are shown in Appendix, TableS1.

RT-qPCR was performed in 96-well plates using a 7500 fast Real-time PCR System and a SYBR Green-based PCR assay. Each reaction contained 3 µl diluted cDNA, 10 µl Maxima® SYBR Green/ROX qPCR Master Mix, and each primer at 0.4 µM to a final volume of 20 µl. The reaction mix was subjected to the following conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were then analyzed at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. In addition, a reverse transcription negative control was included to assess potential genomic DNA contamination. The qRT-PCR experiments were performed with two independent sets of RNA samples. For each RNA sample, three technical replicates were used in a final volume of 20 µl, and the average was used for RT-qPCR analysis. Relative expression of the P5cs1 gene was calculated according to the 2-ΔΔCt method (Livak and Schmittgen 2001). Threshold cycle (CT) values for both the target and internal control genes were the means of triplicate independent PCR reactions. Primers used for real time PCR analysis are provided in Appendix, TableS1.

2.18 Phenotyping S42IL-143 and Scarlett under drought condition for physiological parameters

Both genotypes were sown in climate chamber under control conditions. Three biological replicates were sown for various sensors were used in order to detect early drought stress response on the genotypes under investigation. For all measurements the fully expanded third leaf was analyzed non-destructively in order to detect the moisture content of plant tissue and parameters affecting photosynthetic activity.

Photosynthetic parameters

The infra-red gas analyzer, LI-6400 XT (LICOR Inc., Lincoln, NE, USA), was used to measure the photosynthetic parameters, namely stomatal conductance (Cond), transpiration rate (Trans), intercellular CO_2 concentration (Ci) and photosynthesis rate (Photo). To take continuous measurements the center of the third leaf was positioned in a leaf chamber which was attached with the sensor. The environmental settings of the leaf chamber were following: Temperature = 20°C, reference CO_2 stream = 500 µmol/mol, light intensity = 200 PAR (Photosynthetically active radiation). The air humidity was set to 'full by pass' to ensure measurements with the ambient air humidity set for the climate chamber (Biosciences 2008). Ten measurements were performed for each leaf without changing the position to calculate the mean (technical replicates).

Photosynthetic activity

The Photosynthesis yield analyzer Mini Pam II (Walz, Effeltrich, Germany) was used to measure the effective quantum yield of photosystem II (ΦII) which is a light adapted parameter that allows measurements of plants at steady-state of photosynthesis lightning conditions (Klughammer and Schreiber 2008). The ΦII parameter is calculated by Mini Pam II according Genty *et al.,* (1989):

$$\Phi II = \frac{Fm' - F}{Fm'}$$

Where F is the fluorescence yield measured briefly before application of a Saturation Pulse,

And Fm´ is maximal fluorescence yield of illuminated sample with all ΦII centers closed (Klughammer and Schreiber 2008). Reduction of effective quantum yield of photosystem II reflects the negative effect of drought stress on photochemical light

use efficiency of the tested plant. Five measurements were performed for different positions of each leaf to calculate the mean.

Estimation of leaf water status

A dual mode cavity microwave resonator (EMISENS GmbH, Juelich, Germany) as described by Dadshani *et al.,* (2015), was used to estimate the water status of barley leaves non-destructively. For the leaves being investigated, the signals of Mode 0 (150 MHz) were very small, therefore only Mode 1 (2.4 GHz) was used for the analysis. During the assessment of a leaf, the change of the quality factor *Q* and the resonant frequency, *fr* with respect to the empty resonator were recorded. According to Dadshani *et al.,* (2015) the microwave sensor parameters *FRS* and *IQS*, which are the negative relative frequency shift of *fr* and *Q*, respectively, highly correlate with the water content in tested plant material. Five measurements were performed for each leave without changing the position to calculate the mean (technical replicates).

SPAD value

Leaf chlorophyll concentration is an important parameter that is frequently measured as an indicator of chloroplast development, photosynthetic capacity, leaf nitrogen content or general plant health (Ling et al. 2011). The SPAD meter value is highly correlated with chlorophyll content in leaves and also is closely linked to drought stress (Markwell et al. 1995, Del Pozo et al. 2012). To measure the SPAD value we employed the Minolta SPAD-502 Chlorophyll Meter (Minolta Camera Co., Osaka, Japan) which is measuring leaf absorbance in red and near-infrared wavebands to estimate the amount of chlorophyll in the leaf.

3. RESULTS

3.1 Detection of QTL for shoot traits under drought stress conditions

A set of 54 and 73 introgression lines (S42ILs) was analyzed to categorize the QTLs for plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS). These phenotypic traits were genotyped with the 1536-SNP barley BOPA1 set (Close *et al.* 2009) based on the single nucleotide polymorphic markers in the years 2012 and 2013, respectively.

3.2 Variance analyses

Two-way analysis of variance (ANOVA) was employed to analyze trait variation among genotypes and across the replications and interaction between genotype and treatment across both years 2012 and 2013. The ANOVA described that genotypes showed significant ($P < 0.05$) variation for all traits except for CC, which is showing non-significant variation in the year of 2012. Whereas, replication showed non-significant ($P > 0.05$) variation for all studied traits in both years except for WS which showed significant variation even within replication in the year of 2012. Interaction between genotype and replication revealed non-significant ($P > 0.05$) variation only for HE across both years. Table 3 and Table 4 show the analysis of variance of the eight studied traits in both control and drought conditions across the years of 2012 and 2013, respectively.

Table 3: Variance analysis of eight investigated traits among 54 common S42ILs across years 2012 under control and drought conditions. Phenotypic traits evaluated are heading (HE), wilting score (WS), plant height (PH), number of leaves (NL), number of spikes (NS), chlorophyll content (CC), shoot fresh weight (SFW) and shoot dry weight (SDW).

Trait[a]	SOV[b]	DF[c]	MS[d]	F value	P value	CV[e]	h^{2f}
HE	Genotype	53	12.2	2.4	<.0001	4.5	73.2
	Replication	2	0.4	0.0	0.91		
	Treatment	1	1.7	1.0	0.31		
	Genotype x Treatment	53	5.3	1.0	0.35		
WS	Genotype	53	1.1	2.8	<.0001	24.3	64.8
	Replication	2	5.4	13.1	<.0001		
	Treatment	1	1280.7	9648.3	<.0001		
	Genotype x Treatment	53	18.0	43.9	<.0001		
PH	Genotype	53	332.2	10.4	<.0001	7.5	61.6
	Replication	2	7.0	0.2	0.80		
	Treatment	1	134878	89.6	<.0001		
	Genotype x Treatment	53	1490.2	46.7	<.0001		
NL	Genotype	53	0.6	4.7	<.0001	7.7	68.7
	Replication	2	0.0	0.4	0.64		
	Treatment	1	5.9	62.0	<.0001		
	Genotype x Treatment	53	0.1	0.8	0.0016		
NS	Genotype	53	19.2	12.3	<.0001	18.6	52.4
	Replication	2	0.6	0.3	0.67		
	Treatment	1	6556.4	51.8	<.0001		
	Genotype x Treatment	53	35.7	23.0	<.0001		
CC	Genotype	53	20.3	1.3	0.09	9.4	79.8
	Replication	2	3.1	0.2	0.81		
	Treatment	1	20497	667.2	<.0001		
	Genotype x Treatment	53	222.8	14.3	<.0001		
SFW	Genotype	53	222.4	2.1	<.0001	24.4	95.7
	Replication	2	0.2	0.0	0.9973		
	Treatment	1	87488	5429.6	<.0001		
	Genotype x Treatment	53	2645.1	25.5	<.0001		
SDW	Genotype	53	10.7	1.8	0.0012	14.1	80.4
	Replication	2	0.8	0.1	0.80		
	Treatment	1	1959.6	601.4	<.0001		
	Genotype x Treatment	53	94.5	16.1	<.0001		

[a] The phenotypic traits are defined in Table 1

[b] Source of variations

[c] Degrees of freedom

[d] Mean sum of square

[e] Coefficient of variation in %

[f] Heritability in %

Table 4: Variance analysis of eight investigated traits among 73 common S42ILs across years 2013 under control and drought conditions. Phenotypic traits evaluated are heading (HE), wilting score (WS), plant height (PH), number of leaves (NL), number of spikes (NS), chlorophyll content (CC), shoot fresh weight (SFW) and shoot dry weight (SDW)

Trait[a]	SOV[b]	DF[c]	MS[d]	F value	P value	CV[e]	h^{2f}
HE	Genotype	72	21.7	12.4	<.0001	2.6	72.7
	Replication	3	0.1	0.0	0.90		
	Treatment	1	0.8	0.1	0.86		
	Genotype x Treatment	72	1.4	0.8	0.8570		
WS	Genotype	72	0.6	5.0	<.0001	14.8	58.5
	Replication	3	0.1	1.1	0.30		
	Treatment	1	222.4	2.15	<.0001		
	Genotype x Treatment	72	17.5	132.1	<.0001		
PH	Genotype	72	464.0	9.3	<.0001	11.4	56.2
	Replication	3	43.6	0.8	0.40		
	Treatment	1	19.2	12.3	<.0001		
	Genotype x Treatment	72	1565.1	31.4	<.0001		
NL	Genotype	72	0.9	10.3	<.0001	5.9	63.6
	Replication	3	0.0	0.2	0.80		
	Treatment	1	0.6	4.7	<.0001		
	Genotype x Treatment	72	0.1	1.7	0.0005		
NS	Genotype	72	19.2	12.3	<.0001	18.6	61.8
	Replication	3	0.61	0.2	0.77		
	Treatment	1	332.2	10.4	<.0001		
	Genotype x Treatment	72	35.7	23.0	<.0001		
CC	Genotype	72	70.0	2.2	<.0001	13.3	76.4
	Replication	3	18.9	0.6	0.60		
	Treatment	1	12.2	2.4	<.0001		
	Genotype x Treatment	72	336.4	10.9	<.0001		
SFW	Genotype	72	70.9	4.4	<.0001	13.3	99.2
	Replication	3	12.6	0.7	0.50		
	Treatment	1	19.2	12.3	<.0001		
	Genotype x Treatment	72	1202.1	74.6	<.0001		

SDW	Genotype	72	83.1	25.5	<.0001	12.4	79.5
	Replication	3	2.2	0.6	0.50		
	Treatment	1	1.1	2.8	<.0001		
	Genotype x Treatment	72	35.2	10.8	<.0001		

[a] The phenotypic traits are defined in Table 1

[b] Source of variations

[c] Degrees of freedom

[d] Mean sum of square

[e] Coefficient of variation in %

[f] Heritability in %

3.3 Phenotypic characterization

Mean comparison of eight investigated traits among S42ILs, Scarlett and ISR42-8 is presented in Table 5. S42ILs revealed a significant variation in PH ranged from 73.0 to 120.0 cm in control conditions and mean was 90.3 cm, whereas the mean PH in Scarlett was 86.0 cm. Scarlett is erect type by nature, while wild barley ISR42-8 is bushy type, That's why data is not collected for PH of ISR42-8. Under drought conditions, a moderate reduction of mean PH observed in S42ILs compared to control, where Scarlett revealed significant reduction of PH. 'ISR42-8' revealed remarkable mean NL (6.8) under control conditions producing 8 maximum and 6.5 minimum NL where S42ILs and Scarlett produced 4.8 mean NL. Under drought conditions, increase of NL observed for all genotypes than control. 'ISR42-8' produced the highest mean NL (6.9) whereas Scarlett and S42ILs produced 3.1 and 5.2 NL, respectively. For HE, 'ISR42-8' showed the maximum delay in heading with an average of 58.0 days under control and 56.8 under drought stress condition. S42ILs showed wide range (40-60 days) in HE under drought stress conditions with the mean value of 49.1 days. Where, Scarlett showed no significant difference between control and drought treatment. A significant variation in NS was observed for S42ILs under control conditions which ranged from 5.2 to 21.0 spikes per plant where Scarlett and ISR42-8 ranged from 6.5 to 10.0 and 12.5 to 18.0 NS, respectively. Similarly, S42ILs revealed a wide range of NS under drought conditions with a mean of 11.3 where 'ISR42-8' showed mean NS 18.5 and Scarlett had lowest mean NS (6.9). Likewise shoot traits, 'ISR42-8' produced the highest SFW under control conditions with the mean

value of 56.7 g. S42IL exhibited a wide range of SFW ranging from 36.0 to 106.0 g with a mean of 62.8 g in control conditions where Scarlett produced lowest mean SFW in both control (44.3 g) and drought (14.2 g) conditions. A clear reduction was observed in SDW under drought stress condition. For SDW, again S42IL exhibited a wide range in both treatment conditions where Scarlett is not significantly different under control and drought conditions. S42ILs revealed a range of SDW giving a mean 20.8 g in control and 12.0 g in drought conditions. CC revealed a wide difference in S42ILs under control and drought treatment. Where, Scarlett showed the minimum CC in both treatments with an average value of 47.3 ug-cm2 in control and 22.4 ug-cm2 in drought stress condition. ISR42-8 revealed minimum score for WS with the mean value of 0.8 and Scarlett showed maximum WS (3) under control (3.5) condition. A significant variation in WS was observed for S42ILs under control conditions which ranged from 0.25 to 5.0 and drought condition which ranged from 5.0 to 8.0 with the mean of 6.5 for WS.

Table 5: Mean comparison of shoot traits among 73 S42ILs lines, Scarlett and ISR42-8 under control and drought conditions. Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

Trait[a]	Genotype	Mean[b] ± SE[c]		Control		Drought	
		Control	Drought	Minimum	Maximum	Minimum	Maximum
PH	S42IL	90.3 ± 0.6	58.1 ± 0.5	73.0	120.0	37.0	80.0
	ISR42-8*						
	Scarlett	86.0 ± 2.7	44.1 ± 3.1	80.0	89.0	39.8	53.4
NL	S42IL	4.8± 0.0	5.2± 0.0	4.0	7.0	4.0	7.75
	ISR42-8	6.8 ± 0.1	6.9± 2.2	6.5	8.0	6.0	6.0
	Scarlett	4.8 ± 0.1	3.1± 1.3	4.0	4.5	4.5	5.0
HE	S42IL	49.0± 0.2	49.1± 0.1	41.0	59.0	40	60
	ISR42-8	58.0 ± 0.5	56.8 ± 2.2	57.0	59.0	50	60
	Scarlett	51.3 ± 0.8	49.5± 0.2	53.0	50.0	49	50.0
NS	S42IL	8.3 ± 0.1	11.3 ± 0.8	5.2	21.0	8.5	14.0
	ISR42-8	15.4 ± 1.7	18.5 ± 0.7	12.5	18.0	12.0	8.3
	Scarlett	8.4 ± 1.2	6.8 ± 0.1	6.5	10.0	6.2	7.7
SFW	S42IL	62.8 ± 1.2	18.0 ± 0.2	36.0	106.0	5.0	5.0
	ISR42-8	56.7 ± 1.3	16.6 ± 0.6	47.5	67.2	18.0	21.0
	Scarlett	44.3 ± 0.2	12.3 ± 1.2	42.5	46.9	10.0	16.0
SDW	S42IL	20.8 ± 0.2	12.0 ± 0.1	14.6	41.0	10.0	24.0
	ISR42-8	20.9 ± 0.7	16.0 ± 0.0	17.5	23.2	15.0	18.0
	Scarlett	14.2 ± 1.5	11.0 ± 0.0	14.5	15.2	12.0	10.0
CC	S42IL	51.7 ± 0.2	45.7 ± 0.4	35.2	66.9	30.0	61.6
	ISR42-8	58.3 ± 0.2	42.4 ± 0.9	58.5	59.2	41.0	44.0
	Scarlett	47.3 ± 1.2	22.4 ± 2.5	45.0	48.3	20.0	25.0
WS	S42IL	2.6 ± 0.0	6.5 ± 0.0	0.25	5.0	5.0	8.0
	ISR42-8	0.8 ± 0.2	3.3 ± 0.1	0.5	1.0	3.0	3.5
	Scarlett	3 ± 0.3	4.7 ± 0.1	2.5	3.5	4.7	5.0

[a] The phenotypic traits are defined in Table 5, [b] The Lsmeans of 73 S42ILs, ISR42-8 and Scarlett were calculated as an average of the phenotypic data for each trait across 2012 and 2013 for each treatment separately, [c] Standard error * Data not taken

3.4 Comparison of the S42ILs with the parents

The population S42ILs which consists of 73 ILs lines was tested for tolerance to drought. Analysis of variance revealed high significant variation among S42ILs lines and genotype*treatments interaction in most of investigated traits across the year 2012 and 2013. For detailed description, results ANOVA of the investigated traits in S42 population are shown in (Table 6 and 7) and discussed separately for each trait. Table 6 and 7 show the summary statistics of all the studied traits across both control and drought conditions in the years 2012 and 2013.

Table 6:.Means and simple statistics in 54 S42ILs lines across control and drought conditions in 2012. Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

	Traits	Mean	SD	Minimum	Median	Maximum
Control	PH	90.35	8.347	73.10	88.9	120.2
	NL	4.762	0.391	4.000	4.75	6.00
	HE	48.90	2.6	41.00	49.0	55.0
	NS	8.798	1.405	5.250	8.50	12.5
	SFW	62.33	15.05	36.85	56.5	106.2
	SDW	20.98	3.381	15.35	20.1	41.20
	CC	47.51	3.103	35.55	47.2	56.9
	WS	0.915	0.563	0.250	1.00	5.00
Drought	PH	59.3	9.96	39.6	57.6	91.7
	NL	4.85	0.41	3.50	5.00	6.00
	HE	48.6	2.40	40.0	49.0	53
	NS	4.07	1.24	2.250	3.75	9.00
	SFW	21.1	5.10	15.5	19.8	51.0
	SDW	13.3	1.50	8.69	13.3	20.5
	CC	35.6	4.83	20.1	35.9	46.7
	WS	4.36	0.90	2.00	4.00	6.50

Table 7: Means and simple statistics in 73 S42ILs lines across control and drought conditions in 2013. Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

	Traits	Mean	SD	Minimum	Median	Maximum
Control	PH	75.4	10.8	54.0	74.7	104.7
	NL	5.06	0.42	3.75	5.00	7.00
	HE	49.1	2.00	42.0	49.0	55.0
	NS	8.16	2.50	4.00	7.50	21.0
	SFW	42.2	5.90	25.7	42.6	58.8
	SDW	16.3	4.21	11.9	15.4	38.3
	CC	47.6	3.60	31.5	47.9	58.4
	WS	0.97	0.26	0.25	1.00	2.00
Drought	PH	48.4	9.10	23.5	48.0	73.2
	NL	5.25	0.38	4.00	5.25	6.75
	HE	48.9	2.10	40.0	49.0	54.0
	NS	8.21	2.70	4.25	7.50	25.7
	SFW	18.1	3.32	13.5	17.3	34.6
	SDW	12.7	3.04	10.5	12.1	44.7
	CC	35.8	7.98	8.70	37.0	51.6
	WS	3.86	0.68	1.25	4.00	6.00

3.5 Genetic correlation among investigated traits

Mutual correlation of selected shoot traits for 54 S42ILs in the year 2012 and 73 S42ILs in the year 2013 have been presented in Table 8, which were computed using the LS-mean of a trait for all accessions across tested years 2012 and 2013 separately.

Strong positive correlations were found between PH with, SFW, SDW and CC in the both years 2012 with r values 0.81, 0.80 and 0.72 respectively and in 2013 with r values 0.81, 0.70 and 0.72 respectively. PH had strong positive correlation with NS as well but only in the first year of research with correlation coefficients value of 0.80. For the correlation between PH and NL and WS a strong negative correlation found in the years 2012 and 2013, where the r values were -0.55 and -0.45 for NL and -0.83 and -0.78 for WS across the both years. For correlation between NL with SFW and SDW, a positive and strong correlation was found only in the year 2012 with r values 0.55 and 0.53 respectively. Strong, positive and highly significant correlations were detected for NS with SFW, SDW and CC with the r values 0.92, 0.90 and 0.73 in the year 2012 and 0.54, 0.67and 0.80 across the year 2013 respectively. While NS was found negatively correlated with WS, where the correlation coefficients were -0.79 and -0.54 in 2012 and 2013, respectively. For the correlation of SFW with SDW and CC, a significant strong positive correlation was found, while it had negative significant correlation with WS. Where the correlation coefficients values are 0.96, 0.74 and -0.80 in the year 2012 and 0.63, 0.64 and -0.89 in the year 2013 for SDW, CC and WS respectively. SDW was also found positively correlated with CC (0.69) in the year 2012 and negatively correlated with WS (-0.75 and -0.50) across the both years. For the correlation among CC and WS a significant negative correlation was found in the year 2012 (-0.79) and 2013 (-0.68).

Table 8: Pearson correlation coefficients (r) calculated by averaging the Lsmeans of a trait performance for each trait separately, under control and under drought stress conditions in the years 2012 and 2013. Blue color indicates positive correlation while pink color indicates negative correlation among the traits. Darker shade indicates strong significant value. Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

2012

	PH	HE	NL	NS	SFW	SDW	CC	WS
PH	-							
HE	0.084	-						
NL	-0.550	0.048	-					
NS	0.801	0.057	-0.165	-				
SFW	0.819	0.094	0.553	0.922	-			
SDW	0.800	0.094	0.535	0.903	0.961	-		
CC	0.722	0.057	-0.106	0.734	0.747	0.696	-	
WS	-0.830	-0.011	0.085	-0.797	-0.806	-0.753	-0.797	-

2013

	PH	NL	HE	NS	SFW	SDW	CC	WS
PH	-							
HE	-0.268	-						
NL	-0.450	0.464	-					
NS	0.002	-0.134	-0.062	-				
SFW	0.810	-0.150	0.020	0.548	-			
SDW	0.704	-0.029	-0.078	0.679	0.632	-		
CC	0.724	-0.128	-0.018	0.803	0.648	0.298	-	
WS	-0.780	0.235	0.048	-0.547	-0.891	-0.501	-0.689	-

Plant height (PH)

ANOVA of S42ILs population for plant height revealed highly significant differences among genotypes and interaction between genotypes and treatments across both the years. S42ILs population showed the variation among plant height ranging from 61.25-101.75 cm under control and 25.75-69.81 cm under drought stress conditions. Figure 5 is showing variation for plant height in S42ILs population under control and drought stress conditions.

The population has influenced by drought stress condition, the plants were shorter under drought treatment compared to control (Figure 6 (A) and (B)). Comparing PH of S42ILs lines to the parents under control and drought conditions, 67 lines were shorter, equal or non-significantly longer than the elite parent Scarlett while there

were six ILs which showed significantly higher plant height than Scarlett (Figure 6C). S42IL-140 showed highest plant height (101.76 cm) under control condition followed by S42IL-137 (101.39 cm). While, under drought stress condition S42IL-137 showed highest plant height (69.81 cm) followed by S42IL-148 (68.5 cm), whereas S42IL-140 was 63.62 cm high under drought stress conditions. Whereas, S42IL154 and S42IL-155 also showed significantly higher plant height than Scarlett with the value 78.5 and 100.56 cm under control and 51.18 and 63.37 cm under drought stress condition.

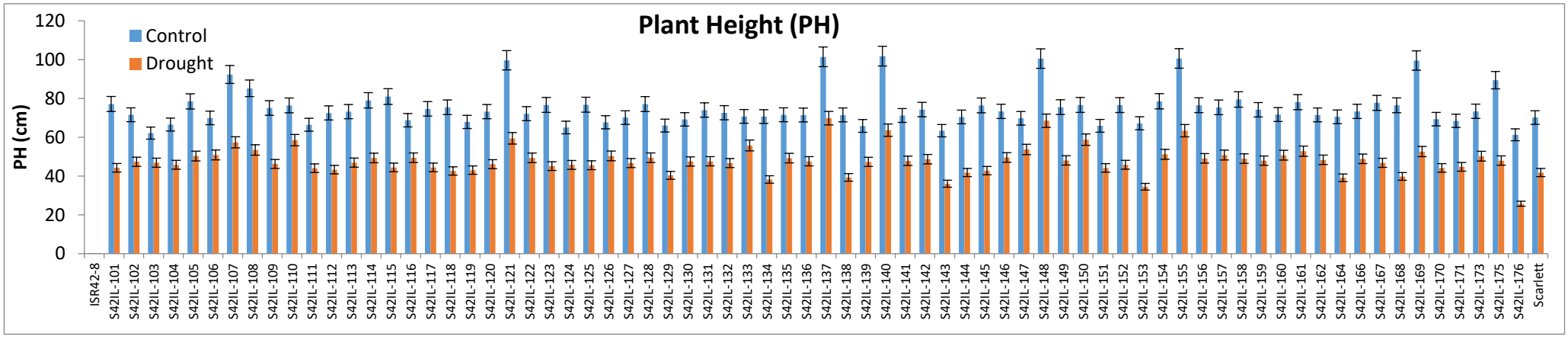


Figure 5: Variation for plant height in S42ILs population under control and drought conditions. Plant height was measured in cm. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

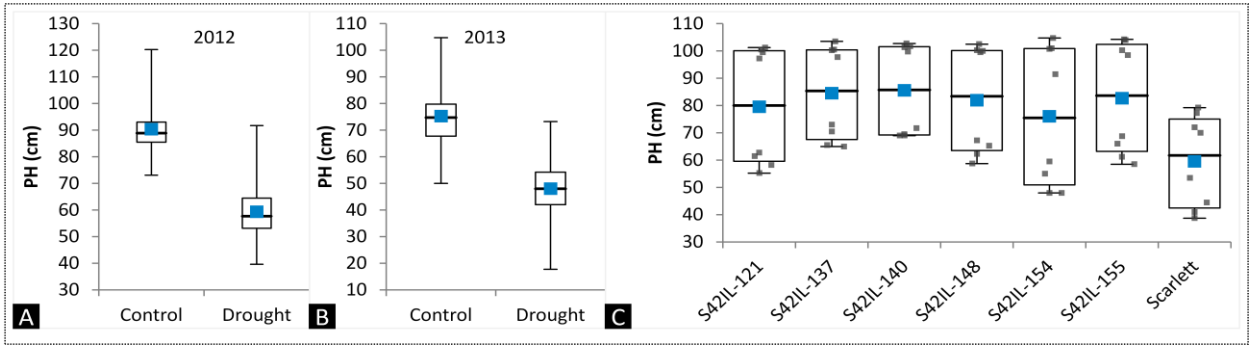


Figure 6: Frequency distribution of plant height in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Number of Leaves (NL)

Highly significant differences were observed among genotypes in both studied years and a significant difference was detected among genotypes * treatments interaction. S42ILs population was influenced by drought and gave more number of leaves under drought stress conditions. The number of leaves per main tiller ranged from 4 to 6.9 under control and from 4.12 to 7.2 under drought stress conditions (Figure 7).

Frequency distribution of S42ILs under control and drought condition is shown in (Figure 8 (A) and (B)). S42IL-176 gave maximum number of leave under control (6.9) and drought (7.25) stress conditions. S42Il-133 and S42IL-143 produced more number of leaves than Scarlett with the value of 6.25 and 6.18 under control and 6.3 and 6.0 under drought stress conditions. These two S42ILs gave more number of leaves in both years with an average of 5.5 and 6.25 leaves/main tiller more than the parent Scarlett under drought stress conditions (Figure 8C).

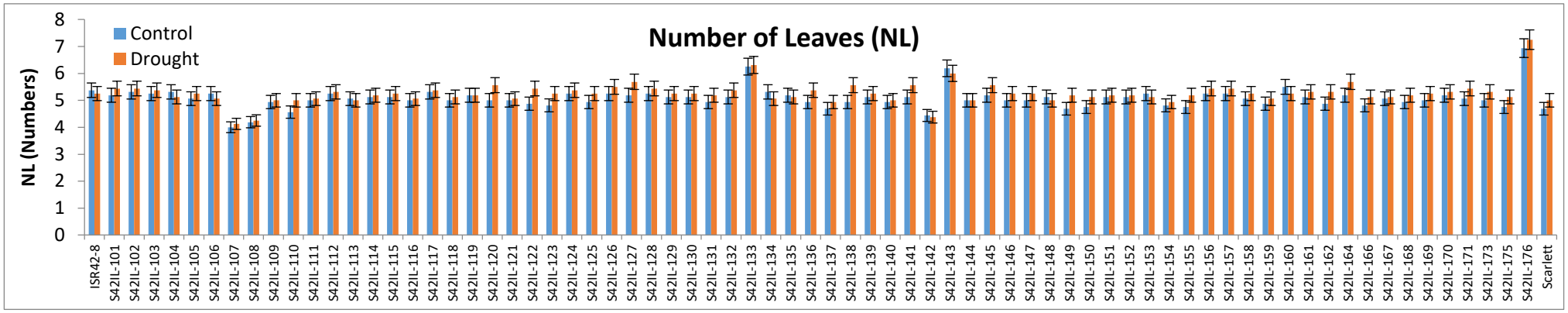


Figure 7: Variation for number of leaves in S42ILs population under control and drought conditions. Number of leaves was measured from main tiller of each plant. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

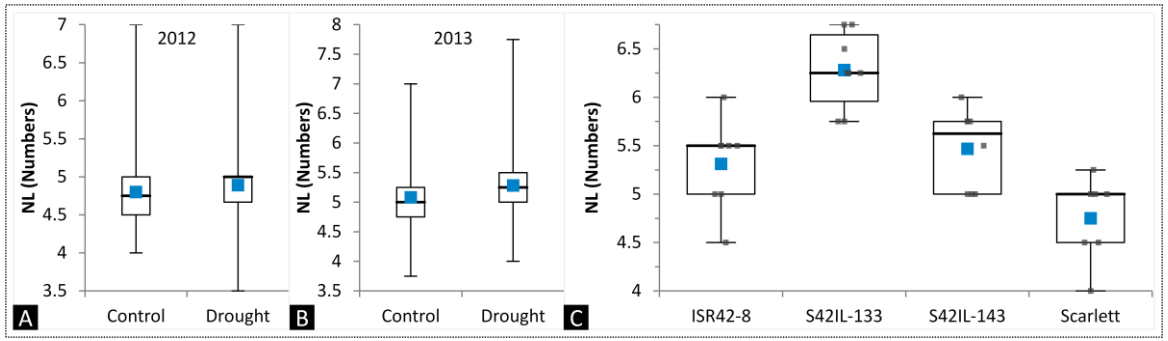


Figure 8: Frequency distribution of number of leaves in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Heading (HE)

Days to heading (HE) was highly significantly different among genotypes but non-significant among replication and genotypes * treatments interaction (Table 3 and 4). Drought treatment has no significant influence on S42ILs population ranged from 42 to 58 days under control and from 41 to 56 days after sowing under drought stress condition (Figure 9).

S42ILs population distribution for heading date over years is presented in Figure 10 (A) and (B). Under drought conditions, as an average over years, two S42ILs lines gave heading earlier as compare to the parent Scarlett under control as well as under drought stress conditions (Figure 10C). S42IL-107 and S42IL-108 gave earliest heading under control (42 and 44 days after sowing) and drought (41 and 42 days after sowing), respectively.

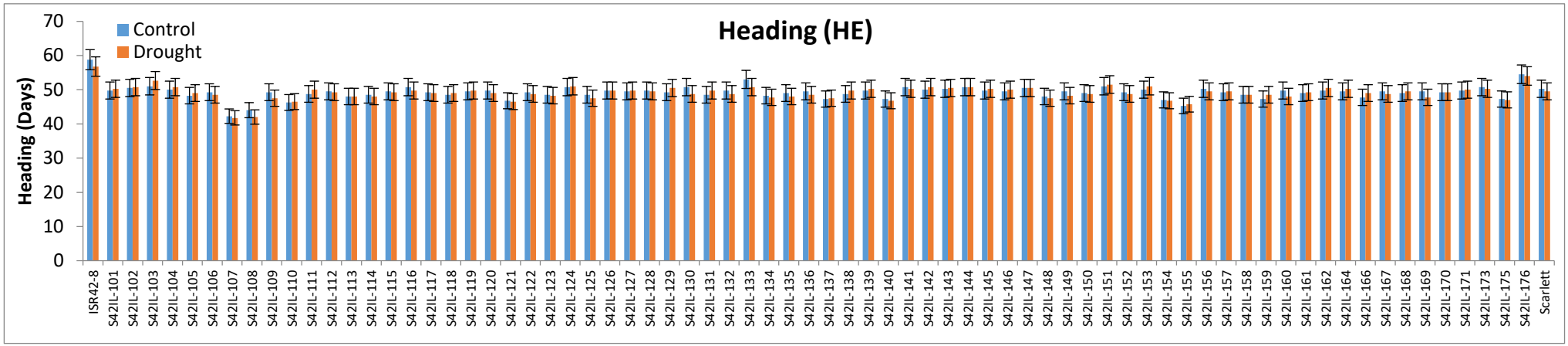


Figure 9: Variation for heading in S42ILs population under control and drought conditions. Heading was counted in number of days from sowing date. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

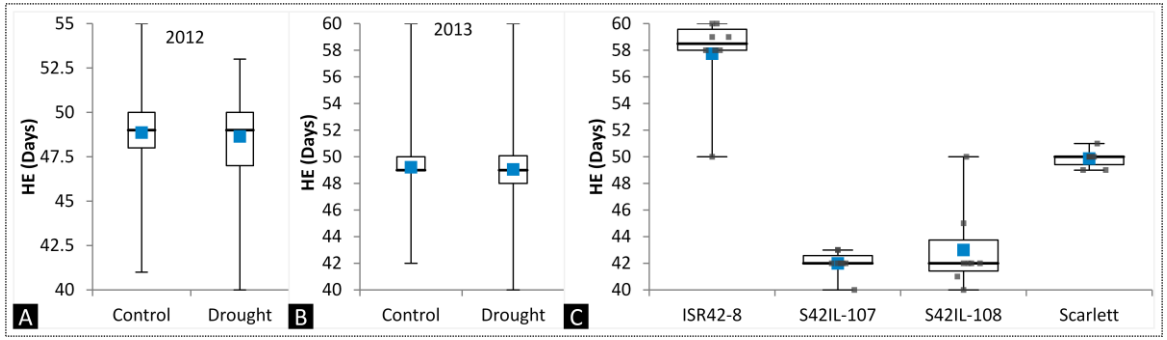


Figure 10: Frequency distribution of heading in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Number of spikes (NS)

For population S42, the ILs lines were evaluated for number of spikes and revealed highly significant differences among the genotypes and treatments over years. Number of spikes per plant ranged from 4.9 to 17.19 under control and from 5.19 to 22.63 under drought stress condition as shown in Figure 11. Population was effected more by drought stress in the year 2012 and produced less number of spikes under drought stress condition.

Figure 12 (A) and (B) is showing distribution of S42ILs population over the year 2012 and 2013 respectively. An introgression line S42Il-124 gave maximum number of spikes under control (17.19) and drought (22.63) condition with general average of 20 spikes per plant as compared to all S42ILs and both parents under control and drought stress conditions (Figure 12C).

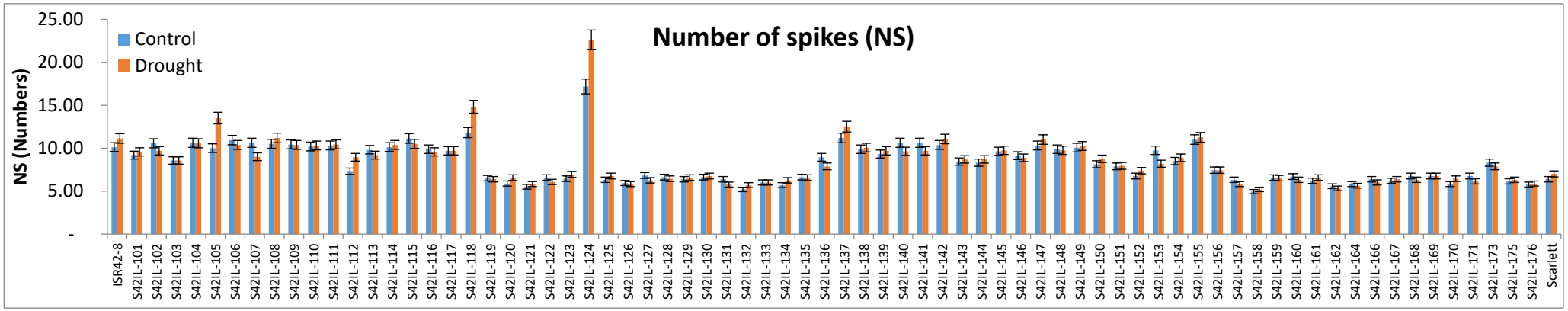


Figure 11: Variation for number of spikes in S42ILs population under control and drought conditions. Before harvesting total number of spikes were counted for each plant. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

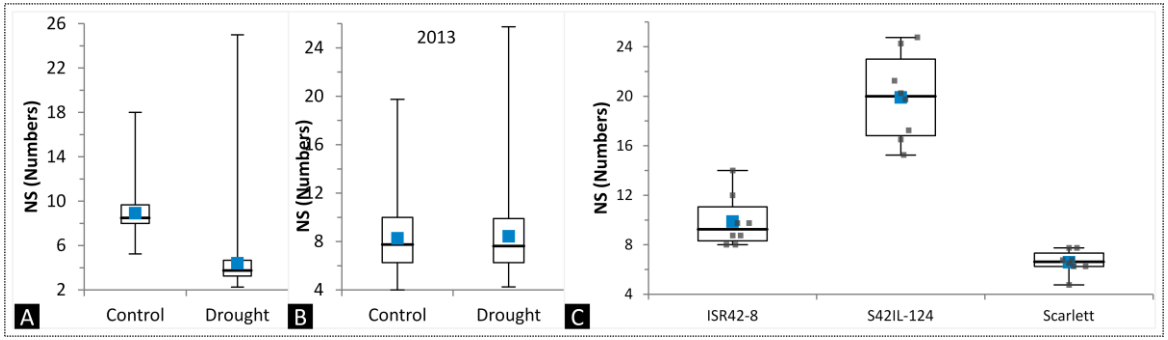


Figure 12: Frequency distribution of number of spikes in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Shoot fresh weight (SFW)

Highly significant differences were detected for shoot fresh weight in relation to genotypes and interaction between genotype and treatments under control and drought stress conditions across both the year 2012 and 2013 (Table 3 and 4). The minimum shoot fresh weight under control was 24.23 g/plant and decreased to 15.05 g/plant under drought stress condition. Similarly, the maximum shoot fresh weight under control was 56.69 g/plant that decreased to 37.02 g/plant under drought stress condition. The differences of shoot fresh weight among ILs as well as treatments is shown in Figure 13.

Drought stress condition influenced S42 population significantly and decrease the shoot fresh weight under drought condition (Figure 14 (A) and (B)). A total of six ILs lines yielded more SFW than the elite parent Scarlett (Figure 14C). S42IL-133 produced highest shoot fresh weight under control stress condition with the value of 56.69 g/plant followed by S42IL 124 (56.0 g/plant), S42IL-155 (52.48 g/plant), S42IL-154 (52.13 g/plant), S42IL-143 (50.39 g/plant) and S42IL-110 (46.48 g/plant). While, the maximum shoot fresh under drought stress condition was shown by S42IL-124 (37.0 g/plant). The shoot fresh weight in S42IL-143, S42IL-154, S42IL-155, S42IL-133 and S424IL110 was 29.13 g/plant, 28.8 g/plant, 28.5 g/plant, 27.4 g/plant and 26.9 g/plant respectively, under drought stress conditions.

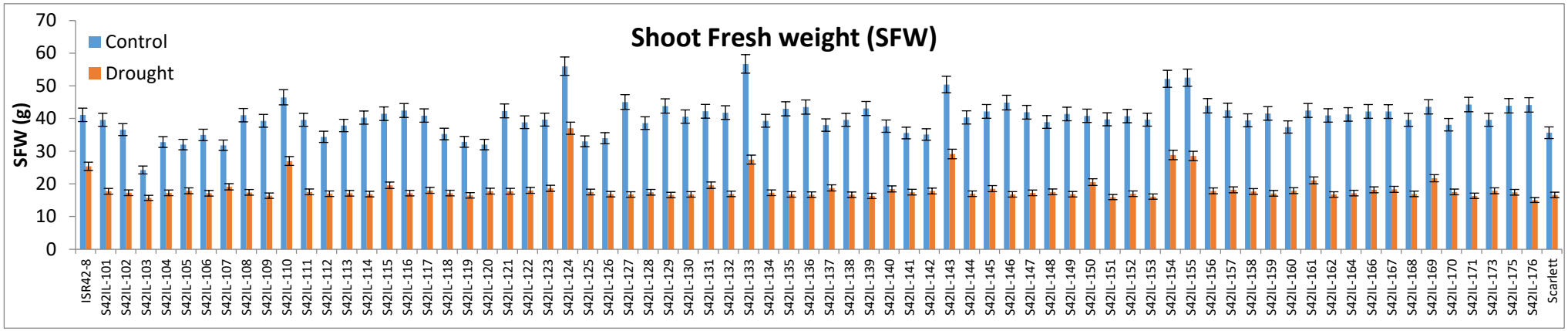


Figure 13: Variation for shoot fresh weight in S42ILs population under control and drought conditions. Shoot fresh weight of each plant was measured in grams. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

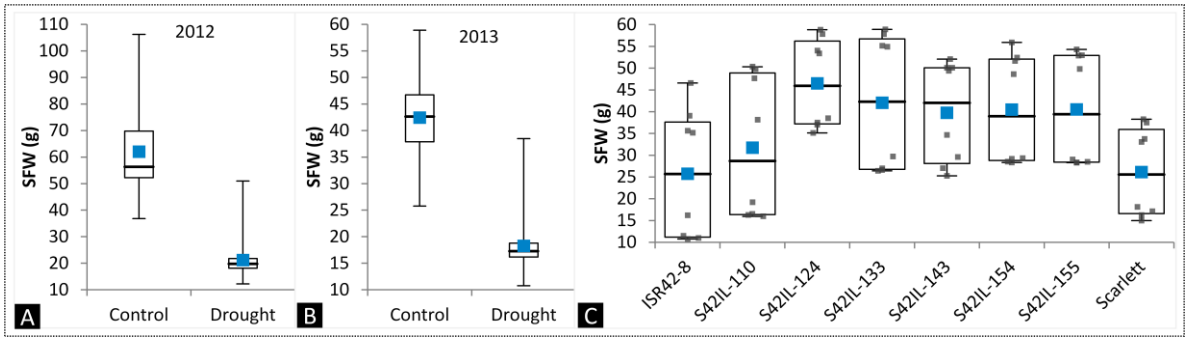


Figure 14: Frequency distribution of shoot fresh weight in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Shoot dry weight (SDW)

For the population, the same trend of shoot dry weight has been observed as for shoot fresh weight because of the strong correlation between them. Shoot dry weight was less under drought stress condition as compare to control condition. The minimum shoot dry weight under control condition was 13.98 g/plant that reduced to 11.54 g/plant under drought stress condition. Figure 15 is showing the differences among S42Ils population over the treatment and as well as within the population.

The differences of SDW among accessions as well as treatments over the years 2012 and 2013 were shown in Figure 16 (A) and (B). Same six S42ILs lines gave more SDW than the elite parent Scarlett and the exotic parent ISR 42-8 (Figure 16C). S42IL-155 produced highest shoot dry weight under control stress condition with the value of 35.15 g/plant followed by S42IL 124 (34.98 g/plant), S42IL-154 (33.96 g/plant), S42IL-133 (32.21 g/plant), S42IL-143 (30.37 g/plant) and S42IL-110 (25.94 g/plant). While, the maximum shoot dry under drought stress conditions was shown by S42IL-155 (22.75 g/plant). The shoot dry weight in S42IL-154, S42IL-133, S42IL-124, S42IL-143 and S424IL110 was 22.28 g/plant, 21.68 g/plant, 21.51 g/plant, 21.39 g/plant and 20.04 g/plant respectively, under drought stress condition.

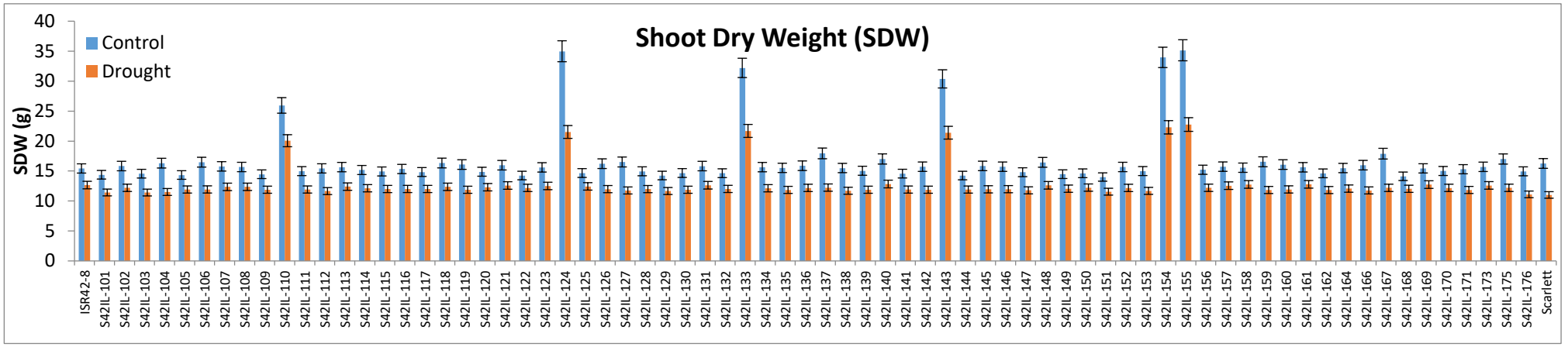


Figure 15: Variation for shoot dry weight in S42ILs population under control and drought conditions. Plants were dried in the oven at 50^0 C and then shoot dry weight of each plant was measured in grams. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

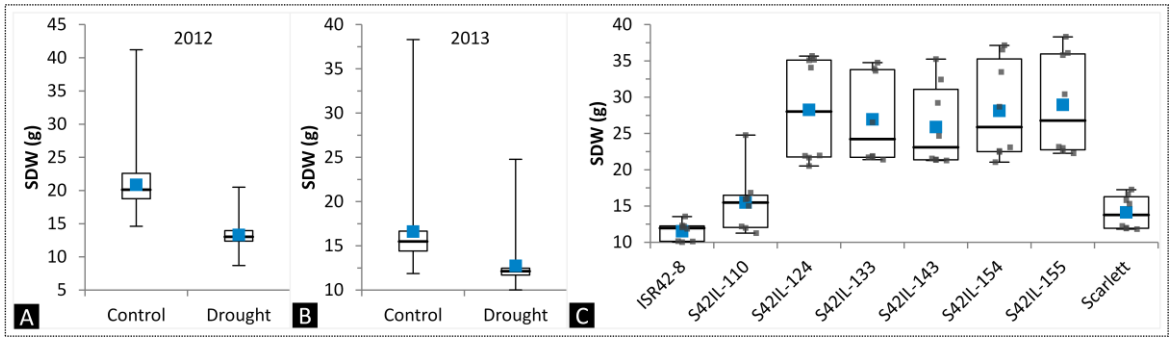


Figure 16: Frequency distribution of shoot dry weight in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Chlorophyll content (CC)

The S42ILs population showed wide range for chlorophyll content under drought and control stress conditions across the years (Figure 17). A drastic effect of drought stress is observed for chlorophyll content ranged from 29.36 ug-cm^2 to 56.51 ug-cm^2 under control and from 24.88 ug-cm^2 to 45.35 ug-cm^2 under drought stress conditions.

Distribution of S42ILs population under control and drought stress condition across both the years 2012 and 2013 is shown in Figure 18 (A) and (B). A total of four S42ILs lines showed more chlorophyll content than the recurrent parent Scarlett and exotic donor ISR42-8 under control and drought conditions (Figure 18C). The highest chlorophyll content was 56.51 ug-cm^2 in S42IL-107 followed by 55.16 ug-cm^2 in S42Il-108, 53.31 ug-cm^2 in S42IL143 and 52.44 ug-cm^2 in S42IL-141 under control conditions.

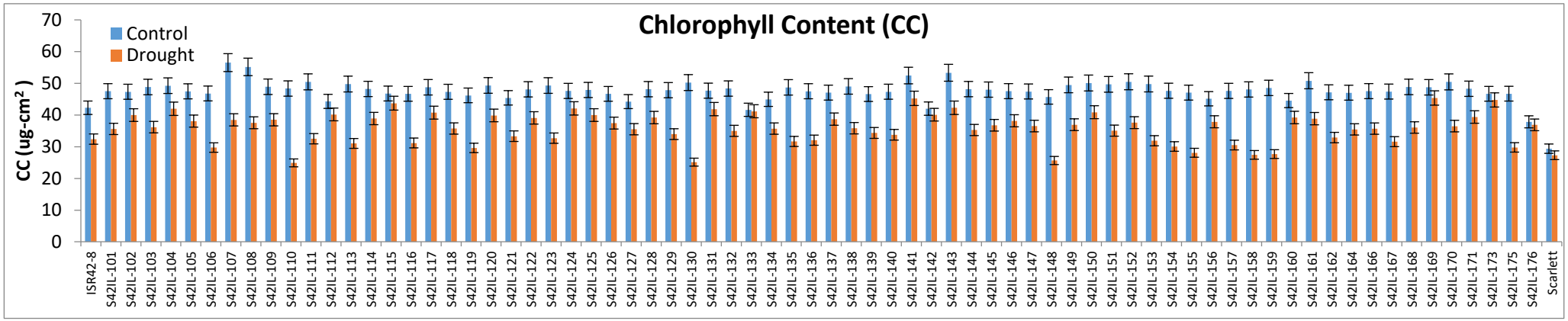


Figure 17: Variation for chlorophyll content in S42ILs population under control and drought conditions. Chlorophyll content was measured using SPAD meter. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

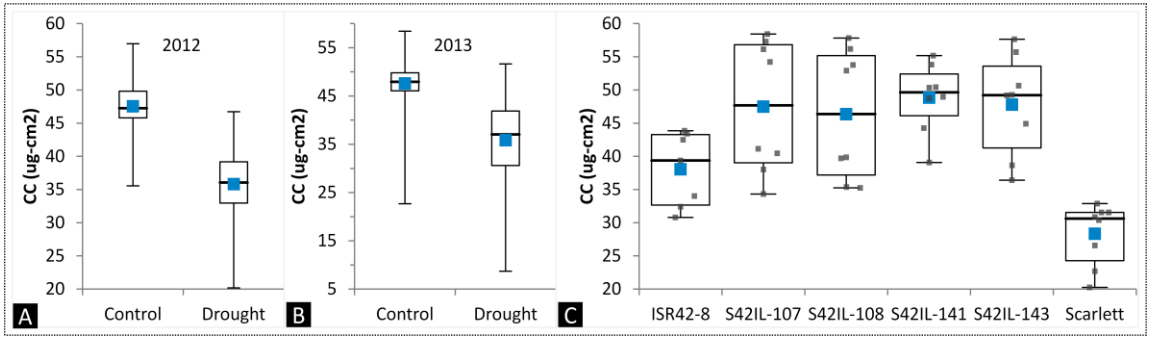


Figure 18: Frequency distribution of chlorophyll content in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

Wilting Score (WS)

The population S42ILs showed a significant variation in leaf wilting under control and drought conditions (Figure 19). Drought stress influenced the plants and wilted more as compared to control condition. The score for wilting score ranged from 0.3 to 1.63 and from 1.18 to 4.68 under drought stress condition.

Figure 20(A) and (B) is showing frequency distribution of S42ILs population under both treatments in 2012 and 2013, respectively. Treatment made a clear significant effect for wilting score on parents as well as S42ILs population. Eight S42ILs lines presented wilting scores ranged between 1 and 1.5 as resistant lines to drought (Figure 20C). S42IL-107 showed lowest wilting while under drought stress with the value of 1.18, followed by s42IL-108, S42IL-143, S42IL-141, S42IL 176, S42IL 154 and S42Il-155 with the value of 1.25, 1.68, 1.8, 2.3, 3.4 and 3.6 respectively.

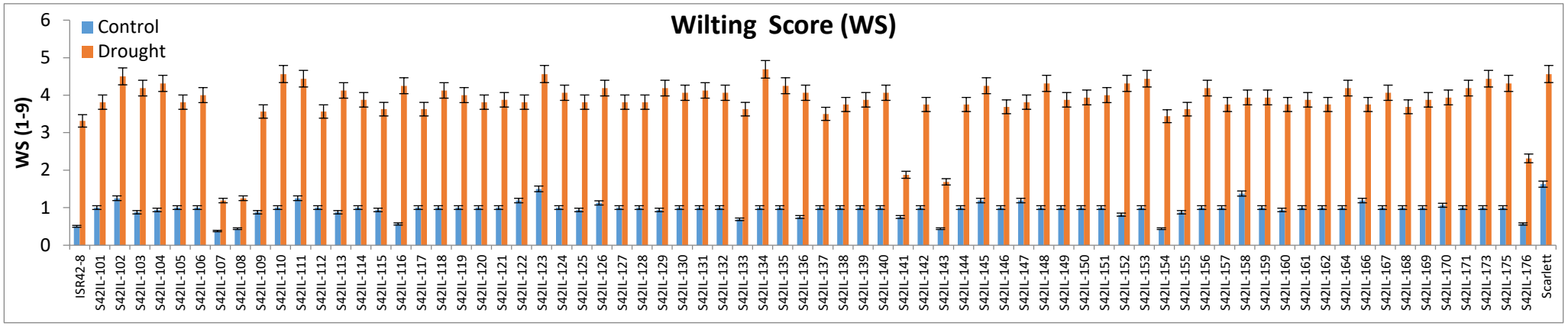


Figure 19: Variation for wilting score in S42ILs population under control and drought conditions. wilting score was measured using `standard evaluation system' (SES) for rice (IRRI, 1980). Blue and orange colors indicate plants under control and drought stress conditions, respectively.

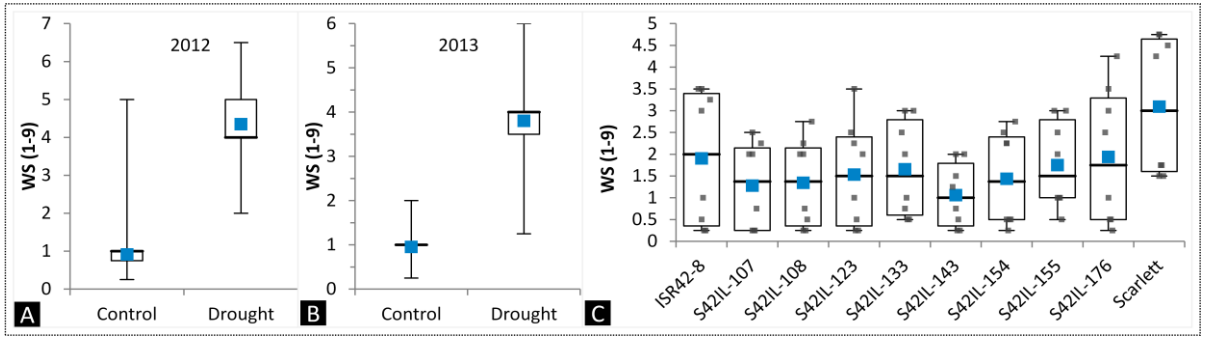


Figure 20: Frequency distribution of wilting score in S42ILs population under control and drought conditions with compared to the parents over the year 2012 (A) and 2013 (B) which indicate the differences among the treatment and population. S42ILs lines differ significantly from recurrent parent Scarlett is showing in (C). Blue dots represent the mean value for overall population in (A) and (B) and for the particular ILs in (C).

3.6 QTL detection

The present study reports on the genetic dissection of shoot-related traits of 72 S42ILs from a cross between barley wild accession ISR42-8 and cultivar Scarlett. The aim of the study was to validate the use of non-destructive high-throughput phenotyping to measure drought response in barley and to identify QTL derived from wild barley that control physiological traits related to drought stress. The QTL map was drawn according to Schmalenbach *et al*., 2011, high-throughput marker defined SNP locations. The ILs considered as a valuable genetic resource of complex QTL, fine mapping and positional cloning of underlying genes (Eshed *et al*., 1994, Szalma ,. 2007 and Schmalenbach *et al*., 2011). With respect to heritabilities, the phenotypic and genptypic data has been subjected to QTL analysis. Considering the position and corresponding target introgressions, all together 15 QTL were detected for five traits.

QTL for plant height

Six significant line treatment interactions were observed for PH with Scarlett which was summarized to 2 QTL located on chromosome 3H and 1H (Table 9 and Figure 21). Higher PH was recorded under control conditions than drought for selected ILs. The Hsp introgression increased PH from 33.3 to 43.2% (Table 9). Considering the Lsmeans of line treatment associations, S42IL-148 revealed maximum PH which possesses an introgression on 3H chromosome reaching from 198.32 cM at QPH.S42.3H. These QTL effects are localized to two chromosomal regions across all chromosomes (Figure 22).

QTL for number of leaves

For NL, two significant line treatment associations were summarized to one QTL located on chromosome 5H (Figure 21). The S42IL-143 and S42Il-133 revealed maximum NL score 6.4 and 6.2 respectively and exhibited Hsp introgression on chromosome 5H (QNL.S42IL.5H). The Hsp introgression increased NL from 31.9 to 36.1% (Table 9). The QTL for NL is localized to one chromosomal regions across all chromosomes (Figure 22).

QTL for Heading

The QTL analysis revealed one strong QTL for HE located on chromosome 2H. A total of two lines were found significant line by treatment associations with Scarlett Figure 21 for HE. According to the Table 9, the exotic introgression increased the trait value from -16.1 to -18.1%. The QTL for HE is localized to one chromosomal regions across all chromosomes (Figure 22). The highest HE differences between an IL and control were exhibited at QHE.S42IL.2H.a for S42IL-108, containing Hsp introgression in 47.4 to 58.5 cM of 2H chromosome which decreased number of days to HE by 18.1 % (Table 9).

QTL for number of spikes

Single significant line treatment interactions were observed for NS with Scarlett which were summarized to one QTL located on chromosomes 4H (Figure 21). More NS observed under drought conditions than control for selected ILs (Figure 21). The Hsp introgression increased NS 95.4 % (Table 9). Figure 22 shows the QTL for NS localized to one chromosomal region on 4H across all chromosomes.

QTL for shoot fresh and dry weight

Altogether six S42ILs showed significant associations for SFW and SDW. Due to overlapping of introgressions these associations were summed to putative three QTL which were located on chromosomes 1H, 2H and 5H. All the genotypes showed higher SFW and SDW in control than drought conditions in Figure 21. According to the Table 9, the exotic introgression increased the trait value from 19.1 to 95.4% for SFW and 12.7 to 97.8% for SDW. At *QSFW.S42IL.5H,* overlapping introgressions was observed by two ILs where S42IL-133 exhibited moderate SFW (60.9%) in positions 4.2 cM of 5H. However, S42IL-110 exhibited minimum SDW (12.7%) on 2H in the position 155.9cM. The highest SDW differences between an IL and control were exhibited at QSDW.S42IL.4H for S42IL-124, containing Hsp introgression in 176.5 to183.5 cM of 4H chromosome which increased SDW by 97.8 % (Table 9). Figure 22 shows the QTLs for SFW and SDW localized to four chromosomal regions on 1H, 2H, 4H and 5H across all chromosomes.

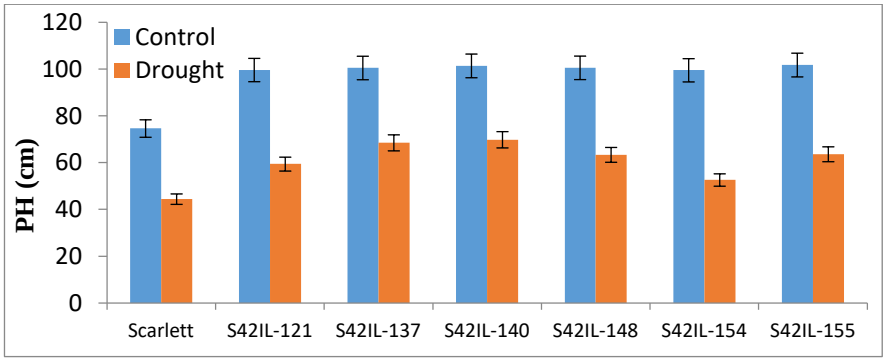
QTL for chlorophyll content

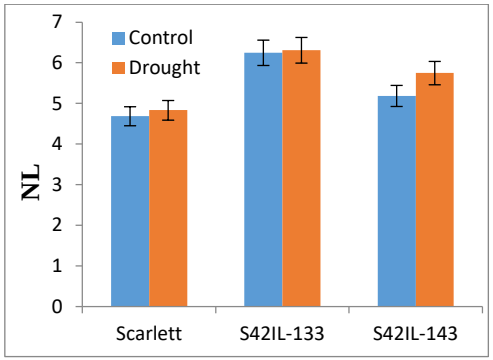
Four significant line treatment interactions were observed for CC with Scarlett which were summarized to two QTL located on chromosomes 1H and 2H (Figure 21). More CC observed under control conditions than drought for selected ILs (Figure 21). The *Hsp* introgression increased CC from 63.6 to 72.4 % (Table 9). The QTL for CC is localized to two chromosomal regions on 1H and 2H across all chromosomes (Figure 22).

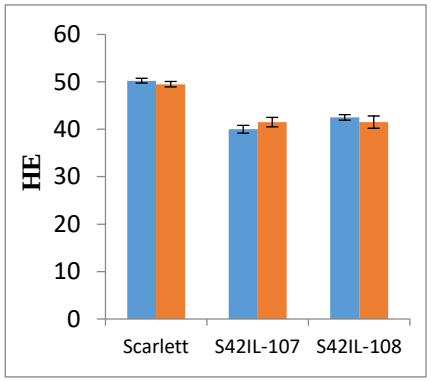
QTL for wilting score

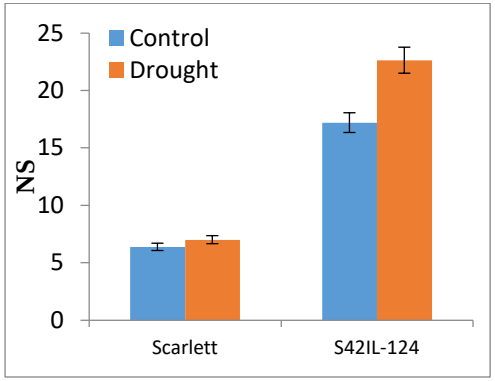
A total of five lines were significant lines by treatment associations with Scarlett (Figure 20 C) for WS. The effects were summarized to 5 putative QTL located on chromosome 1H, 2H, 4H and 5H. According to Figure 21, all the genotype exhibited improved WS values under control and drought as compared to recurrent parent Scarlett. The highest WS differences between an IL and control were exhibited at *QWS.S42IL.2H.a* for S42IL-107, containing *Hsp* introgression in 47.8 to 58.5 cM of 2H chromosome which decreased WS by -63.6 %, While S42IL-143 showed the minimal difference in WS by -27.3 % (Table 9). The QTL for WS is localized to five chromosomal regions across all chromosomes (Figure 22).

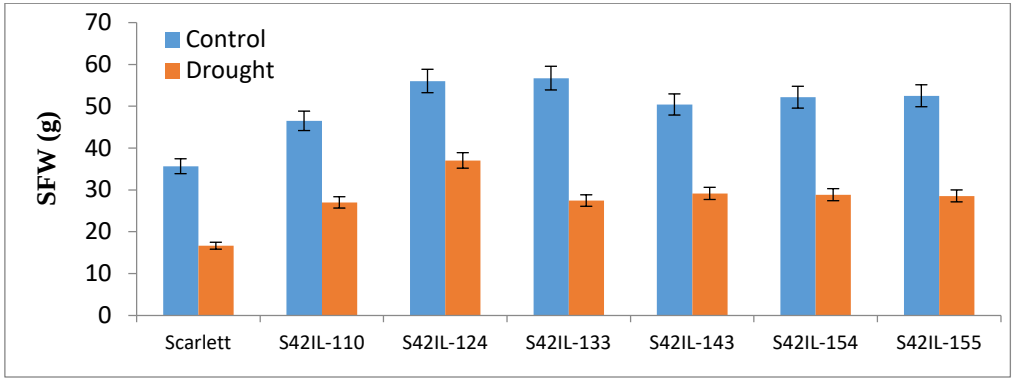
Figure 21: Comparison of selected introgression lines (ILs) for eight shoot traits with the recurrent parent Scarlett under control and drought stress conditions across the years 2012 and 2013. Each bar shows the mean value of three replicates.

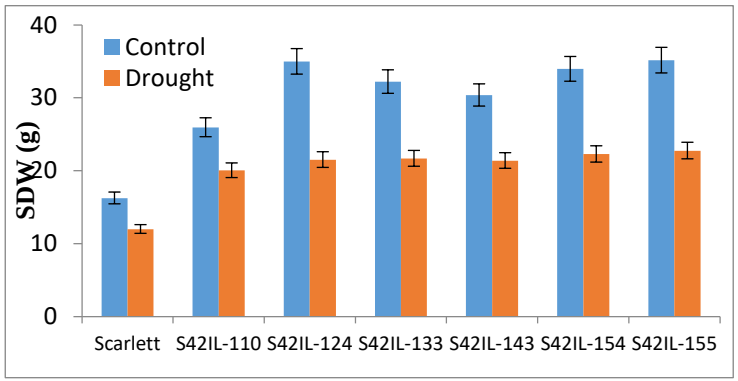


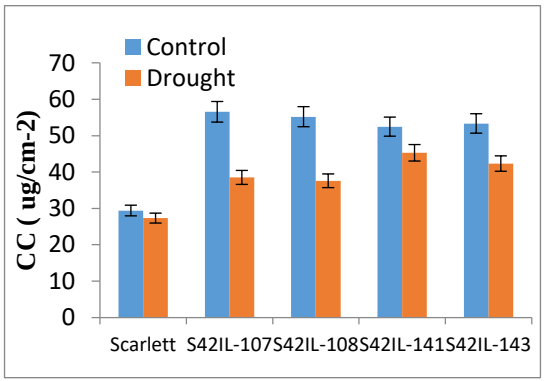


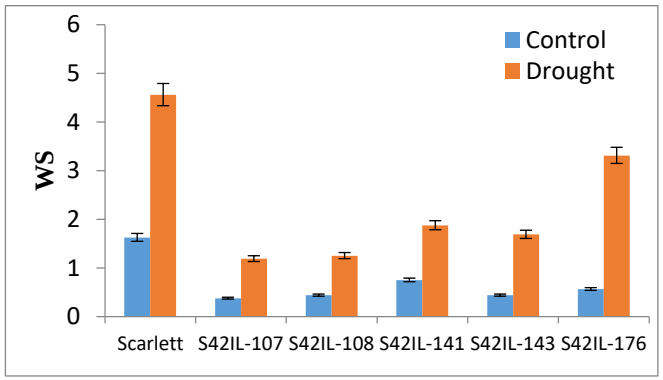












Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

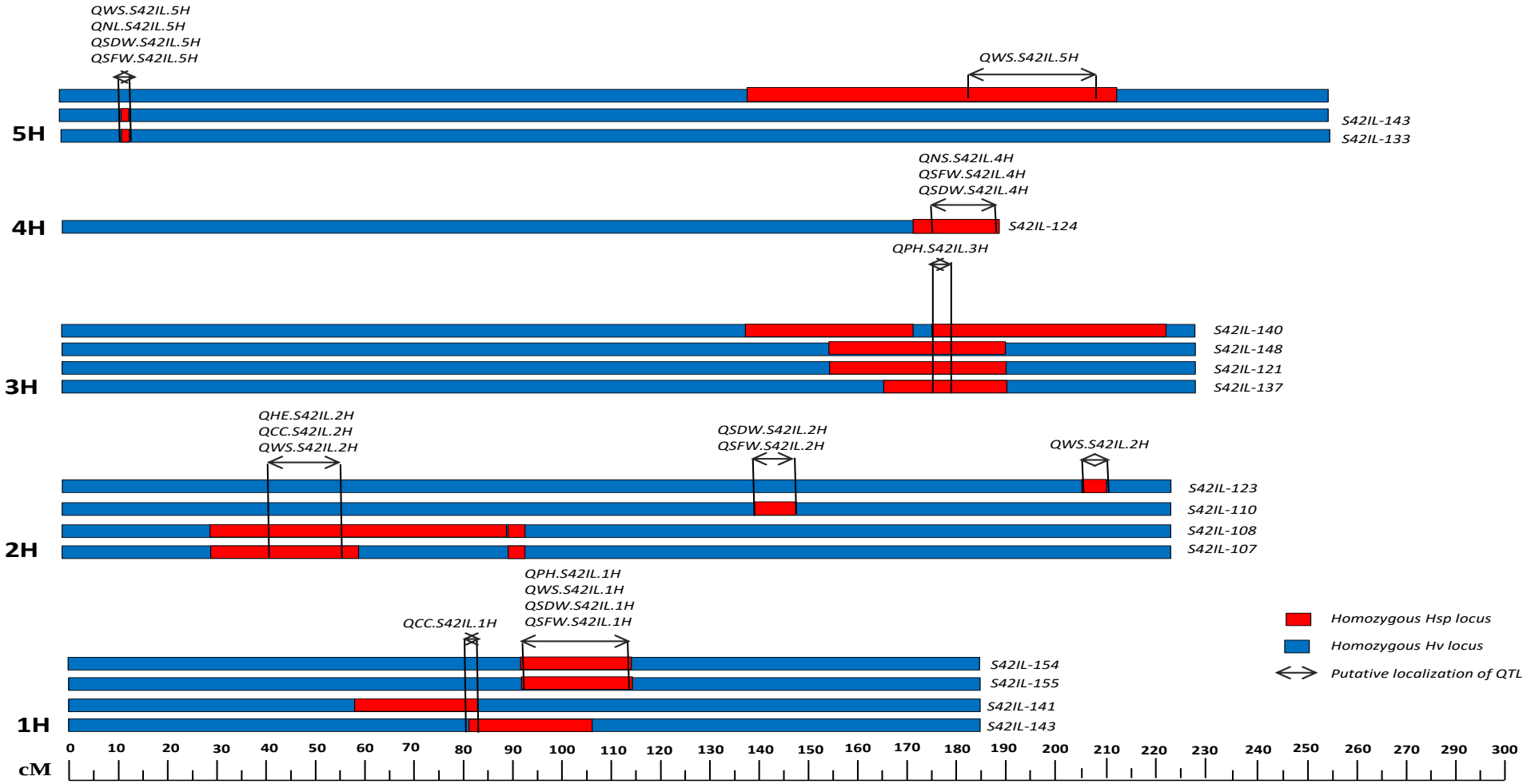


Figure 22: Chromosomal map of the selected introgression lines showing the validation of exotic QTL alleles. The red regions showed the location of wild introgressions according to Schmalenbach et al., (2011). The QTL regions are narrowed by comparing the common overlapping introgression across the S42IL population as well as by comparing QTL bearing wild introgression with the chromosomal regions having no QTL.

Table 9: List of significant QTL effects for eight studied traits detected among S42IL population. Phenotypic traits evaluated are plant height (PH), number of leaves (NL), heading (HE), number of spikes (NS), shoot fresh weight (SFW), shoot dry weight (SDW), chlorophyll content (CC) and wilting score (WS).

Trait[a]	QTL name	Chr.[b]	Introgression (cM)	S42ILs	Lsmeans S42ILs[c]	Lsmeans Scarlett[c]	RP(IL)[d] (%)
PH	*QPH.S42IL.1H*	1H	104.39-106.46	S42IL-154	82.5	59.5	38.6
				S42IL-155	76.4	59.5	28.4
	QPH.S42IL.3H	3H	185.1-190.8	S42IL-137	84.5	59.5	41.7
				S42IL-121	79.6	59.5	33.7
				S42IL-148	81.9	59.5	37.6
				S42IL-140	85.6	59.5	43.8
NL	*QNL.S42IL.5H*	5H	4.2	S42IL-133	6.2	4.7	31.9
				S42IL-143	6.4	4.7	36.1
HE	*QHE.S42IL.2H*	2H	47.4-58.5	S42IL-107	42	51.3	-18.1
				S42IL-108	43	51.3	-16.1
NS	*QNS.S42IL.4H*	4H	176.5-183.5	S42IL-124	12.9	6.6	95.4
SFW	*QSFW .S42IL.1H*	1H	102.3-127.7	S42IL-154	40.8	26.1	56.3
				S42IL-155	40.5	26.1	55.1
	QSFW.S42IL.2H	2H	155.9	S42IL-110	31.1	26.1	19.1
	QSFW .S42IL.4H	4H	176.5-183.5	S42IL-124	46.5	26.1	78.1
	QSFW .S42IL.5H	5H	4.21	S42IL-133	42.0	26.1	60.9
				S42IL-143	39.7	26.1	52.1
SDW	*QSDW .S42IL.1H*	1H	102.3-127.7	S42IL-154	27.2	14.1	92.0
				S42IL-155	27.7	14.1	96.4
	QSDW.S42IL.2H	2H	155.9	S42IL-110	15.9	14.1	12.7
	QSDW .S42IL.4H	4H	176.5-183.5	S42IL-124	27.9	14.1	97.8
	QSDW .S42IL.5H	5H	4.2	S42IL-133	26.9	14.1	90.7
				S42IL-143	25.8	14.1	82.9
CC	*QCC.S42IL.1H*	1H	82.51-84.14	S42IL-141	47.8	28.3	68.9
				S42IL-143	48.8	28.3	72.4
	QCC.S42IL.2H	2H	47.4-58.5	S42IL-107	47.4	28.3	67.4
				S42IL-108	46.3	28.3	63.6
WS	*QWS.S42IL.1H*	1H	102.3-127.7	S42IL-154	1.4	3.3	-30.3
				S42IL-155	1.7	3.3	-30.3
	QWS.S42IL.2H	2H	47.4-58.5	S42IL-107	1.2	3.3	-63.6
				S42IL-108	1.3	3.3	-60.6
	QWS.S42IL.4H	4H	99.5-110.2	S42IL-123	1.5	3.3	-54.5
	QWS.S42IL.5H	5H	4.2	S42IL-133	1.6	3.3	-36.4
				S42IL-143	1.1	3.3	-27.3
	QWS.S42IL.5H	5H	203.8-231.7	S42IL-176	1.9	3.3	-42.4

[a] The phenotypic traits are defined in Table2

[b] Chromosome number

[c] Least square means of the S42IL and Scarlett, respectively

[d] Relative trait performance of the S42IL compared to Scarlett, calculated as RP(S42IL)= [Lsmeans(S42IL) – Lsmeans(Scarlett)]/ Lsmeans(Scarlett)

3.7 Positional cloning of a major QTL for proline that modulates drought stress tolerance in cultivated barley

A set of 73 wild barley introgression lines was genotyped with high resolution using the Illumina Golden Gate assay. Out of 1536 BOPA1 SNPs, 1148 markers gave useful genotype information in the S42IL set. Of these, a total of 636 SNPs (55.4%) were polymorphic between Scarlett and ISR42-8 and were finally used for characterizing the S42ILs.

To determine the relationship between drought tolerance for proline content in *H. vulgare*, we first examined whether drought stress would enhance the proline levels in contrasting parents i.e.; german spring barley cultivar Scarlett (*H. vulgare ssp. vulgare*) and a wild barley accession ISR42-8 (*H. vulgare ssp. spontaneum*). The parents, Scarlett and ISR42-8 showed significant variation for proline content (PC) under control and drought stress conditions. ISR42-8 revealed a remarkable increase of proline content from 30.6 µg/g under control condition to 879.3 µg/g under drought stress within 9 hours, whereas Scarlett showed a modest increase in PC in drought block as compared to control. On average, ISR42-8 accumulated around 762.2 µg/g more proline content than Scarlett under drought stress conditions in 9 hours (Figure 23).

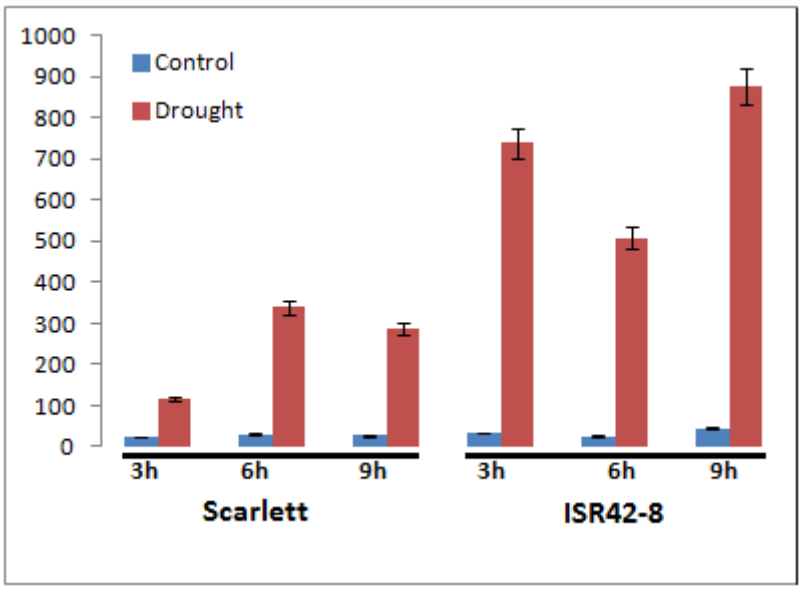


Figure 23. Effect of drought stress on proline accumulation in leaves of Scarlett and ISR42-8 within 3, 6 and 9 hours. Blue color shows the leaves under control

condition, while red color shows the leaves of plants under drought stress condition.

15 days old *H.vulgare* plantlets were treated with water stress for 3, 6 and 9 hours, respectively. All treatments had three biological replicates. Proline contents were measured by ninhydrin assay at A520 nm. Values represent means value of three independent experiments.

To extract the proline from samples, a standard curve was made using the series of proline standers i.e.; 1ppm, 2ppm, 5ppm, 10ppm and 20ppm showing in Figure 24 A, to calibrate the spectrophotometer which came up with a standard curve, linear regression with proline concentration on the x-axis and the measured absorbance at 520 nm on the Y-axis Figure 24 B.

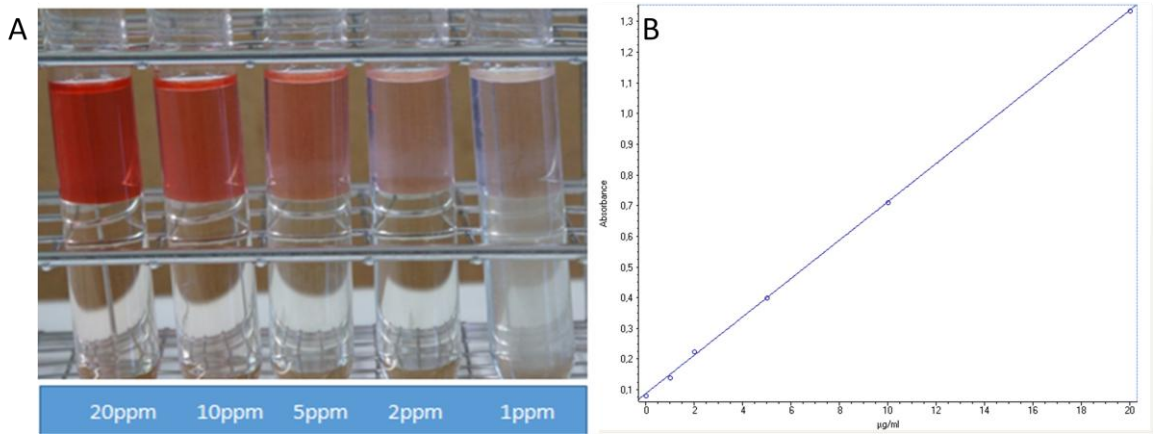


Figure 24. Proline standards ranging from 1ppm to 20ppm in the same medium i.e.; toluene as the one used for the extraction from samples (A). Calibration curve obtained with the spectrophotometer procedure with the cuvette (B).

3.8 Major QTL for proline accumulation

Genetic mapping of proline accumulation was performed using a library of introgression lines having chromosomal segments of wild barley accession ISR42-8 in the Scarlett background. Drought treatment has significant influence on S42ILs population for proline content. S42ILs population was sown in tunnel and fully expanded flag leaf was taken for proline measurement. The population S42ILs showed a wide range of proline content values with a mean of 0.3-822.0

µg/g under control and 7.8-4466.3 µg/g under drought stress conditions comparing with the elite parent Scarlett and the exotic parent ISR42-8 (Figure 25). Most of the S42ILs behave differently for proline under control and drought stress conditions. Among S42ILs population, S42IL-143 showed the highest accumulation for proline content under drought stress condition followed by S42IL-108 while S42IL-167 and S42IL-159 showed the minimal value for proline content while comparing with the elite parent Scarlett and the exotic parent ISR42-8.

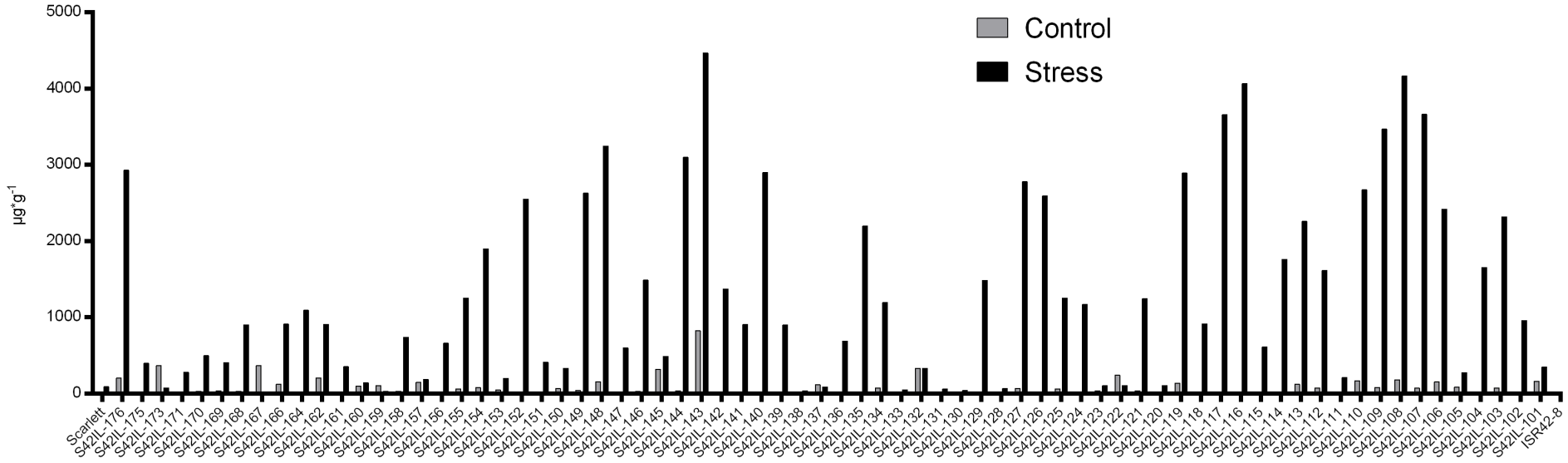


Figure 25. Variation of proline accumulation in 73 S42ILs population under drought stress and control conditions; proline content was measured in µg/g from fresh leaf material. Grey and black colors indicate plants under control and drought stress conditions, respectively.

For mapping, a comparison of individual IL with the recurrent parent Scarlett for variation in proline content was made using the Dunnett-test. A total of thirteen lines were revealed significant line by treatment associations with Scarlett for proline content. The effects were summarized to five putative QTL located on chromosome 1H, 2H, 3H, 4H and 5H that exhibited significant association with increased proline accumulation and were regarded as QTL based on their variation among the S42ILs population (Figure 26).

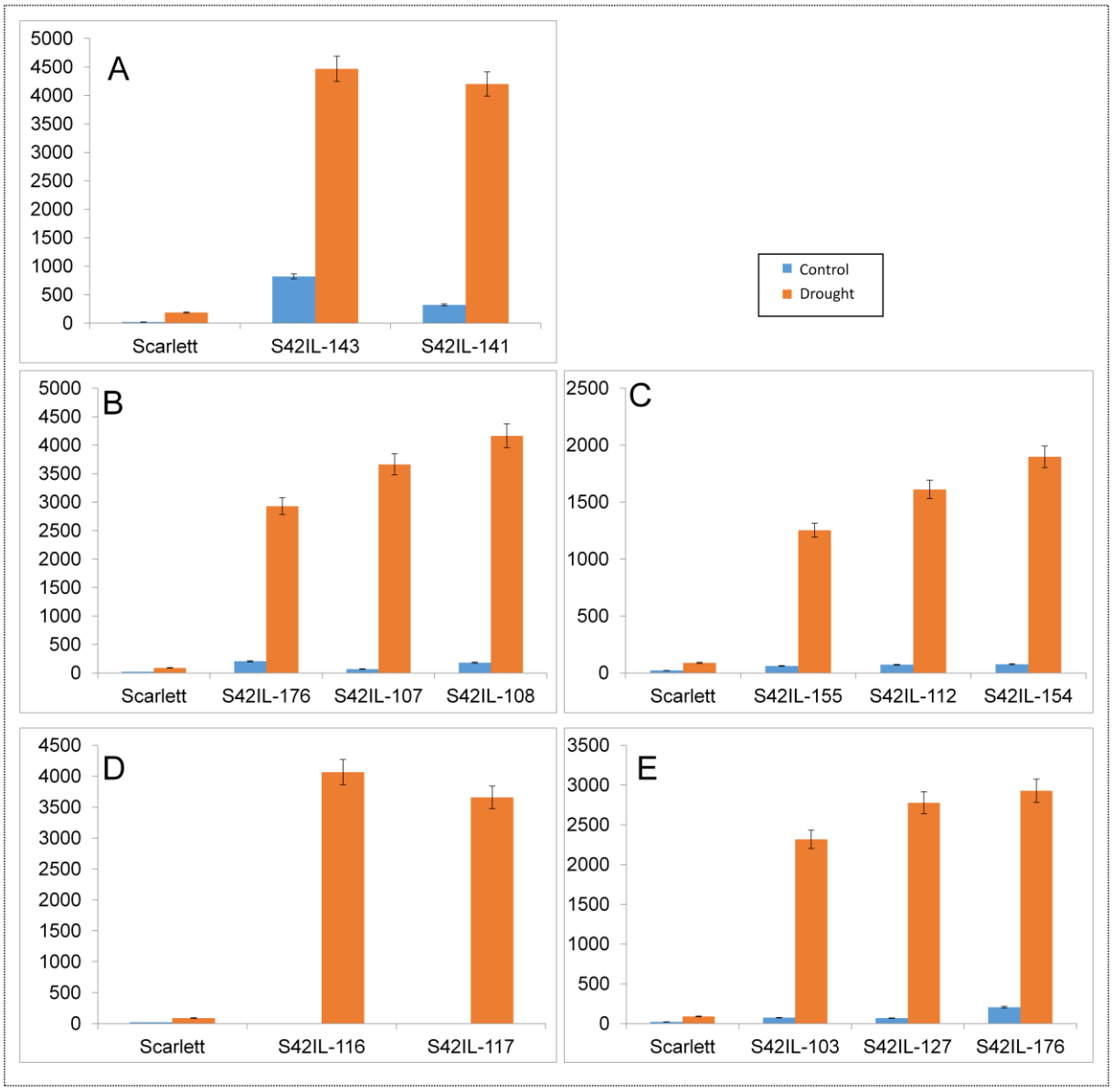


Figure 26: Quantification of five QTLs alleles for proline content on chromosome 1H (A), 2H (B), 3H (C), 4H (D) and 5H (E). proline content is measured in ug/g. Blue and orange colors indicate plants under control and drought stress conditions, respectively.

A total of five QTLs were distributed for proline content on chromosomes 1H, 2H, 3H, 4H and 5H. Three ILs, S42IL-176, S42IL-107 and S42IL-108 shared the common introgression on chromosome 2H and revealed a QTL between 47.45 - 55.52 cM according to SNP map by Schmalenbach *et al.,* (2011) and Honsdorf *et al.,* (2014). Similarly S42IL-112, S42IL-154 and S42IL-155 shared common introgression on chromosome 3H between 104.39-135.80 cM and summarized to single QTL at this locus. S42IL-116 and S42IL-117 also summed to a QTL for proline on 4H by sharing an introgression between 27.52-47.80 cM. Altogether three S42ILs, S42IL-103, S42IL-127 and S42IL-176 revealed significant association for proline on chromosome 5H. Due to overlapping of single common introgression these lines were summed to a single putative QTL between 139.93-140.07 cM. But the strongest QTL was found on chromosome 1H on S42IL-143 and S42IL-141 between 82.51- 84.14cM which is quite unique among large introgression in these two independent ILs and resulted in a remarkable increase in proline accumulation under drought stress conditions. These ILs revealed drought inducible proline accumulation up to 4,500 µg/g. The recurrent parent Scarlett and the ILs carrying Scarlett alleles at this locus exhibited a minor increase in proline accumulation under drought stress conditions. Five QTLs for proline content on different chromosomes in a circus plot are shown in Figure 27.

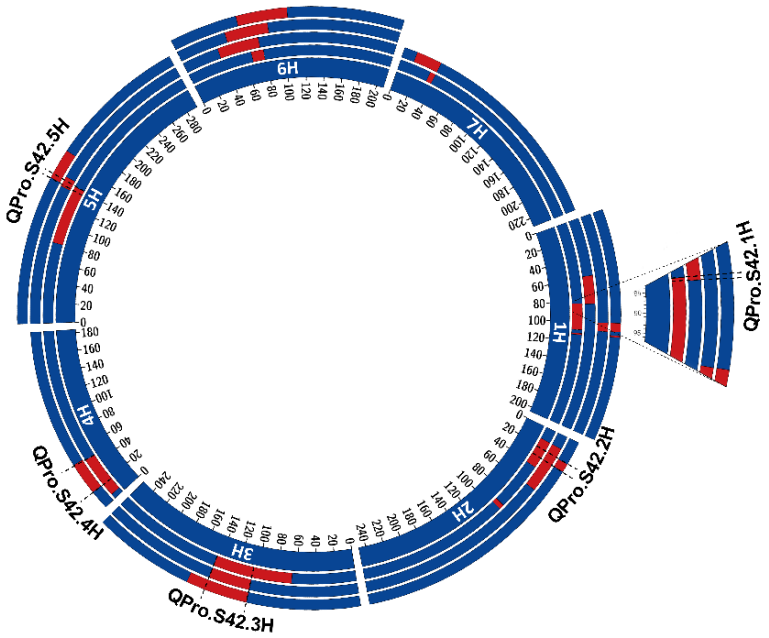


Figure 27: Circus plot showing five major QTLs in the S42ILs population. Five major QTLs for PC are shown in chromosome 1H, 2H, 3H, 4H and 5H. Blue color indicates the Scarlett genome. The red regions is showing the location of wild introgressions according to Schmalenbach *et al.,* (2011).

Considering that high proline accumulation becomes visible as it exhibits a red color after reaction with ninhydrine. Proline content accumulation under control and drought stress condition is shown in Figure 28. A darker color indicated more proline in the major QTL allele-bearing ILs S42IL-143 and S42IL-141 on chromosome 1H.

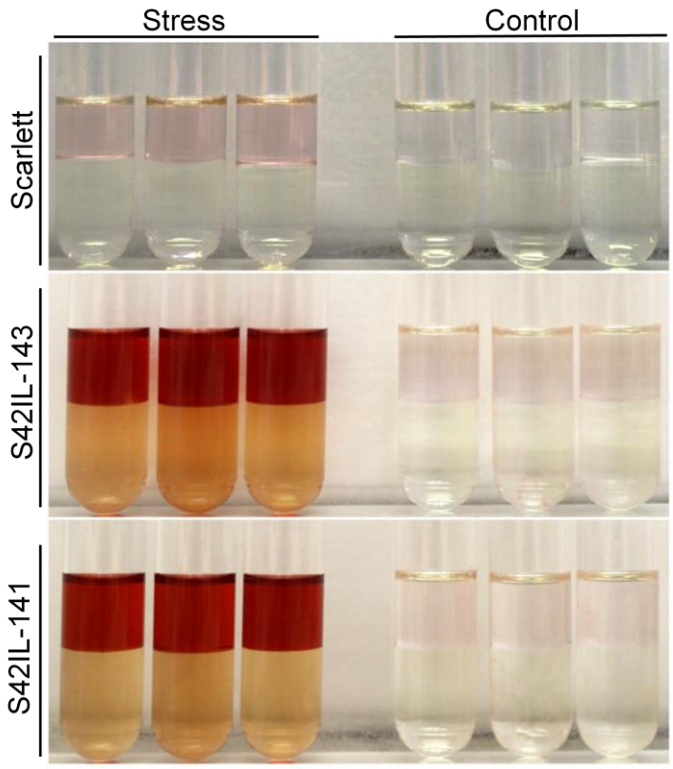


Figure 28: Free proline accumulation becomes visible after reacting with ninhydrine. Figure shows a clear difference of proline content accumulation under stress and control condition in Scarlett, S42IL-143 and S42IL-141. S42IL-143 and S42IL-141 are showing more proline content under stress condition as compared to recurrent parent Scarlett. A darker color indicates more proline.

Taking the information about SNP and their sequence maker allele at the position of part of introgression which was overlapping in both S42ILs provided by Schmalenbach *et al.,* (2011) and Honsdorf *et al.,* (2014), position was confirmed on physical map with the help of ensembl genome browser. The physical map revealed nineteen more genes between these SNPs; one of them was MLOC_57545, which is responsible for delta 1-pyrroline-5-carboxylate synthase1 enzyme.

List of all nineteen genes is given in table 10. (The MLOC numbers and their physical position are according to Ensembl Genomes: Extending Ensembl across the taxonomic space, which was last visited at 21.08.2017).

Table 10: Candidate genes in the P5cs1 locus in barley on chromosome 1H.

	Genes	Position (bp)	Gene ontology terms (molecular functions/ biological process)
1	MLOC_58251	402,450,680-402,458,484	hypothetical protein
2	MLOC_72250	402,475,895-402,478,271	60S ribosomal protein L36
3	MLOC_22683	402,570,395-402,573,145	kinase interacting family protein
4	MLOC_65624	402,576,749-402,579,517	uncharacterized protein
5	MLOC_69899	402,744,784-402,747,323	laccase 17 for Lignin Polymerization during Vascular Development Arabidopsis
6	MLOC_10200	402,748,178-402,753,742	uncharacterized protein
7	MLOC_63739	403,374,395-403,383,767	respiratory burst oxidase
8	MLOC_10769	403,409,297-403,411,094	uncharacterized protein
9	MLOC_77700	403,506,189-403,509,989	putative F-box/FBD/LRR-repeat protein
10	MLOC_50459	403,510,136-403,512,832	ATP-dependent peptidase/ ATPase
11	MLOC_77143	403,514,940-403,516,631	domain of unknown function DUF1618
12	MLOC_24040	403,529,842-403,530,644	uncharacterized protein
13	MLOC_57545	403,769,721-403,773,264	delta 1-pyrroline-5-carboxylate synthase1
14	MLOC_60455	404,064,748-404,067,341	bHLH transcription factor, putative
15	MLOC_16792	404,069,059-404,072,279	uracil phosphoribosyltransferase
16	MLOC_16794	404,073,093-404,074,443	prenylated rab acceptor family protein (it plays a role in vesicular trafficki lipid transport and cell migration)
17	MLOC_58017	404,203,623-404,207,667	copper-binding family protein
18	MLOC_50701	404,212,049-404,217,276	putative MYB family transcription factor
19	MLOC_6058	404,274,992-404,277,967	GINS complex subunit 1-like protein (GINS complex is essential for t initiation of DNA replication in yeast)

The nucleotide sequence and the deduced amino acid sequence were analyzed using the BLAST software online (http://www.ncbi.nlm.gov/blast). Blast analysis and multiple sequence alignments revealed that this MLOC_57545 gene had high homology with known genes in GenBank involved in proline metabolism in different species. The deduced amino acid sequence of MLOC_57545 was more than 90% identical to *Hordeum vulgare* homologues in GenBank and shared the highest identity of 95% as shown in Figure 29. We named that *Hordeum vulgare* homologues as HvP5cs1 gene. AK249154 is the accession number for homologues in *Hordeum vulgare* and was used as a candidate for proline content in *Hordeum vulgare* for further study.

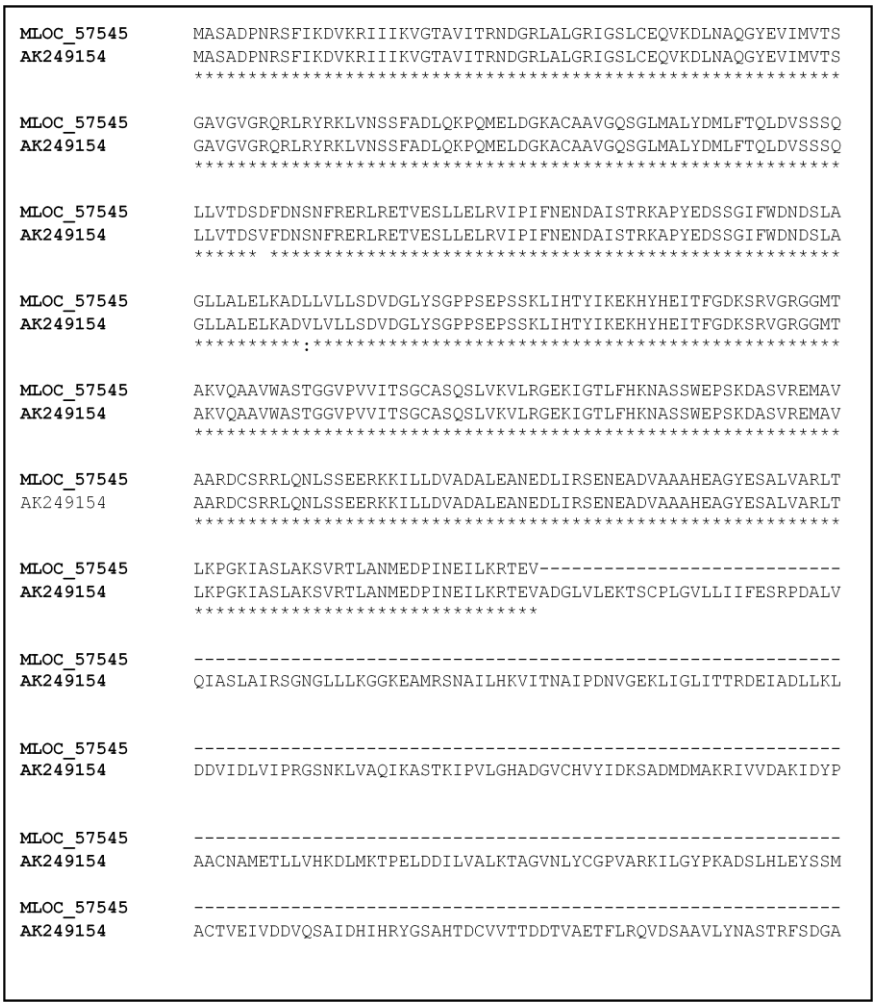


Figure 29: Alignment of MLOC_57545, responsible for delta 1-pyrroline-5-carboxylate synthase1 with candidate gene HvP5cs1 (AK249154) showing its similarity with each other.

Then the HvP5cs1gene was sequenced in Scarlett and ISR42-8 to identify the putative mutation associated with the variation in proline accumulation among the parents. Here, substitution mutations were found between Scarlett and ISR42-8 in exons 7, 9 and 13, of which only the C/T mutation in exon 13 resulted in an amino acid substitution from histidine (ISR42-8) to arginine (Scarlett) (Figure 30).

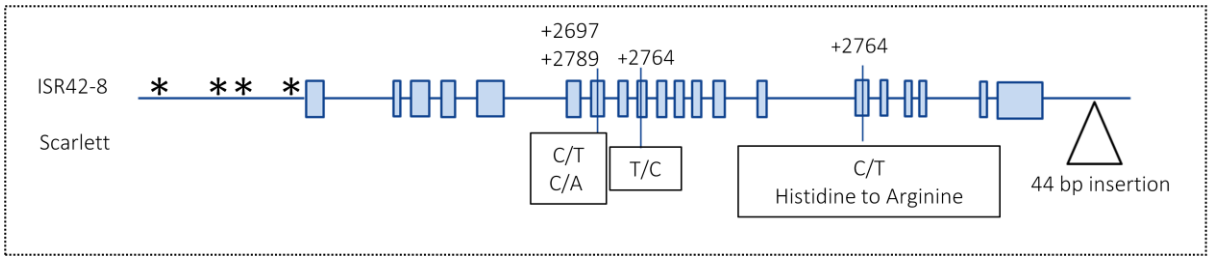


Figure 30: HvP5cs1 gene structure showing critical mutations between cultivar Scarlett and wild barley ISR42-8 within the gene.

The full-length barley HvP5cs1 gene comprised 20 exons and had a protein length of 716 aa. A phylogenetic tree was generated based on the ClustalW Protein alignment analysis using a Neighbor-Joining method in the MEGA 4 program. The following sequences with corresponding accession numbers were used for bioinformatics analysis: AtP5CS1 (NP_181510.1), AtP5CS2 (NP_191120.2); OsP5CS1 (NP_001055723.1), OsP5CS2 (NP_001044802.1); BdP5CS1 (XP_003568327.1), BdP5CS2 (XP_003564608.1); HvP5CS1 (Ak249154.1), HvP5CS2 (MLOC_37763.1). The phylogenetic analysis demonstrated conservation of the HvP5CS1 protein among monocots is shown in Figures 31.

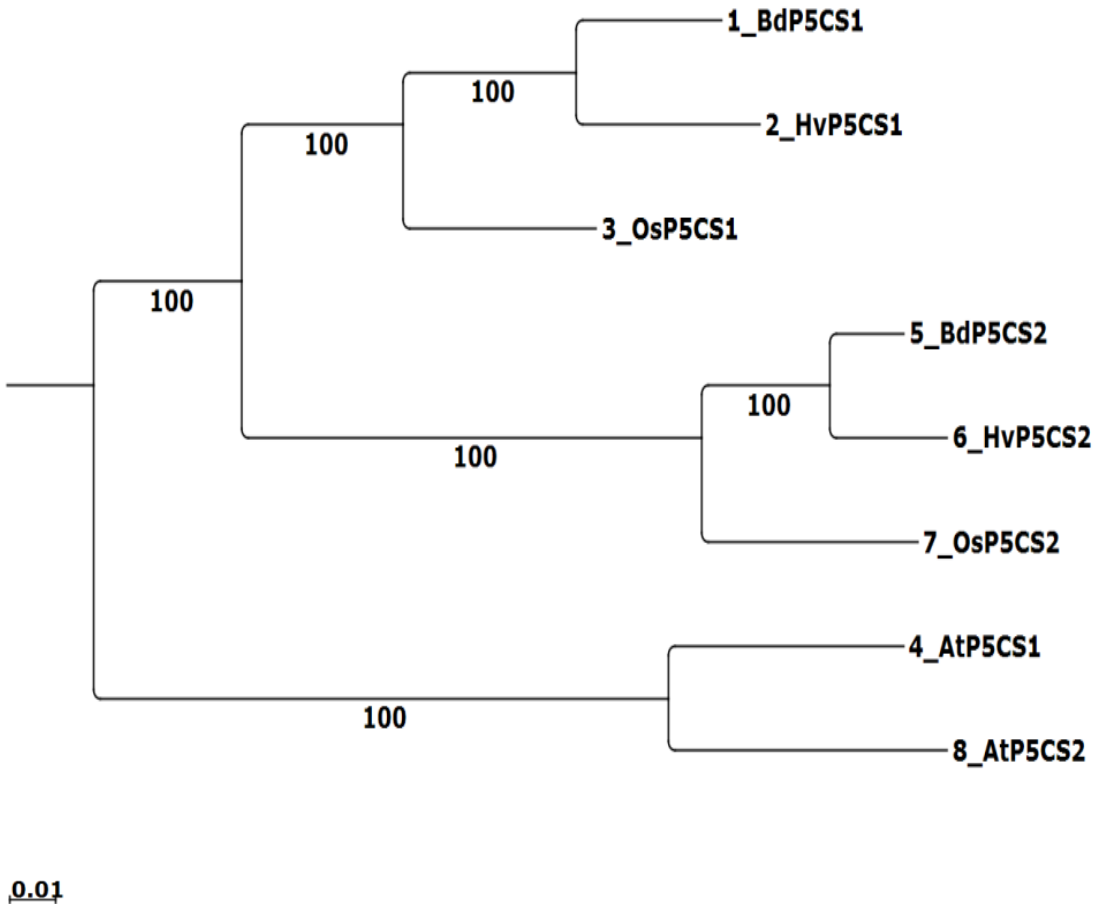


Figure 31: The phylogenetic analysis of P5cs1 and P5cs2 gene of four plant species (At – *Arabidopsis thaliana*, Bd – *Brachypodium distachyon*, Hv – *Hordeum vulgare*, Os – *Oryza sativa*). Nucleotide sequences were used for the construction of the phylogenetic tree.

The Protein sequences of target genes from NCBI were used for sequence alignment analysis by the ClustalW method in the MegAlign program (DNASTAR, Inc., Madison, WI). Aminoacids alignment of HvP5CS1 with other species was also made showed the conservation within the gene (Figure 32).

Taken together, this recombinant analysis suggested that wild barley ISR42-8 carried a novel P5cs1a allele that mediates a major drought-inducible QTL effect on proline accumulation in the cultivated barley background.

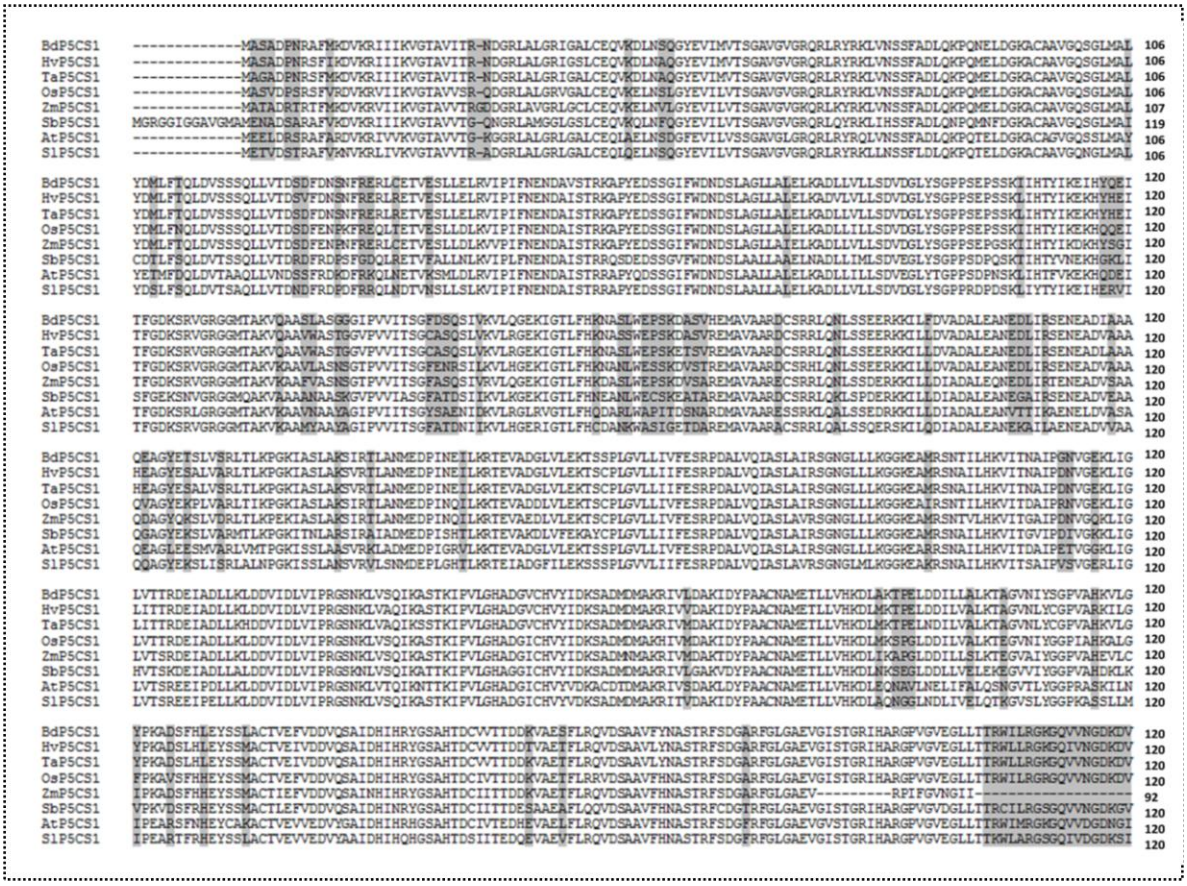


Figure 32: HvP5cs1 aminoacid alignment with *Brachypodium distachyon* (BdP5CS1), *Triticum aestivum* (TaP5CS1), *Oryza sativa* OsP5CS1, *Zea mays (*ZmP5CS1), *Sorghum bicolor (*SbP5CS1), *Arabidopsis thaliana* AtP5CS1 and *Solanum lycopersicum* SlP5CS1 showing high conservation within the gene.

In addition to these critical mutations8 in exons 7, 9 and 13, a 44-bp insertion was identified in Scarlett at the 3´UTR region compared with ISR42-8 (Figure 33). This 44 bp insertion was used as a diagnostic SSLP-marker to genotype ILs population in further study.

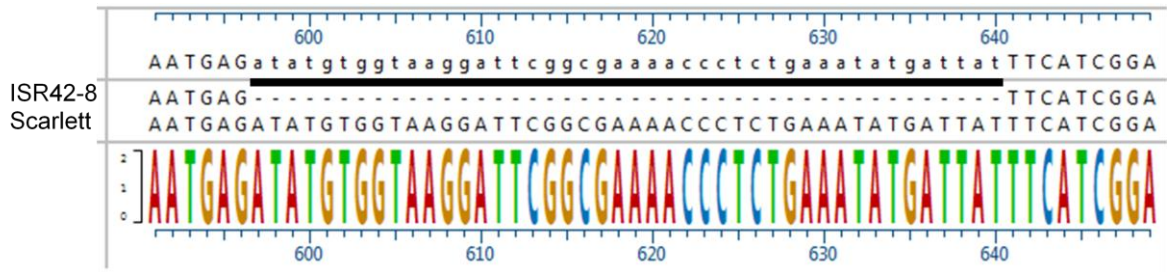


Figure 33: Nucleotides sequence comparison of Scarlett 3´UTR with ISR42-8. The alignment was performed using CLUSTAL W showing 44 bp insertion only in German cultivar Scarlett.

To verify again if S42IL-143 and S42IL-141 carried a common wild barley introgression, a test was made to genotype a diagnostic SSLP-marker which confirmed that both ILs harbor wild barley chromosomal segment at the QTL region (Figure 34).

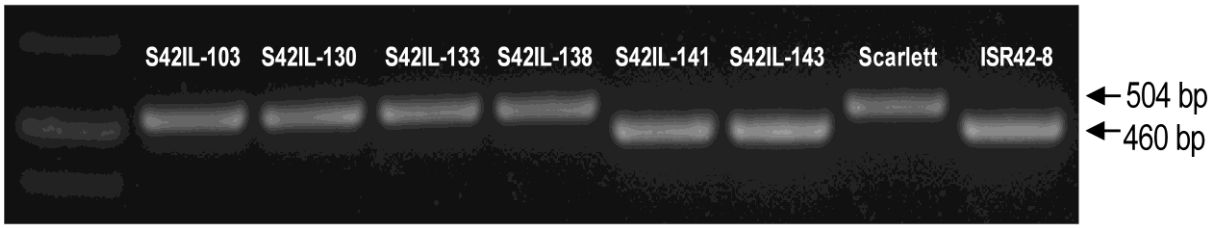


Figure 34: Confirmation of common wild barley introgression in ILs S42IL-143 and S42IL-141 using a SSLP-marker from the QTL region on chromosome 1H. Fragment 460 bp and 504 bp represents the ISR42-8 and Scarlett alleles, respectively.

In addition, a validation of this QTL effect in both ILs was carried out and evaluated QTL segregation in a BC4S2 population derived from the QTL bearing IL S42IL-143 using the same SSLP-marker. This population revealed a clear segregation of Scarlett and ISR42-8 allele, which were associated to low and high proline accumulation under drought stress conditions, respectively (Figure 35).

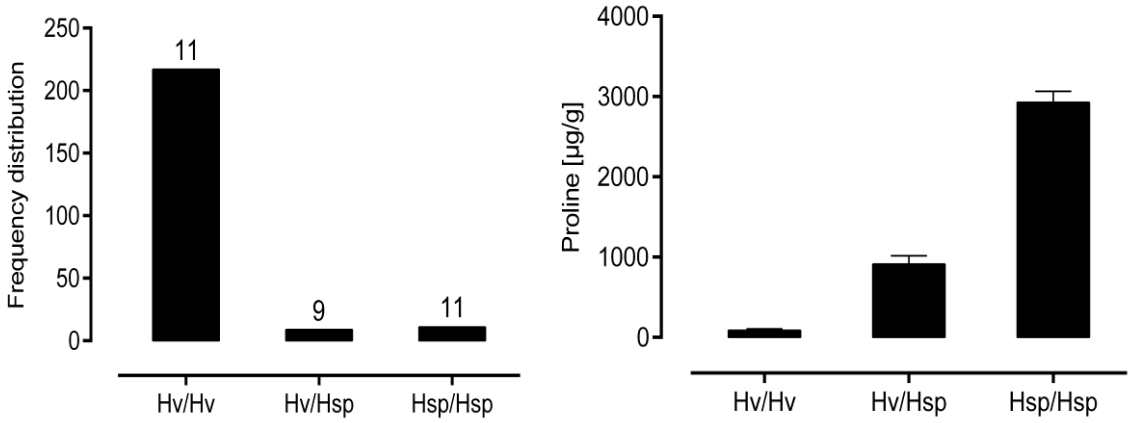


Figure 35: Segregation of QTL alleles in BC4S2 population derived from in QTL bearing IL S42IL-143 (Left). Validation of QTL effect for proline accumulation using BC4S2 population derived from QTL bearing IL S42IL-143 (Right).Hsp/Hsp (*H. vulgare ssp*. *spontaneum*, Hv/Hsp (heterozygous) and Hv/Hv (*H. vulgare*)

To refine the QTL for proline on chromosome 1H (*QPro.S42-1H*) at gene resolution, positional cloning approach using a segregating high-resolution mapping population comprising around 3300 BC4S2 progenies derived from the QTL bearing IL S42IL-143 was followed. Initial mapping among the S42IL population helped us to refine the targeted interval to 1.6 cM from SNP: TP59951 to SNP: TP3687 according to SNP map by Schmalenbach *et al.,* (2011) and Honsdorf *et al.,* (2014). From these SNPs, left and right KASP markers were established for high-throughput genotyping to select informative recombinants among the 3300 BC4S2 progenies. Detailed information of these KASP is presented in Table 11.

Table 11: KASP marker development to identify informative recombinants for fine mapping

	SNP	SNP name	Chr.	Position (bp)	Sequence marker allele
KASP1	(A/G)	TP59951	1H	402386488-402386551 (KASP-L)	Allele 1. TGCAGTTGTCGTCCGCGT CCTCATTTTAAATTATGAG ATG**A**GATGAGATGAGATG CGTTTACTT Allele 2. TGCAGTTGTCGTCCGCGT CCTCATTTTAAATTATGAG ATG**G**GATGAGATGAGATG CGTTTACTT
KASP2	(G/A)	TP3687	1H	404304288-404304351 (KASP-R)	Allele 1. TGCAGAC**G**TAACACAAAC GCAAATGTTCAGGAAAGA AAAGCTTCAGGTGGTAGG CGCAACAAGA Allele 2. TGCAGAC**A**TAACACAAAC GCAAATGTTCAGGAAAGA AAAGCTTCAGGTGGTAGG CGCAACAAGA

Chromosomal map of introgression lines S42IL-141 and S42IL-143 overlapping for the QTL locus controlling drought inducible proline accumulation, fine mapping of QTL and position of KASP makers according to physical map is presented in Figure 36.

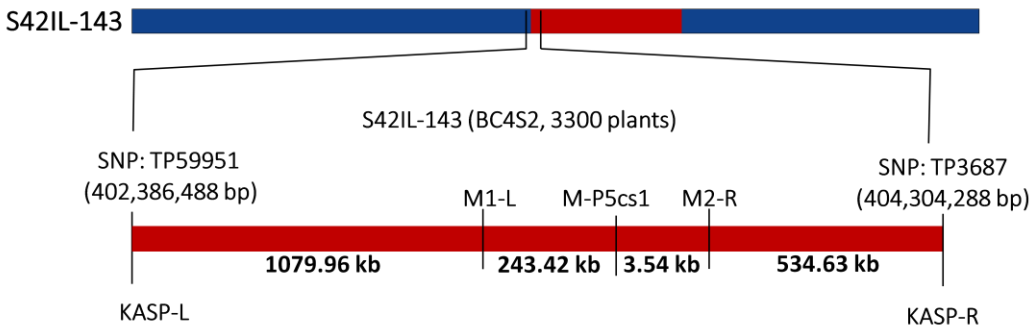


Figure 36: Chromosomal map of introgression line S42IL-143 for the QTL Locus controlling drought inducible proline accumulation and fine mapping of QTL region using high resolution BC4S2 population segregating for the QTL region in barley. Blue color indicates the Scarlett genome. The red regions is showing the location of wild introgressions according to Schmalenbach *et al.,* (2011).

The KASP genotyping was outsourcing at TraitGenetics®, Gatersleben, Germany, which helped us to refine 3300 BC4S2 population into 97 informative recombinants. Later, only these informative recombinants were quantified for proline accumulation under drought stress conditions. Then, two additional markers in the targeted region were developed at left (M1-L) and right (M2-R) (Table 12) border of the most promising candidate gene HvP5cs1. The genotyping of left and right border markers and their comparison with the phenotypic data revealed eight and five recombinants indicating that the casual mutation controlling proline accumulation may lie in the HvP5cs1 gene, which encodes a pyrroline-5-carboxylate synthetase enzyme protein. Notably, three recombinants found which were heterozygous at the 3´ UTR of HvP5cs1 gene but proved to carry ISR42-8 homozygous 5´ promoter of P5cs1 gene. These recombinants exhibited higher proline accumulation similar to ISR42-8 under drought stress condition. Therefore, a hypothesis was made that the causal mutation may lie in the promoter of HvP5cs1 gene. Hence, an additional marker M-P5cs1 was developed (Table 12) at the putative promoter region of the HvP5cs1 gene which

revealed 100% co-segregation of wild allele of ISR42-8 and cultivated allele of Scarlett with high and low proline phenotypes, respectively. The heterozygous recombinants at this marker, exhibited a marginal increase in proline accumulation under drought stress conditions (Figure 37).

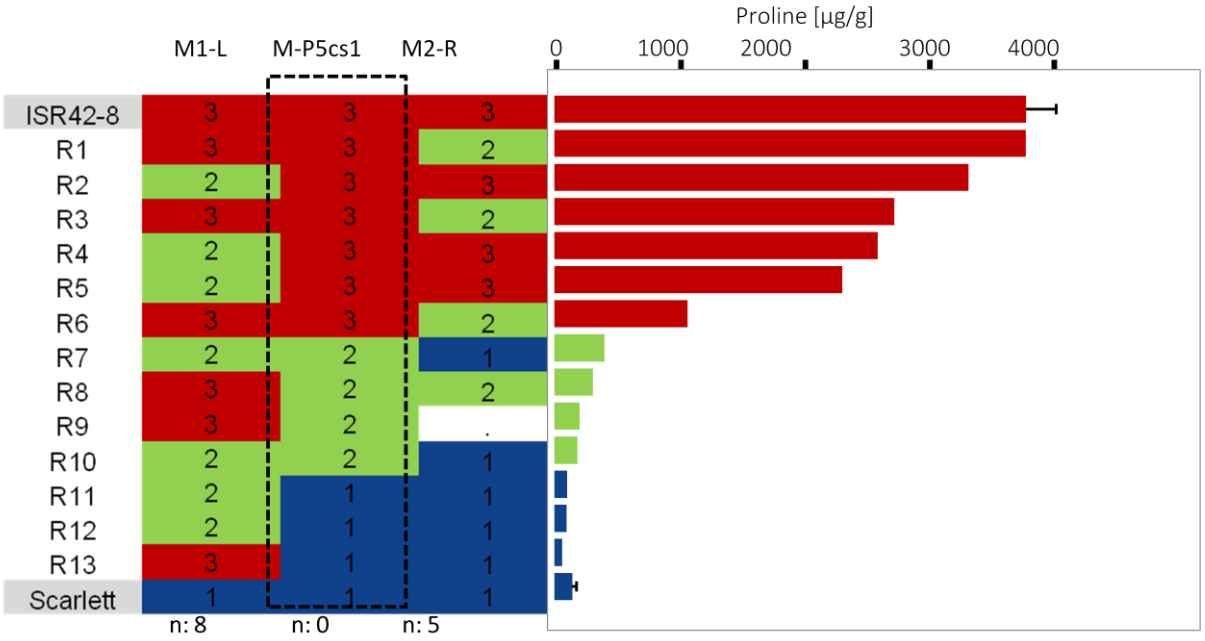


Figure 37: Recombination analysis by comparing the genotyping and phenotyping data of the informative recombinants segregating for the targeted QTL region which underlie P5cs1 gene. Number `3` is genetic score for homozygous ISR42-8, `2` is representing heterozygous and `1` is genetic score for homozygous Scarlett allele. Similarly, the red color shows homozygous ISR42-8 allele. Green is indication of heterozygous, while blue color is depicting homozygous Scarlett allele.

Table 12: Marker development to identify informative recombinants for fine mapping. M-P5cs1 marker was restricted with the help of Accl enzyme resulting in single fragment in Scarlett, two in ISR42-8 and three in heterozygous plants.

Marker	Gene	Region	Primers	Primer sequence (5` to 3`)	Product size (bp)	
					Scarlett	ISR42-8
M1-L	MLOC_60455	4-5 exons	M1-L-F	CCGTGATGTGT TCATACTTCG	502	640
			M1-L-R	TGTGTGGGTTC TGTTGCAGT		
M2-R	MLOC_57545	3´ UTR	M2-R-F	AAAGGGCAAAT TGTGAATGG	504	460
			M2-R-R	TGTGGTTTTGCT TGCTCTTG		
M-P5cs1	MLOC_57545	5´ UTR	M-P5cs1-F	AGTGACCCCGG TTGGAAACT	959	598,361
			M-P5cs1-R	GTGTGATGACG CATTCCTCT		

Furthermore, this allelic polymorphism of cultivated and wild barley alleles at the marker M-P5cs1 was confirmed through restriction fragments and DNA sequencing among the selected informative recombinants which is shown in Figure 38.

All these results suggested that critical mutations may lie on promoter of P5cs1 gene of Scarlett and ISR42-8, which make difference in the expression of proline among these two contrasting parents.

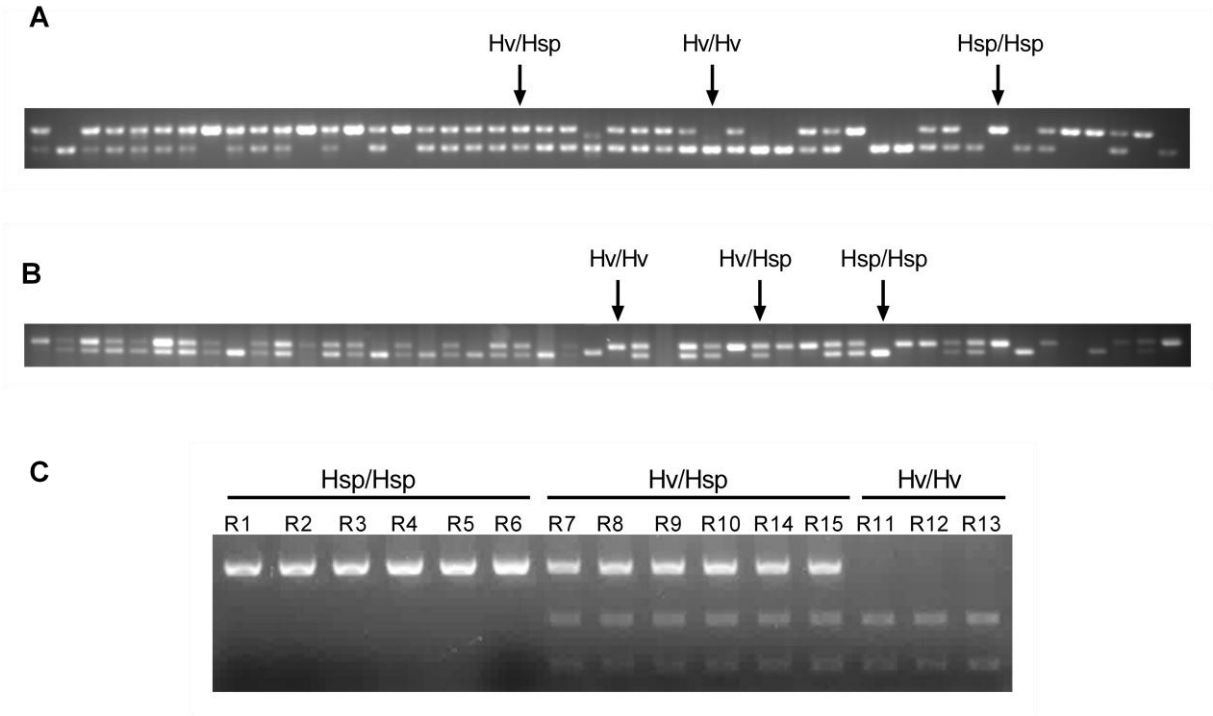


Figure 38: (A) Genotyping of informative recombinants using left border SSLP marker M1-L, (B) right border SSLP marker M2-R and (C) gene specific M-P5cs1 marker analysis through cleaved amplified polymorphic sequence using AccI restriction enzyme. Restriction fragment analysis revealed 420 bp and 580 bp in homozygous Scarlett allele. Hsp/Hsp (*H. vulgare ssp*. *spontaneum*, Hv/Hsp (heterozygous) and Hv/Hv (*H. vulgare*).

3.9 P5CS1 gene carries allele variation in promoter

Based on the recombinant analysis and as the wild barley QTL allele was associated with an incremental increase of proline accumulation under drought, a hypothesis made that a functional mutation may lie in the promoter. Hence, approximately 2 kb upstream of ATG analyzed to scan and estimate the putative promoter of the HvP5cs1 gene. Then, approximately 1.5 kb of the putative promoter region sequenced in ISR42-8 and Scarlett. Sequence analysis between ISR42-8 and Scarlett revealed critical mutations at essential DNA binding motifs (ABRE cis-elements) for the transcription factors ABF1 and ABF2 (Figure 39).

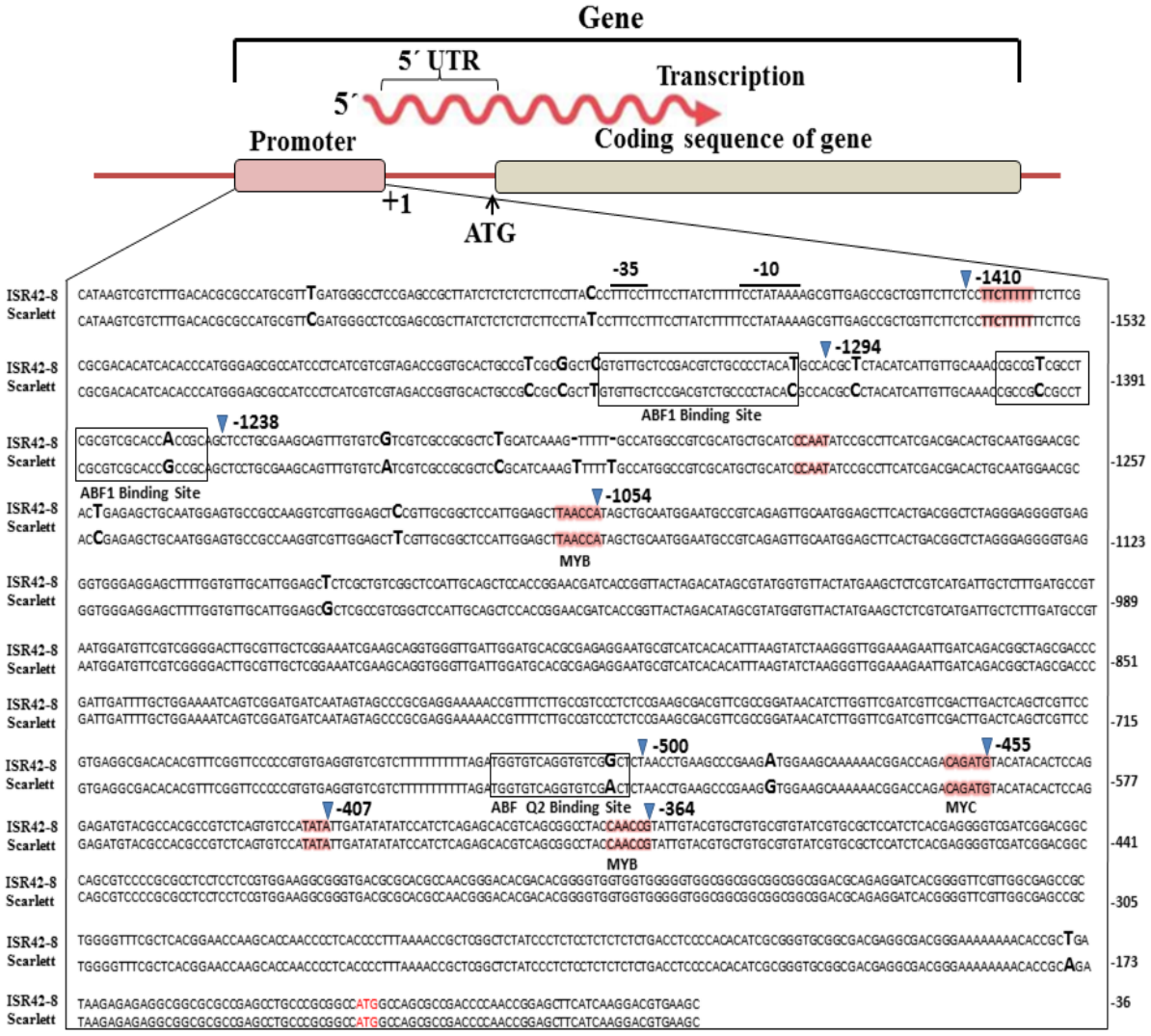


Figure 39: Promoter polymorphism among cultivated Scarlett and wild barley ISR42-8 showing polymorphism at ABF1 and ABF2 (ABRE-binding factors) binding sites.

Considering that transcription factor binding sites may be involved in drought-inducible proline accumulation, the promoter sequence was analyzed further using MULAN analysis (Ovcharenko *et al.,* 2005). This analysis has been designed to effectively perform multiple comparisons of genomic sequences necessary to identify local sequence conservation and to detect evolutionarily conserved transcription factor binding sites (TFBS) shared by all analyzed species located at the same position as defined by the alignment. Interestingly, the SNP mutation across the promoter resulted

in the loss of two essential TFBS for ABF1 and ABF2 in the spring barley cultivar Scarlett, whereas these sites were predicted to harbor active ABRE sites in ISR42-8 (Figure 40). Here, it was believed that the number and arrangement of TFBS across the HvP5cs1 promoter is associated with drought-inducible proline accumulation in ISR42-8.

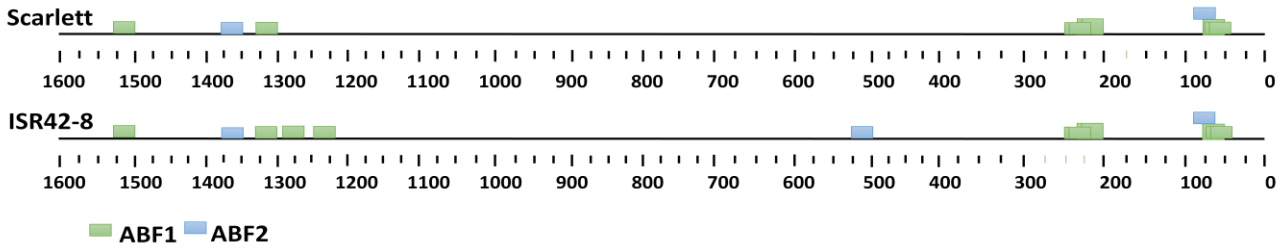


Figure 40: Depiction of DNA binding motifs by MULAN analysis across the promoters of cultivated Scarlett and wild barley ISR42-8. Green and blue color indicates ABRE-binding factors (ABF1 and ABF2), respectively.

Next, the 44-bp insertion mutation was genotyped as a diagnostic marker for the drought-inducible P5cs1a allele among a global diversity set of cultivars, landraces and wild barley (Reinert et al., 2017), accessions for allele mining (Figure 41). This genotyping revealed that all 179 different genotypes that were collected in 38 countries across the globe contain a 44-bp insertion, similar to the cultivar Scarlett, suggesting that ISR42-8 inherited a unique P5cs1 haplotype among the barley genetic resources.

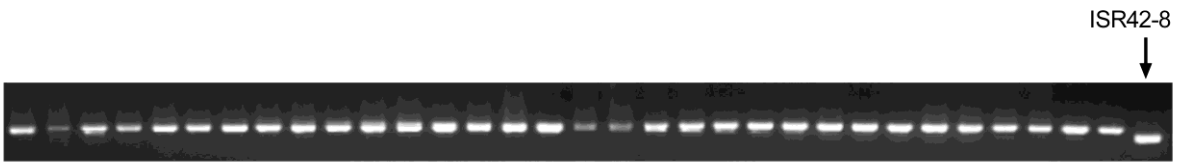


Figure 41: Allele mining for P5cs1 haplotypes among global barley population containing 179 different genotypes, collected from 38 countries. HvP5cs1 gene showed 44 bp insertion in all genotypes except in wild barley ISR42-8 allele.

3.10 Proline accumulation is proportional to up-regulation of the P5cs1 gene

The expression analyses of P5CS1 mRNA in Scarlett and S42IL-143 was perforemed to investigate its association with proline accumulation under varying water stresses. Expression analysis was carried out in barley leaf samples in three biological replicates. Initially, semi-quantitative (sq) RT-PCR was performed to test P5CS1 mRNA expression variation during three drought stress regimes: 3 days after stress (DAS), 6 DAS and 9 DAS. SqRT-PCR analysis revealed that both Scarlett and S42IL-143 minimally induced P5CS1 under control conditions. A modest increase in P5CS1 expression was noted in Scarlett after 6 DAS and 9 DAS. By contrast, a clear up-regulation of P5CS1 mRNA was observed in S42IL-143 at 3 DAS, 6 DAS and 9 DAS (Figure 42).

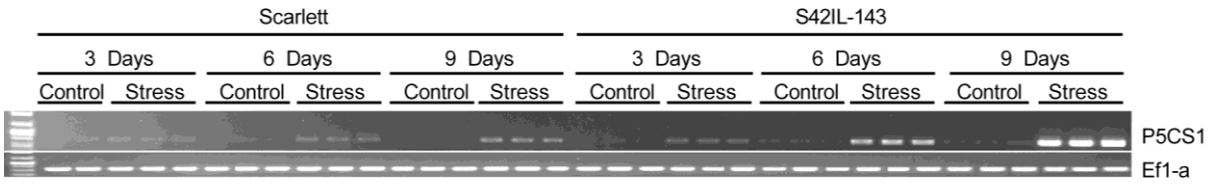


Figure 42: Semi-quantitative RT-PCR analysis of the P5cs1 mRNA under different time frames of control and drought stress conditions. Experiment was conducted using three biological replicates of each genotype in each block.

To investigate these expression differences quantitatively, qRT-PCR carried out using the same samples in three biological replicates. This analysis confirmed a modest induction of P5CS1 transcripts in Scarlett at 6 DAS and 9 DAS. However, S42IL-143 showed a significant drought-inducible up-regulation, exhibiting an approximately 36-fold increase in P5CS1 transcripts at 9 DAS compared with control conditions. This transcript level in S42IL-143 was 8-fold greater than that of Scarlett at 9 DAS (Figure 43).

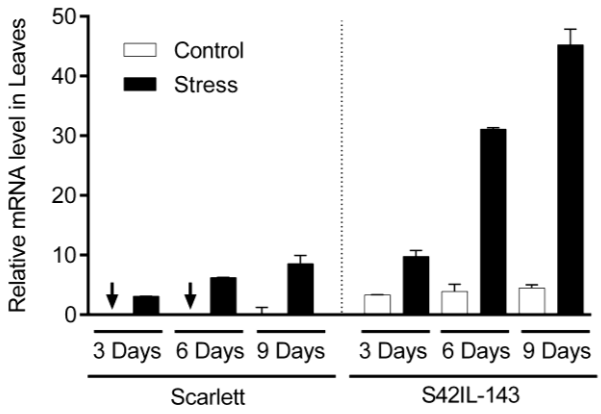


Figure 43: Quantification of relative mRNA levels in leaves of Scarlett and S42IL-143 via qRT-PCR (Arrows are showing minimal values). Experiment was conducted using three biological replicates of each genotype. Bars represent standard error.

Taken together, both experiments revealed a clear drought-inducible up-regulation of P5CS1 mRNA in S42IL-143. To test whether the up-regulation of P5CS1 mRNA was proportional to proline accumulation, the proline content of the same leaf samples quantified that were utilized for the sqRT-PCR and qRT-PCR analyses. It was notable that increased P5CS1 mRNA expression was in direct proportion to excessive proline accumulation among the leaf samples of Scarlett and S42IL-143 (Figure 44). These data indicate that drought-inducible proline accumulation is under the control of an incremental up-regulation of P5cs1 mRNA in different drought stress regimes.

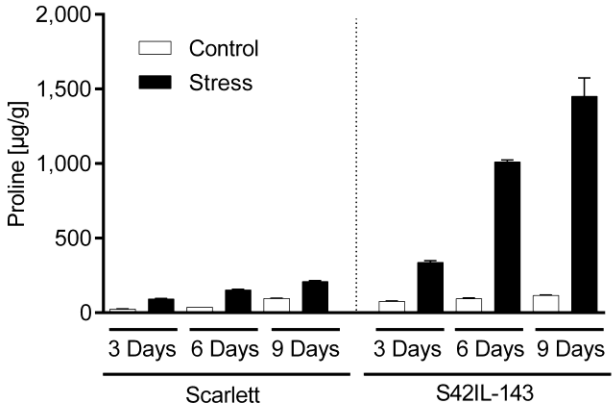


Figure 44: Proline accumulation in leaves of Scarlett and S42IL-143 under control and drought stress conditions. Experiment was conducted using three biological replicates of each genotype. Bars represent standard error.

In vitro grown 10 days seedling of Scarlett was subjected to immediate drought stress by exposing them on dry paper towels. The control block was kept on continuous supply of water. Leaf and root tissues were harvested from the control and drought blocks after 2 and 3 hours. The experiment was carried out in two replications via semi-quantitative RT-PCR analysis.

3.11 Higher proline accumulation maintains the water status in leaves

To test whether higher proline accumulation has a role in water conservation in Scarlett and S42IL-143, the dynamics of the water status assessed in leaves under control and 3 DAS, 6 DAS and 9 DAS using an EMISENS dual mode cavity microwave resonator. According to Dadshani *et al.,* (2015), the microwave parameters inverse quality factor shift (IQS) and resonant frequency shift (FRS) strongly correlate with the amount of water stored in plant tissues. For the experimental duration, the gap between the FRS values of Scarlett plants under well-watered conditions and stress conditions increased gradually from 6.7% (3 DAS) to 40.4% at 9 DAS (Figure 45-A). In contrast to Scarlett, the FRS values of S42IL-143 did not exhibit significant differences between the control and drought stress treatments during the entire experimental period (Figure 45-B). Similar to the FRS values, the IQS values of Scarlett plants under stress conditions were reduced from 17.2% (3 DAS) to 40.4% (9 DAS) compared with control conditions (Figure 45-C). In contrast to Scarlett, S42IL-143 plants exhibited no significant difference between control and stress treatments regarding the IQS values supported by the microwave resonator, indicating the ability of S42IL-143 to maintain leaf water levels (Figure 45-D).

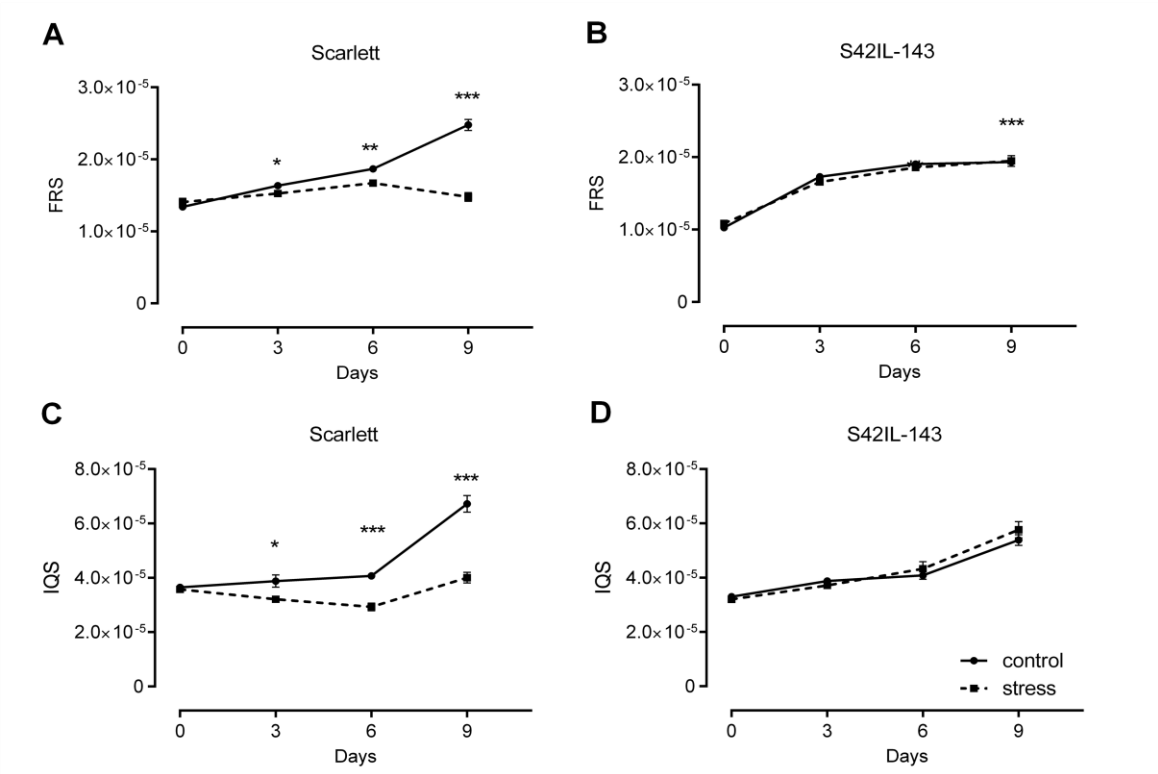


Figure 45: FRS values in Scarlett (A) and S42IL-143 (B) and IQS values in Scarlett (C) and S42IL-143 (D) under control and drought stress condition. Solid line control; dashed line stress condition; Significance level * p=0.05, ** p=0.01, *** p=0.001, while FRS indicates resonant frequency shift and IQS indicates inverse quality factor shift.

3.12 Higher proline expression mediates the photosynthetic rate and effective quantum yield of photosystem II under extreme drought stress conditions

Plants affected by drought stress undergo changes in physiological processes involved in photosynthesis, such as stomatal conductance, transpiration rate, and intercellular CO_2 concentration. These parameters can be quantified using a LICOR 6400XT infrared gas exchange analyzer. These analyses revealed that in response to drought stress, Scarlett reduces the stomatal conductance (*gs*) to inhibit loss of water by transpiration. Compared with well-watered plants the stomatal conductance of Scarlett under drought stress declined gradually by 20% (3 DAS) to 83% (9 DAS) (Figure 46-A). However, S42IL-143 plants exposed to drought stress maintained their stomatal conductance at 3 DAS and 6 DAS, but a significant reduction of *gs* was

detectable after 9 DAS in S42IL-143 (Figure 46-B). The measured transpiration rates (*E*) support the data obtained from measurements of stomatal conductance. Under drought stress conditions, the transpiration rate of Scarlett was reduced from 11.6% (3 DAS) to 77.1% (9 DAS) (Figure 46-C) compared with control conditions. Analogous to stomatal conductance, no significant difference in *E* was detectable between S42IL-143 plants under controlled and stressed conditions between 0 and 6 DAS, whereas the transpiration rate of S42IL-143 plants was 46% lower than S42IL-143 plants under control conditions at 9 DAS (Figure 46-D). The influx of CO_2, which is essential for carbon assimilation and photosynthetic activity, is directly affected by stomatal conductance. We found that the internal CO_2 concentration of the mesophyll (*Ci*) remained constant for both genotypes under control conditions and between 0 and 6 DAS. However, the slope of *Ci* in Scarlett was 40% less than that of S42IL-143, which was reduced by 22.4% at 9 DAS (Figure 46-E and F). Consequently, the photosynthetic rate (*A*) was measured to assess photosynthetic activity using the difference in CO_2 and H_2O between the reference and the sample streams, respectively, according to (Long *et al.,* 1996). The photosynthetic rate decreased by 9.9% in Scarlett at 3 DAS and was gradually reduced by 69% at 9 DAS (Figure 46-G). Interestingly, no significant difference in the net photosynthetic rate was observed in S42IL-143 at 3 DAS and 6 DAS, but a slight reduction in A was observed at 9 DAS. Overall, the photosynthetic rate of S42IL-143 was 3-fold higher in extreme drought conditions at 9 DAS (Figure 46-H).

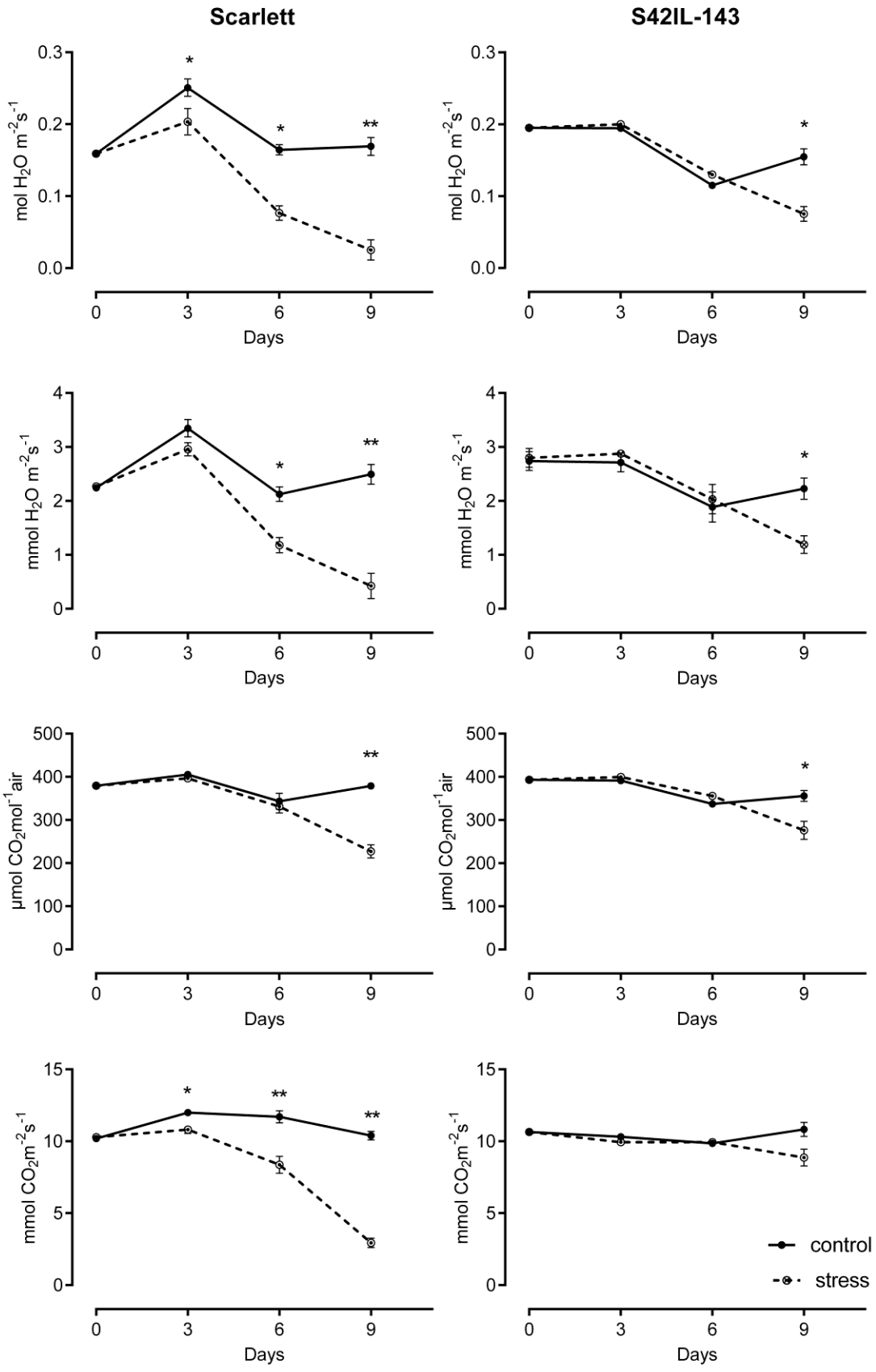

Scarlett
S42IL-143
mol H2O m-2s-1
mmol H2O m-2s-1
µmol CO2mol-1air
mmol CO2m-2s-1
Days
control
stress

Figure 46: Gas exchange parameters of Scarlett and S42IL-143 under control and drought conditions. Stomatal conductance of Scarlett and S42IL-143 (A and B) Transpiration rate in Scarlett and S42IL-143 (C and D) Intercellular CO_2 concentration in Scarlett and S42IL-143 (E and F) Photosynthetic rate in Scarlett and S42IL-143 (G and H). Solid line control; dashed line stress condition; Significance level * p= 0.05, ** p=0.01

In addition, the effective quantum yield of photosystem II (Y(II)) measured at steady-state photosynthesis under light using a MINI-PAM-II to confirm the photosynthetic activity. Notably, the effective quantum yield of photosystem II in Scarlett was significantly reduced at 9 DAS, whereas no significant difference was observed in S42IL-143 (Figure 47-A and B), thus suggesting increased photosynthetic activity putatively due to excessive proline accumulation under drought stress conditions in *P5cs1a* allele bearing near isogenic line.

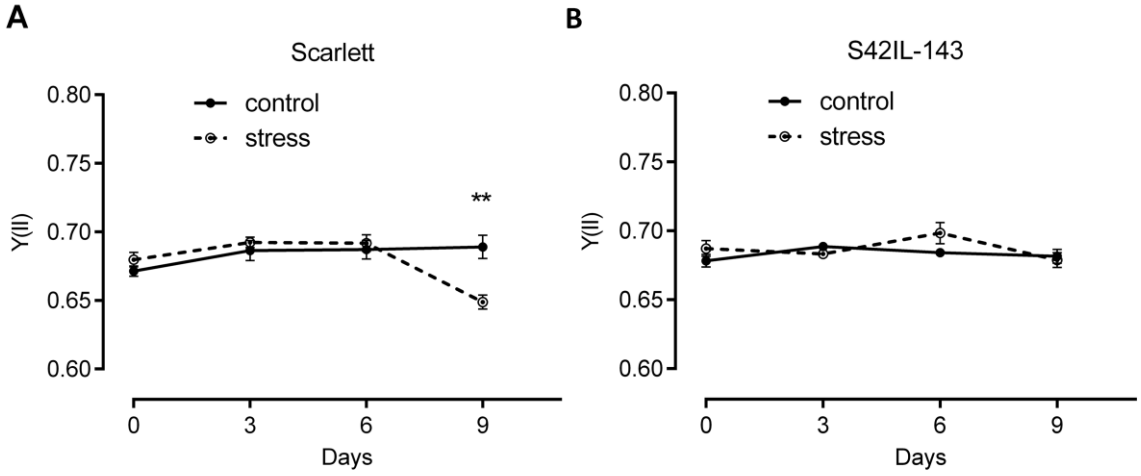


Figure 47: Chlorophyll fluorescence measurement of Scarlett and S42IL-143 under control and drought stress condition. Solid line control; dashed line stress condition; Significance level ** p=0.01

3.13 Higher proline accumulation effect on SPAD value

The SPAD value, which is estimating the amount of chlorophyll in green leaves, increased until 3 DAS for both genotypes under controlled condition reaming constant until 9 DAS. Scarlett plants exposed to drought stress had a reduced SPAD value at 6 DAS by 5.2% ($p<0.05$) relative to controlled condition and 16% ($p<0.01$) 9 DAS. Different to Scarlett the SPAD value of ISR-143 increased under drought stress 6 DAS by 6.5% ($p<0.05$) relative to controlled condition and 10% ($p<0.05$) 9 DAS. (Figure 48)

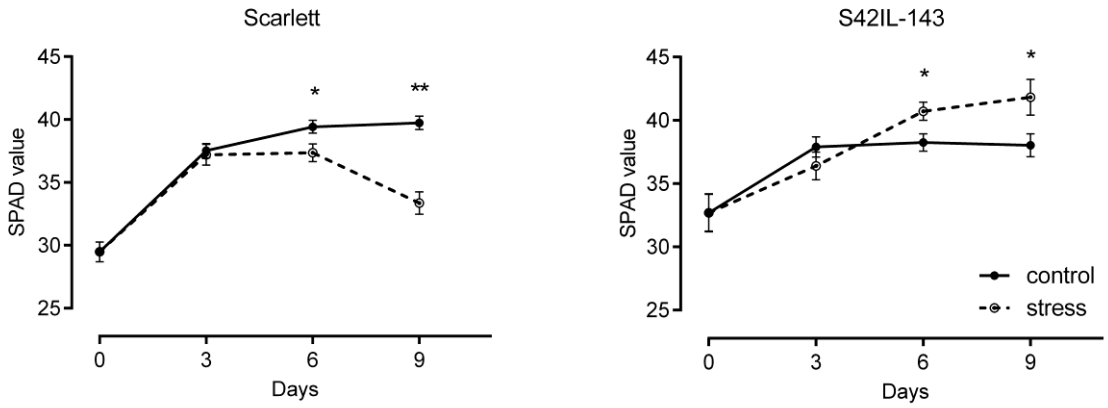


Figure 48: SPAD value of Scarlett and S42IL-143 under control and drought stress condition. Solid line control; dashed line stress condition; Significance level ** p=0.01

4. DISCUSSION

Drought is a major abiotic stress that restricts growth and productivity of many crops. Given that an increased barley production is required to meet the demand of the growing population in the mist of increasing climatic change, there is the need to improve barley adaptation to drought stress. Gaining morphological (agronomic), physiological and molecular insights into how Barley deals and response to drought stress will facilitate efforts toward improving its drought stress adaptation in the breeding programs. Thus, the objectives of this study were to (i) assess the morphological and physiological responses of barley plants at the vegetative stage under controlled and severe drought conditions and, (ii) test the expression of drought-responsive genes over time course period under drought treatment. Moreover, the present study reports the genetic variations of the shoot related traits and proline accumulation due to drought stress among 73 S42ILs from a cross between barley wild accession ISR42-8 and cultivar Scarlett. It was expected that the exotic parental allele make a contribution to barley improvement in general. The S42ILs carrying introgression can be directly used for the development of new elite cultivars. A similar population has already been conducted in barley by Md Arifuzzaman *et al.,* (2014a, 2014b) under same tunnel condition for drought and control conditions to detect QTLs for root and shoot phenotypic traits. Honsdorf *et al.,* (2014) investigated QTLs in same S42ILs for morphological traits under drought and well watered treatments in greenhouse and found exotic parental allele has positive contribution to barley improvement. High-Resolution (HR) population is valuable source for map-based cloning of genes. Currently, it was used to clone the thresh-1 locus detected in S42IL-HR (Schmalenbach *et al.,* 2011). Specific S42IL-HR population was used to clone proline content gene and proved as a valuable source for fine mapping of QTL towards map based cloning of genes.

4.1 QTL identification

Different QTLs have been identified for the shoot and physiological traits, and located on the whole barley genome. The number of markers associated with the traits and the QTLs for each trait will discuss as follow

Plant height

In our study, two QTLs were detected for PH and mapped on chromosome 3H from 185.1 cM to 190.8 cM and 1H from 104.39 cM to 106.46 cM. QTLs exhibited very strong effects on traits, that why it seems to be very important to analysis of genetic determination of the observed traits. Our results showed that the significant QTL for plant height was positioned in 3H. Malosetti *et al.,* (2011), worked on barley inbred lines (RILs) derived from three way cross of barley genotypes (Candela, 915006, and Plaisant) and found two major QTLs for plant height, i.e. one on 3H and second on 5H. QTL found on 3H was localized at the same region and linked to SNP 6716–823 in the 3H.1 linkage group (105.75 cM). Honsdorf *et.al.,* (2014), worked with same S42IL library and detected QTLs associated with PH on all chromosomes except 5H. QTL on 3H was already detected in previous field study with the S42 population by von Korff *et.al.,* (2006).

Plant height is regulated by several genes in barley (Araus *et al.,* 2008 ; Kuczyńska *et al.,* 2013). The Hv20ox2 gene was identified by Jia *et al.,* (2011) and noticed a reduced expression of this gene in semi dwarf plants.
Association between different trait like plant height and other traits, including grain yield, was studied by several researcher and found that the results are not consistent. Yin *et al.,* (1999) and Jia *et al.,* (2011) observed increased yield, whereas Thomas *et al.,* (1991) and Hellewell *et al.,* (2000) observed decreased yield of semi-dwarf plants. They concluded that with decreased expression of the Hv20ox2 gene lowers gibberellins, apical meristem growth and produce more tillers per plant.

Number of leaves

For NL, only one strong QTL was detected on chromosome 5H. The growth, development and yield could be co-regulated through the control of leaf number as the photosynthetic capacity of the plant depends on the leaf numbers. Hoffmann *et al.,* (2012) worked on the same plant material and observed significant difference between S42ILs and Scarlett for NL and summed up with four different QTLs for NL on 2H, 4H, 6H and 7H. QTL for NL on 2H was also discovered by Cuesta-Marcos *et al.,* (2010)

who worked with 102 barley accessions to estimated the number of leaves until heading and found a significant QTL effect near by the earliness per se locusEam6 as stated by Franckowiak and Konishi (2002).

Heading

The QTL analysis revealed one strong QTL for HE located on chromosome 2H. According to the Table 9, the exotic introgression increased the trait value from 16.0 to 42.0%.

Sayed *et al.,* (2017) worked with same population and twelve putative QTLs for HE were mapped on chromosomes 1H, 2H, 3H, 6H and 7H. Pillen *et al.,* (2003), and von Korff *et al.,* (2006) also worked with same plant material and detected the marker locus EBmac415 on 2H where the exotic allele decreased time to heading and coincided with the major flowering QTL on chromosome arm 2HS, which supported our finding as well. While Beales *et al.,* (2007) worked with chinese spring wheat and identified the contig sequence of IWB54033 located in the 2A QTL corresponded to the Ppd-1A sequence, confirming previous results on the relationship between photoperiod response and heading in barley. Marcotuli *et al.,* (2017) revealed a single region on chromosome 2A in wheat associated with HE that was consistent in both environments i.e.; control and drought with a high LOD score of 25.5. The largest-effect association identified was the Ppd-H1 locus on chromosome 2H, which was also shown to be influential throughout barley development (Maurer *et al.,* 2015, 2016).

Number of spikes

Single significant line treatment interactions were observed for NS with Scarlett which were summarized to one QTL located on chromosomes 4H.

Spikes number is one of the most important grain yield-related traits in cereal crops. Shamasbi *et al.,* 2017 found eight putative QTLs on different chromosomes while working with DH barley population derived from a cross between the Australian cultivar 2-rowed Clipper and Algerian 6-rowed Sahara 3771. One of the major QTL (ANIONT1A-TACMD) was on chromosome 4H that affects spike length and number of

spike. Sayed *et al.,* (2017) worked with same S42ILs population and summarized six QTLs for NS but on different chromosomes (2H, 6H and 7H).

Saal *et al.,* (2010) also worked on same spring barley BC2DH population S42 and found 3 QTLs for NS on chromosomes 1H, 6H and 7H. While Wang et al., (2016) mapped a total of 18 QTLs for NS for five consecutive years in 122 doubled haploid (DH) lines derived from a cross between the six-rowed dwarfing barley cultivar Huaai 11 and the two-rowed barley cultivar Huadamai 6. He found one reliable QTL qSP5-1 located on chromosome 5H for NS in year 2011, 2012 and 2013, with increasing spike number per plant. Several QTLs were previously reported on the 1H, 2H, 5H, 6H and 7H (Chutimanitsakun *et al.,* 2011, Li *et al.,* 2006, Peighambari *et al.,* 2005). While, Ibrahim *et al.,* (2010) detected five QTLs in wheat but found one with increased NS by 10.8% and 16.3% under well-watered and drought stress, respectively.

Shoot fresh and dry weight

Five S42ILs showed significant associations for shoot fresh weight (SFW) and shoot dry weight (SDW). Due to overlapping of introgressions, these associations were summed to three putative QTL which were located on chromosomes 1H, 2H and 5H.

In our studies, a significant effect of the treatments and genotypes and also significant interactions of genotypes and treatments were detected. Therefore, a high correlation was detected between SFW and SDW for the control and drought treatment across both the years. Drought stress on barley genotypes were estimated primarily as the reduction in SFW and SDW (Wehner *et al.,* 2015). Reduced biomass production under drought was reported in barley during different developmental stages of barley (Jamieson *et al.,* 1995), even in field experiments (Varshney *et al.,* 2011), greenhouse experiments (Honsdorf *et al.,* 2014, Wehner *et al.,* 2015) and hydroponics (Zhao *et al.,* 2010).

Pillen *et al.,(*2003 and 2004) also worked with same S42ILs population and found two QTLs for SDW on on different chromosomes (4H and 7H).Whereas, Chloupek and Forster (2006) worked with 12 diverse barley genotypes and found one similar QTL for SFW on 5H in addition to QTLs on 3H and 7H. Teulat (1997) worked with 187 barley

(*H. vulgare* L.) recombinant inbred lines from a cross between two Mediterranean varieties, Tadmor and Er/Apm and found QTLs for SFW on chromosomes 1H and 6H. Bálint *et al.,* (2008) also detected 3 QTLs on 1H, 5H and 7H, respectively under Osmotic stress and two QTLs on chromosomes 2H and 7H, under control and under osmotic stress together for shoot dry weight in 94 double haploid lines of Oregon-Wolfe Barley (OWB).

With regard to candidate genes, Vinod *et al.,* (2006) identified a candidate gene (EXP13) on chromosome 1 controlling shoot dry weight in rice under well-watered conditions, which can be a good candidate for barley as well. While, Ibrahim (2007) mapped eighteen QTLs in two wheat populations (D84 and T84) for dry weight of biomass on chromosomes 2A, 4A, 2B, 6B, 7B, 3D and 6D, and five QTLs again in 2010 for biomass, out of which one was linked with exotic allele QBm.D84-3D on 3D chromosome, which were found to increase dry weight of biomass under drought stress condition and well water condition.

Chlorophyll content

Four significant line treatment interactions were observed for CC with Scarlett which were summarized to two QTL located on chromosomes 1H and 2H.

Sayed et al., 2017 mapped four QTLs associated significantly with CC, located on chromosomes 4H, 5H and 6H, while working on the same population. Mousavi et al., 2016 detected two QTLs for chlorophyll content, one on chromosome 2H and other on 7H in 72 F1 derived doubled haploid lines (DH) from the cross between Steptoe and Murex. Eshghi et al., (2013) found 5 QTL associated with CC in BC3 population of six rowed spring barley (Azhul) with wild barley (H.vulgare subsp. spontaneum). Guo et al., (2008) also identified five QTLs associated with CC on chromosomes 2H and 4H using 194 line of RILs population. Xue et al., (2008) also worked with different barley genotypes and detected four putative QTLs for CC on 2H, 3H and 6H.

Wilting score

A total of eight lines were significant lines by treatment associations with Scarlett (Figure 20 C) for WS. The effects were summarized to 5 putative QTL located on chromosome 1H, 2H, 5H. Sayed *et al.,* (2012) performed an advanced backcross quantitative trait locus (AB-QTL) analysis in same S42ILs population to clarify genetic mechanisms controlling proline content (PC) and leaf wilting (WS) in barley under drought stress conditions and detected several QTL for WS on chromosome 1H, 2H, 3H, and 4H. Out of which, QWS.S42.1H and QWS.S42.4H were associated to decrease in WS due to the introgression of exotic alleles.

Von Korff *et al.,* (2008) also worked with the same population of barely and found a QTL for wilting score at position (195.7- 206.5 cM) on 1H. Whereas, In rice 5 QTL on chromosomes 5 (at 57.5 and 85.2 cM), 9 (at 65.6 cM) and 11 (at 46.3 and 103.9 cM) for leaf rolling and four QTLs for leaf drying, distributed on chromosomes 1 (at 76.7 cM), 3 (at 14.1 and 91.4 cM) and 11 (at 29.5 cM) were isolated by Gomez *et al.,* (2006).

Yue *et al.,* (2006) also mapped six QTLs in rice for leaf drying score (LDS) on chromosomes 1, 2, 3 (two QTLs), 8 and 9. Champoux *et al.,* (1995) conducted an early QTL study and found twelve of the 14 QTL associated with leaf rolling in rice.

4.2 Proline accumulation under drought stress condition

Over 40 years of intensive research on proline metabolism has revealed its roles in plant development in general and drought adaptation in particular. In the process of drought tolerance, it acts as a compatible solute that serves as a key osmotic regulator and protects against cell-membrane ruptures, contributes to the maintenance of redox balance and cell homeostasis and acts as a signaling molecule during severe drought. In addition, proline is involved in post drought stress recovery as a radial source of energy (Bartels and Sunkar 2005, Szabados and Savoure 2010). Although considerable information has been reported on its metabolism in plants, thus far, its broader regulation and utility have not been realized in crop plants. In the present study, we explored the unique genetic resources of barley adapted to dry climates to

screen its adaptive intelligence, which had evolved over time, and tested their utility in the cultivated gene pool. We employed a forward quantitative genetics approach to identify, validate and introgress natural variant of proline determination using a library of wild barley introgression lines. We believe that early domestication and post-Mendelian intensive breeding and selection caused fundamental losses to vital alleles for drought adaptive traits such as proline accumulation. It therefore seems inevitable to explore the natural genetic resources of crop plants and employ vital genetic resources to meet the present and future challenges of water scarcity.

The present genetic mapping identified five QTL for proline accumulation under drought stress conditions. In the present study, we focused on QTL *QPro.S42-1H* as it accounted the strongest drought-inducible effect on proline accumulation. Secondly, two independent ILs, S42IL-143 and S42IL-141 complemented this QTL effect due to the introgression of wild P5cs1a allele in the Scarlett background. Generally, the confirmation of gene function by introgressing wild allele in the cultivated background via classical crossings is direct and reliable than gene transfer via transgenic approaches, but there exists a considerable criticism on the genetic background of the allele bearing near isogenic lines. Therefore, it is notable to focus on the fact that ILs, S42IL-143 and S42IL-141 carried a less likely but a special arrangement of wild introgressions that run antiparallel but shared a small common segment at the P5cs1 gene. Hence, this arrangement was highly advantageous to exclude the background effects of additional genes as the extent of QTL *QPro.S42-1H* was almost similar in both ILs. Additionally, high resolution recombination analysis provides an unequivocal evidence of functional confirmation of wild barley P5cs1a allele for drought inducible proline accumulation among the independent BC4S2 progenies in the Scarlett background. Previous studies of proline metabolism in the model plant Arabidopsis and related higher plants suggest a vital role an enzyme-encoding genes, P5CS1 under drought stress conditions (Liang et al., 2013).

In the next step, we investigated vital sequence polymorphisms underlying the genetic and molecular regulation of the drought-inducible P5cs1a allele of ISR42-8. As no significant polymorphisms were identified in the coding region, and allele bearing IL S42IL-143 and recombinant analysis exhibited incremental up-regulation of P5cs1

mRNA, we hypothesize that this QTL allele may imply regulation at the level of transcription. Promoter analysis revealed critical mutations across the transcription binding motifs of the ABF1 and ABF2 transcription factors, which resulted in the establishment of two additional ABF (abscisic acid-responsive element binding protein) binding sites in the drought-inducible P5cs1a allele of wild barley. These motifs were missing along with an insertion mutation at the motif adjoining sequences which created the change of number and arrangement of essential DNA binding motifs in Scarlett. Different reports have suggested that ABFs are major transcription factors that bind to ABREs and regulate ABA-responsive gene expression (Choi *et al.,* 2000, Uno *et al.,* 2000). ABREs (ABA-responsive elements) are 8-bp long conserved sequences (PyACGTGG/TC) with a core sequence of ACGT (Nakashima *et al.,* 2009, Fujita *et al.,* 2011). The ABF gene family is expressed in vegetative tissues in response to ABA and osmotic stress in Arabidopsis, suggesting its fundamental role in ABA-mediated drought stress tolerance (Fujita *et al.,* 2011). All ABF transcription factors carry four conserved domains in addition to the bZIP domain (Fujita *et al.,* 2011, Fujita *et al.,* 2013). Transcription factors having a bZIP domain target DNA duplex sites as homodimers or heterodimers and bind to related but distinct palindromic sequences (Ellenberger 1994, Hurst 1995). *Shen et al., (1996)* shed interesting insight on the ABRE cis-elements; they found that these elements require other copies of ABREs or the combination of an ABRE with one of several coupling elements across the promoter region. These researchers also claimed that a single copy of an ABRE element was insufficient to activate ABA-responsive genes (Riley *et al.,* 2008). The role of multiple TFBs is well documented in other systems, e.g., G-box factors, in substantiating transcriptional up-regulation in plants (Schulze-Lefert *et al.,* 1989, Toniatti *et al.,* 1990), which suggests that the active role of multiple TFBs in gene up-regulation depends primarily on the targeting TF itself, the inter-TFB distance and the adjoining sequence of the TFBs. Recently, Wang *et al.,* (2016) discovered a promoter mutation across MYB *cis*-elements associated to drought-inducible expression of ZmVPP1 gene, which confers drought stress tolerance in maize genotypes.

In the light of these findings, we believe that the number and an arrangement of ABFs binding sites seems a unique evolutionary signature in wild barley accession ISR42-8 that modulate incremental up-regulation of P5cs1 gene expression and the subsequent proline accumulation under extreme drought stress conditions.

Finally, we tested whether drought-inducible proline accumulation has a role in mediating drought stress tolerance in S42IL-143. Previously, several physiological measurements, such as leaf water status (Bolanos and Edmeades 1996, Fischer *et al.,* 1998, Jones 2007), stomatal conductance (Fischer *et al.,* 1998, Medrano *et al.,* 2002), photosynthetic parameters (Pei *et al.,* 1998, Li *et al.,* 2006, Pinheiro and Chaves 2011) and efficiency of photochemistry (Epron *et al.,* 1992, Souza *et al.,* 2004), have been widely used as markers for evaluating drought stress tolerance in various plant species (Chaves *et al.,* 2009, Liu *et al., 2015)*. Hence, we measured these parameters in S42IL-143 and Scarlett under varying water stress conditions. Water status measured through the non-destructive microwave parameters FRS and IQS demonstrated that S42IL-143 was able to maintain water status compared with Scarlett under drought conditions. Using non-destructive measurement, Dadshani *et al.,* (2015) found that the microwave parameters FRS and IQS were highly correlated with water status in leaves in barley. Triggered by the drought signaling cascade activity of the stomatal aperture, the gas exchange and photosynthetic rates are considered important parameters in the determination of drought stress tolerance for rain-fed agriculture. It is perhaps logical that reduced stomatal conductance may result in the reduction of the photosynthetic rate in plants (Lawlor and Tezara 2009, Brestic and Zivcak 2013, Hossain *et al.,* 2015). However, several reports confirm that drought-tolerant genotypes maintain open stomata and active photosynthesis, even under dehydration conditions, while drought-sensitive genotypes immediately reduce the stomata aperture under drought conditions (Benešová *et al.,* 2012, Hossain *et al.,* 2015). In the present study, we observed that S42IL-143 exhibited increased stomatal conductance but still showed an increased photosynthetic rate under drought stress compared with Scarlett based on active gas exchange parameters and chlorophyll fluorescence yield measurements, which are direct and ideal indicators to characterize the efficiency of photochemistry under varying environmental conditions (Rascher *et*

al., 2000, Fracheboud *et al.,* 2004). According Yuan *et al.,* (2016), plants that maintain their effective quantum yield of PSII photochemistry under drought stress are recognized as stress-tolerant. The assessment of the effective quantum yield of photosystem II (Y(II)) in this analysis also confirmed that the photosynthetic activity of S42IL-143 is not reduced by drought stress as dramatically as in the sensitive cultivar Scarlett. These data suggest that increased proline accumulation modulates physiological parameters and drought stress tolerance in S42IL-143 due to the introgression of a novel P5cs1a allele from wild barley accession ISR42-8. This unique accession seems to carry special adaptive mechanisms against drought because of its natural adaptation to semi-dessert condition of the Middle-East.

Taken together, the present study successfully demonstrated the isolation of a new P5cs1a allele of wild origin that implies transcriptional up-regulation for excessive proline accumulation and subsequent drought stress tolerance. We believe the discovery of a unique P5cs1a allele among the diversity of natural barley is a promising step toward determining the molecular basis of drought physiology in an important agriculture crop. Future research will help to clarify molecular and evolutionary diversification of the ABA cascade in mediating drought tolerance from the cell to whole plant level in term of yield advantage under drought stress conditions. Additionally, this favorable P5cs1a allele has been introgressed in an isogenic background of cultivated barley, which provides an opportunity for straightforward transfer in developing drought-resilient barley cultivars and extending its utility among the related crop species through cis-genesis or transgenic approaches.

References:

1. Åberg, E. (1938). *Hordeum agriocrithon* nova sp., a wild six-rowed barley. Ann. R. Agric. Col. Swed. 6:159–216.
2. Ahmad, P., Prasad M. N. (2012). Abiotic Stress Responses in Plants - Metabolism, Productivity and Sustainability (P Ahmad and MN. Prasad, Eds.). New York: Springer Berlin Heidelberg.
3. Ahmadi, A., Baker, D. A. (2001). The effect of water stress on the activities of key regulatory enzymes of the sucrose to starch pathway in wheat, Plant Growth Regul. 35, 81–91.
4. Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24: 1337–1344.
5. Anjum, F., Yaseen, M., Rasul, E., Wahid, A., Anjum, S. (2003). Water stress in barley (Hordeum vulgare L.). I. Effect on chemical composition and chlorophyll contents, Pakistan J. Agr. Sci. 40, 45–49.
6. Araus, J. L., Slafer, G. A., Reynolds, M. P., and Royo, C. (2002). Plant breeding and water relations in C3 cereals: what should we breed for? Ann. Bot-London 89: 925–940.
7. Araus, J.L., Slafer, G.A., Royo, C., Serret, M.D. (2008). Breeding for yield potential and stress adaptation in cereals. Crit Rev Plant Sci 27: 377–412.
8. Armengaud, P., Thiery, L,. Buhot, N., Grenier-DeMarch, G,, Savouré, A. (2004). Transcriptional regulation of proline biosynthesis in Medicago truncatula reveals developmental and environmental specific features. Physiol Plantarum 120: 442–450.DOI: 10.1111/j.0031-9317.2004.00251.
9. Aroca, R. (2012). Plant Responses to Drought Stress - From Morphological to Molecular Features (R Aroca, Ed.). New York: Springer.
10. Ayadi, M., Cavez, D., Miled, N., Chaumont, F., Masmoudi, K. (2011). Identification and characterization of two plasma membrane aquaporins in durum wheat (Triticum turgidum L. subsp. durum) and their role in abiotic stress tolerance. Plant Physiology et Biochemistry 49, 1029–1039.

11. Bagci, S,A., Ekiz, H., Yilmaz, A., Cakmak, I. (2007). Effects of zinc deficiency and drought on grain yield of field-grown wheat cultivars in Central Anatolia J. Agron. Crop Sci., 193, pp. 198-206.
12. Balint, A.F., Vagujfalvi, A., Szira, F., Galiba, G., Borner, A., Cattivelli, L., and Dubcovsky, J. (2008). QTLs and genes for abiotic stress tolerance in cereals: their general role in the environmental adaptation and their developmental-stage specificity. Options Mediterraneennes 81:197-200.
13. Bartels, D., Sunkar, R. (2005). Drought and salt tolerance in plants. Critical reviews in plant sciences 24(1):23-58.
14. Bates, L.S., Waldron, R.P., and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-208.
15. Beales, J., Turner, A., Griffiths, S., Snape, J.W., Laurie, D.A. (2007). A pseudo-response regulator is misexpressed in the photoperiod insensitive Ppd-D1a mutant of wheat (Triticum aestivum L.). Theor. Appl. Genet, 115, 721–733.
16. Bekele, E. (1983). A differential rate of regional distribution of barley flavonoid patterns in Ethiopia, and aview on the center of origin of barley. Hereditas 98:269–280.
17. Benešová, M., et al. (2012). The physiology and proteomics of drought tolerance in maize: early stomatal closure as a cause of lower tolerance to short-term dehydration? Plos One 7(6):e38017.
18. Bolanos, J. Edmeades, G. (1996). The importance of the anthesis-silking interval in breeding for drought tolerance in tropical maize. Field Crops Research 48(1):65-80.
19. Boyer, J.S. (1982). Plant productivity and environment. Science, 218, 443–448.
20. Brestic, M., Zivcak, M. (2013). PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications. Molecular Stress Physiology of Plants, (Springer), pp 87-131.
21. Canady, M.A., Meglic, V., Chetelat, R.T. (2005). A library of Solanum lycopersicoides introgression lines in cultivated tomato. Genome 48:685–697.

22. Cattivelli, L., Rizza, F., Badeck, F,W., Mazzucotelli, E., Mastrangelo, A.M., Francia, E., Mare, C., Tondelli, A., Stanca, A.M. (2008). Drought tolerance improvement in crop plants:An integrated view from breading to genomics. Field Crops Res 105:1-14.
23. Chaitanya, K.V., Rasineni, G.K., Reddy, A.R. (2009). Biochemical responses to drought stress in mulberry (Morus alba L.): evaluation of proline, glycine betaine and abscisic acid accumulation in five cultivars. Acta Physiol Plant 31:437–443.
24. Champoux, M.C., Wang, G., Sarkarung, S-, Mackill, D.J., O'Toole, J.C., Huang, N., McCouch, S.R. (1995). Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. Theor. Appl. Genet., 90: 969-981.
25. Chaumont, F., Barrieu, F., Wojcik, E ., Chrispeels, M.J., Jung., R. (2001). Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 125: 1206–1215.
26. Chaves, M.M., Costa, J.M., Saibo, N.J.M. (2011). Recent advances in photosynthesis under drought and salinity. Advances in botanical research, Vol. 57. San Diego: Elsevier Ltd. p50-83.
27. Chaves, M., Flexas, J., Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of botany 103(4):551-560.
28. Chen, T.H.H., Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Current Opinion in Plant Biology 5, 250–257.
29. Chloupek, O., Forster, B.P. and Thomas, W.T.B. (2006). The effect of semi dwarf genes on root system size in field-grown barley. Theoretical and Applied Genetics 112,779-786.
30. Choi, H-i., Hong, J-h., Ha, J-o., Kang, J-y., Kim, S.Y. (2000). ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275(3):1723-1730.
31. Chutimanitsakun, Y., Nipper, R.W., Cuesta-Marcos, A., Cistué, L., Corey, A., Filichkina, T., Johnson, E.A., Hayes, P.M. (2011).Construction and application

for QTL analysis of a Restriction Site Associated DAN (RAD) Linkage map in barley. BMC Genomics12,4.

32. Ciarmiello, L.F., Woodrow, P., Fuggi, A., Pontecorvo, G., Carillo, P. (2011). Plant Genes for Abiotic Stress. Abiotic Stress in Plants—Mechanisms and Adaptations. InTech; Rijeka, Croatia: pp. 283–308.
33. Close, T. J., Bhat, P. R., Lonardi, S., Wu, Y. H., Rostoks, N. (2009). Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10: 582.
34. Cuesta-Marcos, A., Casas, A.M., Yahiaoui, S., Gracia, M.P., Lasa, J.M., Igartua, E. (2008). Joint analysis for heading date QTL in small interconnected barley populations. Mol Breed, 21 (3):383–399.
35. Dadshani, S., et al. (2015). Non-invasive assessment of leaf water status using a dual-mode microwave resonator. Plant methods 11(1):8.
36. Darvasi, A. (1998). Experimental strategies for the genetic dissection of complex traits in animal models. Nature Genetics 18, 19–24.
37. Daryanto, S., Wang, L., Jacinthe, P.A. (2016). Global synthesis of drought effects on maize and wheat production PLoS One, 11 (2016), p. e0156362.
38. Dawood, M.G., Sadak, M.S. (2014). Physiological role of glycinebetaine in alleviating the deleterious effects of drought stress on canola plants (Brassica napus L.). Middle East Journal of Agriculture Research 3(4):943-954.
39. Dawood, M., Taie, H., Nassar, R., Abdelhamid, M., Schmidhalter, U. (2014). The changes induced in the physiological, biochemical and anatomical characteristics of Vicia faba by the exogenous application of proline under seawater stress. South African Journal of Botany 93:54-63.
40. De Datta, S.K., Malabuyoc, J.A., Aragon, E.L. (1988). A field screening technique for evaluating rice germplasm for drought tolerance during vegetative stage. Field Crops Res. 19: 123-124.
41. Del Pozo, A., Castillo, D.,Inostroza, L.,Matus, I., Méndez, A., and Morcuende, R.(2012). Physiological and yield responses of recombinant chromosome substitution lines of barley to terminal drought in a Mediterranean- type environment. Annals of Applied Biology 160(2): 157-167.

42. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. Journal of the American Statistical Association 50(272):1096-1121.
43. Eduardo, I., Arus, P., Monforte, A.J. (2005). Development of a genomic library of near isogenic lines (NILs) in melon (Cucumis melo L.) from the exotic accession PI161375. Theor Appl Genet 112:139–148.
44. Eduardo, I., Arus, P., Monforte, A.J.,Obando, J., Fernandez-Trujillo, J.P., Martınez, J.A., Alarcon, A.L., Alvarez, J.M., and van der Knaap, E. 2007. Estimating the genetic architecture of fruit quality traits in melon (Cucumis melo L.) using a genomic library of near-isogenic lines. J. Amer. Soc. Hort. Sci. 132:1–10.
45. Ellenberger, T. (1994). Getting a grip on DNA recognition: structures of the basic region leucine zipper, and the basic region helix-loop-helix DNA-binding domains. Current Opinion in Structural Biology 4(1):12-21.
46. Epron, D., Dreyer, E., Breda, N. (1992). Photosynthesis of oak trees [Quercus petraea (Matt.) Liebl.] during drought under field conditions: diurnal course of net CO2 assimilation and photochemical efficiency of photosystem II. Plant, Cell & Environment 15(7):809-820.
47. Eshed, Y., Zamir, D. (1994) A genomic library of Lycopersicon pennellii in Lycopersicon esculentum-a tool for fine mapping of genes. Euphytica 79:175-179
48. Eshghi, R., Salayeva, S., Ebrahimpour, F., Rahimi, M., Baraty, M. and Ojaghi, J. (2013). Advancedbackcross QTL analysis in hulless barley: I. Detection of exotic alleles for yield and yield components introgressed from Hordeum vulgare ssp. spontaneum. International Journal of Agriculture and Crop Sciences 5(2),95-100.
49. Estrada-Campuzano, G., Miralles, D.J., Slafer, G.A. (2008). Genotypic variability and response to water stress of pre- and post-anthesis phases in triticale, Eur. J. Agron. 28, 171–177.
50. FAO. (2017) US Department of Agriculture: https://www.statista.com/statistics/272760/barley-harvest-forecast/).

51. Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A. (2009). Plant drought stress: effects, mechanisms and management. Agronomy for Sustainable Development, Springer Verlag/EDP Sciences/INRA, 29 (1), pp.185-212.
52. Fernandez-Trujillo, J.P., Obando, J., Martınez, J.A., Alarcon, A.L., Eduardo, I., Arus, P., Monforte, A.J. (2007). Mapping fruit susceptibility to postharvest physiological disorders and decay using a collection of near-isogenic lines of melon. J. Amer. Soc. Hort. Sci.132:739–748.
53. Finkers, R., van Heusden, A.W., Meijer-Dekens, F., van Kan, J.A.L., Maris, P., Lindhout, P. (2007). The construction of a Solanum habrochaites LYC4 introgression line population and the identification of QTLs for resistance to Botrytis cinerea. Theor Appl Genet 114:1071–1080.
54. Fischer, R., et al. (1998). Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. Crop science 38(6):1467-1475.
55. Fracheboud, Y., Jompuk, C., Ribaut, J., Stamp, P., Leipner, J. (2004). Genetic analysis of cold-tolerance of photosynthesis in maize. Plant molecular biology 56(2):241-253.
56. Franckowiak, J.D., Konishi, T. (2002). Early maturity 6, Eam6. Barley Genet Newsl 32:86–87.
57. Frederick J.R, Camp C.R., Bauer P.J. (2001) Drought-stress effects on branch and main stem seed yield and yield components of determinate soybean, Crop Sci. 41, 759–763.
58. Fu, J., Huang, B. (2001), Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress, Environ. Exp. Bot. 45, 105–114.
59. Fujita, Y., Fujita, M., Shinozaki, K., Yamaguchi-Shinozaki, K. (2011). ABA-mediated transcriptional regulation in response to osmotic stress in plants. Journal of plant research 124(4):509-525.

60. Fujita, Y., Yoshida, T., Yamaguchi-Shinozaki, K. (2013), Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. Physiologia plantarum 147(1):15-27.
61. Funck, D., Eckard, S., Muller, G. (2010). Non-redundant functions of two proline dehydrogenase isoforms in *Arabidopsis*. BMC Plant Biol. 10:70. 10.1186/1471-2229-10-70
62. Genty, B., Briantais, J.M., Baker, N.R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990: 87-92.
63. Gomez, S.M., Kumar, S.S., Jeyaprakash, P., Suresh, R., Biji, K.R., Boopathi, N.M., Price, A.H., Babu, R.C. (2006). Mapping QTLs Linked to Physio-Morphological and Plant Production Traits under Drought Stress in Rice (Oryza sativa L) in the Target Environment American J. Bioch Biotech 2(4):161-169.
64. Guo, P.G., Baum, M., Varshney, R.K., Graner, A., Grando, S., and Ceccarelli, S. 2008. QTLs for chlorophyll and chlorophyll fluorescence parameters in barley under post-flowering drought. Euphytica 163: 203-214.
65. Gupta, P.K., Rustgi, S. (2004). Molecular markers from the transcribed/expressed region of the genome in higher plants. Functional and Integrative Genomics 4, 139–162
66. Hare, P., Cress, W. (1997). Metabolic implications of stress-induced proline accumulation in plants. Plant growth regulation 21(2):79-102.
67. Harris, D., Tripathi, R.S., Joshi, A. (2002). On-farm seed priming to improve crop establishment and yield in dry direct-seeded rice, in: Pandey S., Mortimer M., Wade L., Tuong T.P., Lopes K., Hardy B. (Eds.), Direct seeding: Research Strategies and Opportunities,International Research Institute, Manila, Philippines, pp. 231–240.
68. Henry, R. (2006). J. Plant Conservation Genetics (New York, Haworth Press.
69. Hoffmann, et al. (2012). Detection of nitrogen deficiency QTL in juvenile wild barley introgression lines growing in a hydroponic system. BMC Genetic.13:88.

70. Honsdorf, N., March, T.J., Hecht, A., Eglinton, J., Pillen, K. (2014). Evaluation of juvenile drought stress tolerance and genotyping by sequencing with wild barley introgression lines. Molecular Breeding 34(3):1475-1495.
71. Hossain, M.M., Lam, H-M., Zhang, J. (2015). Responses in gas exchange and water status between drought-tolerant and-susceptible soybean genotypes with ABA application. The Crop Journal 3(6):500-506.
72. Hu, W., Yuan, Q., and Wang, Y. (2012). Overexpression of a wheat aquaporin gene, TaAQP8, enhances salt stress tolerance in transgenic tobacco. Plant and Cell Physiology 53, 2127–2141.
73. Hummel, I., Pantin, F., Sulpice, R., Piques, M., Rolland, G., Dauzat, M., Christophe, A., Pervent M., Bouteillé, M., Stitt, M., Gibon, Y., Muller, B. (2010). Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. Plant Physiology 154: 357-372.
74. Hurst, H. (1995). Protein profile-transcription factors 1: bZIP proteins, vol. 2. (Academic Press, London, UK).
75. Ibrahim, M., Hassan, A., Arshad, M., Tanveer, A. (2010). Variation in root growth and nutrient element of wheat and rice: effect of rate and type of organic materials. Soil and Environment, 29: 47-52.
76. Ibrahim, M.S.E. (2007). QTL analysis of drought tolerance in spring wheat (Triticum aestivum L.) Ph.D. thesis, Crop Science and biotechnology dept., faculty of Agriculture, Bonn University.
77. Ibrahim, S.E., Schubert, A., Pillen, K., Lon, J. (2010). Quantitaive trait loci analysis for drought tolerance in an advanced backcross population of spring wheat. Sudan J Agric Res 15:118.
78. Jajarmi, V. (2009). Effect of water stress on germination indices in seven wheat cultivar. World Acad Sci Eng Technol 49:105–106
79. Jamieson, P.D., Martin, R.J., Francis, G.S., Wilson, D.R. (1995). Drought effects on biomass production and radiation-use efficiency in barley. Field Crop Res. 43, 77–86.
80. Jia, Q., Zhang, X.Q.,Westcott, S., Broughton, S., Cakir, M., Yang, J., Lance, R., Li, C. (2011). Expression level of a gibberellin 20–oxidase gene is

associated with multiple agronomic and quality traits in barley. Theor Appl Genet 122:1451–1460,

81. Johanson, U., Karlsson, M., Gustavsson, S., Sjovall, S., Fraysse, L., Weig, A.R., Kjellbom, P. (2001). The complete set of genes encoding Major Intrinsic Proteins in Arabidopsis provides a framework for a new nomenclature for Major Intrinsic Proteins in plants. Plant Physiol 126: 1358–1369.
82. Jones, H.G. (2007). Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. Journal of experimental botany 58(2):119-130.
83. Jongrungklang, N., Toomsan, B., Vorasoot, N., Jogloy, S., Boote, K.J.,Hoogenboom, G., Patanothai, A. (2013). Drought tolerance mechanisms for yield responses to pre-flowering drought stress of peanut genotypes with different drought tolerant levels. Field Crops Research 144, 34–42.
84. Kano, M., Inukai, Y., Kitano, H.,Yamauchi, A. (2011). Root plasticity as the key root trait for adaptation to various intensities of drought stress in rice. Plant and Soil 342, 117–128.
85. Katoh, K., Misawa, K., Kuma, Ki., Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic acids research 30(14):3059-3066.
86. Kaushal, N., et al. (2011). Proline induces heat tolerance in chickpea (Cicer arietinum L.) plants by protecting vital enzymes of carbon and antioxidative metabolism. Physiology and Molecular Biology of Plants 17(3):203-213.
87. Kavar, T., Maras, M., Kidrič, K., Šuštar-Vozlič, J., Meglič, V. (2008). Identification of genes involved in the response of leaves of Phaseolus vulgaris to drought stress. Molecular Breeding 21, 159–172.
88. Kaya C, SÖNMEZ O, Aydemir S, and DİKİLİTAŞ, M. (2013) Mitigation effects of glycinebetaine on oxidative stress and some key growth parameters of maize exposed to salt stress. Turkish Journal of Agriculture and forestry 37(2):188-194.
89. Kaya, C.O., Sonmez, S., Demir, A.Y., Dikilitas, M. (2013). Mitigation effects of glycinebetaine on oxidative stress and some key growth parameters of maize

exposed to salt stress. Turkish Journal of Agriculture and forestry 37(2):188-194.

90. Kaya, M.D., Okçub, G., Ataka, M., Çıkılıc, Y., Kolsarıcıa, Ö. (2006). Seedtreatments to overcome salt and drought stress during germinationin sunflower (Helianthus annuusL.), Eur. J. Agron. 24, 291–295.
91. Kearsey, M.J., Pooni, H.S. (1996). The Genetic Analysis of Quantitative Traits. Chapman and Hall, London.
92. Kent, N.L., Evers, A.D. (1994). Kent,s technology of ceraeal Elsevier science: Oxford.
93. Keurentjes, J.J.B., Bentsink, L., Alonso-Blanco, C., Hanhart, C.J., Vries, H.B.D., Effgen, S., Vreugdenhil, D., Koornneef, M. (2007). Development of a near-isogenic line population of Arabidopsis thaliana and comparison of mapping power with a recombinant inbred line population. Genetics 175:891–905.
94. Kishor, K.P.B., Sangam, S., Amrutha, R.N., Sri Laxm,i P., Naidu, K.R., Rao, KRSS., Rao, S., Reddy, P., Theriappan, P., Sreenivasulu, N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr Science 88: 424–438.
95. Klughammer, C. Schreiber, U. (2008). Saturation pulse method for assessment of energy conversion in PS I. PAM Application Notes 1: 11-14 http://www.walz.com/e_journal/pdfs/PAN07002.pdf.
96. Kranner, I., Minibayeva, F., Beckett, R.P., Seal C.E. (2010). What is stress? concepts, definitions and applications in seed science. New Phytologist. 188 (3) 655-673.
97. Kuczyńska A, Surma M, Adamski T, Mikołajczak K, Krystkowiak K, Ogrodowicz P (2013) Effects of the semi–dwarfing sdw1/denso gene in barley. J Appl Genet 54:381–390.
98. Kumar, J., Abbo, S. (2001). Genetics of flowering time in chickpea and its bearing on productivity in the semi-arid environments, Adv. Agron.72, 107–138.

99. Lancashire, P.D., Bleiholder, H., Boom, T.V.D., Langelüddecke, P., Stauss, R., Weber, E., Witzenberger, A. (1991). An uniform decimal code for growth stages of crops and weeds. Ann Appl Biol, 119:561-601.

100. Larcher, W. (2003). Physiological plant ecology: Ecophysiology and stress physiology of functional groups. Heidelberg: springer-verlag. p345-437.

101. Lawlor, D.W. Tezara, W. (2009). Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. Annals of botany.

102. Le, B.H., Wagmaister, J.A., Kawashima, T., Bui, A.Q., Harada, J.J., Goldberg, R.B. (2007). Using genomics to study legume seed development. Plant Physiolog. 144(2) 564-572.

103. Lehmann, S., Gumy, C., Blatter, E., Boeffel S., Fricke, W., Rentsch, D. (2011). In planta function of compatible solute transporters of the AtProT family. J. exp. Bot. 62: 787–796.

104. Lesk, C., Rowhani, P., Ramankutty, N. (2016). Influence of extreme weather

105. Levitt, J. (1980). Responses of Plants to Environmental Stresses. Vol. II. Water, Radiation, Salt and Others Stresses.

106. Li, J., Ban, L., Wen, H., Wang, Z., Dzyubenko, N., Chapurin, V., Gao, H.,Wang, X. (2015). An aquaporin protein is associated with drought stress tolerance. Biochemical and Biophysical Research Communications 459, 208–213.

107. Li, J.Z., Huang, X.Q., Heinrichs, F., Ganal, M.W. and Röder, M.S. (2004). Analysis of QTLs for yield, yield components, and malting quality in a BC3-DH population of spring barley. Theoretical and Applied Genetics, 110 (2), 356-363.

108. Li, R.H., Guo, P.G., Michael, B., Stefania, G., Salvatore, C. (2006). Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Agricultural Sciences in China 5(10):751-757.

109. Li, Z.K., Fu, B.Y., Gao, Y.M., Xu, J.L., Ali, J., Lafitte, H.R., Jiang, Y.Z., Rey, J.D., Vijaya kumar, C.H.M., Maghirang, R., Zheng, T.Q., Zhu, L.H. (2005) Genome-wide introgression lines and their use in genetic and molecular

dissection of complex phenotypes on rice (Oryza sativa L.). Plant Mol Biol 59:33–52.

110. Liang, X., Zhang, L., Natarajan, S.K., Becker, D.F. (2013). Proline mechanisms of stress survival. Antioxidants & redox signaling 19(9):998-1011.

111. Ling, Q., Huang, W., and Jarvis, P. (2011). "Use of a SPAD-502 meter to measure leaf chlorophyll concentration in Arabidopsis thaliana." Photosynthesis research 107(2): 209-214.

112. Liu, C., Fukumoto, T., Matsumoto, T. (2013). Plant Physiology and Biochemistry Aquaporin OsPIP1 ; 1 promotes rice salt resistance and seed germination. Plant Physiology and Biochemistry journal 63, 151–158.

113. Liu, Y., et al. (2015). Assessment of drought tolerance of 49 switchgrass (Panicum virgatum) genotypes using physiological and morphological parameters. Biotechnology for biofuels 8(1):1.

114. Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25(4):402-408.

115. Malosetti, M., van Eeuwijk, F.A., Boer, M.P., Casas, A.M., Elia, M. (2011). Gene and QTL detection in a three–way barley cross under selection by a mixed model with kinship information using SNPs. Theor Appl Genet 122:1605–1616.

116. Marcotuli, I., Agata, G., Giacomo, M., Antonio, S., Silvana, Z., Antonio, B., Rosanna, S., Pasqualina, C. (2017). "Development of a High-Density SNP-Based Linkage Map and Detection of QTL for Î²-Glucans, Protein Content, Grain Yield per Spike and Heading Time in Durum Wheat." International Journal of Molecular Sciences 18.6: 1329.

117. Markwell, J., Osterman, J.C., and Mitchell, J.L. (1995). "Calibration of the Minolta SPAD-502 leaf chlorophyll meter." Photosynthesis research 46(3): 467-472.

118. Mather, K. (1949). Biometrical Genetics, 1st Edn. Methuen, London.

119. Mattioli, R., Marchese, D., D'Angeli, S., Altamura, M.M., Costantino, P., Trovato, M. (2008). Modulation of intracellular proline levels affects flowering

time and inflorescence architecture in Arabidopsis. Plant Mol Biol 66: 277–288.DOI: 10.1007/s11103-007-9269-1.

120. Mattioli, R., Marchese, D., D'Angeli, S., Altamura, M.M., Costantino, P., Trovato, M. (2008). Modulation of intracellular proline levels affects flowering time and inflorescence architecture in Arabidopsis. Plant Mol Biol 66: 277–288.DOI: 10.1007/s11103-007-9269-1.

121. Maurer, A., Draba, V., and Pillen, K. (2016). Genomic dissection of plant development and its impact on thousand grain weight in barley through nested association mapping. J. Exp. Bot. 67:2507–2518. doi:10.1093/jxb/er w070.

122. Maurer, A., Draba, V., Jiang, Y., Schnaithmann, F., Sharma, R., Schumann, E. (2015). Modelling the genetic architecture of flowering time control in barley through nested association mapping. BMC Genomics16 :290. doi:10.1186/s12864 - 015-1459-7.

123. Md Arifuzzaman, Muzammil S., Pillen, K., Naz, AA., and Léon, J. (2014). Wild barley introgression lines revealed novel QTL alleles for root and related shoot traits in the cultivated barley (Hordeum vulgare L). BMC Genetics 2014, 15:107).

124. Md Arifuzzaman, Naz, AA., Sayed, M.A., Muzammil, S., Pillen, K., and Léon, J. (2014). Detection and validation of novel QTL for shoot and root traits in barley (Hordeum vulgare L.) Molecular Breeding .Vol. 34, Issue 3, pp 1373-1387.

125. Medrano, H., Escalona, J.M., Bota, J., Gulías, J., Flexas, J. (2002). Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter. Annals of botany 89(7):895-905.

126. Miller, G., Honig, A., Stein, H., Suzuki, N., Mittler, R., Zilberstein, A. (2009). Unraveling Δ1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. Journal of Biological Chemistry, v. 284, n. 39, p. 26482-26492.

127. Miller, G., Stein, H., Honig, A., Kapulnik, Y., Zilberstein, A. (2005). Responsive modes of Medicago sativa proline dehydrogenase genes during

salt stress and recovery dictate free proline accumulation. Planta, v. 222, n. 1, p. 70-79.

128. Mizoi, J., Yamaguchi-Shinozaki, K. (2013). Molecular approaches to improve rice abiotic stress tolerance, in Rice Protocols, ed. Y. Yang (New York, NY: Humana Press), 269–283.

129. Mohammed, A., Tarpley, L. (2011). High night temperature and plant growth regulator effects on spikelet sterility, grain characteristics and yield of rice (Oryza sativa L.) plants. Canadian journal of plant science 91(2):283-291.

130. Molina-Cano, J. L., Fra Mon, P., Salcedo, G., Aragoncillo, G., Roca De Togores, F., Garcia-Olmedo, F. (1987). Morocco as a possible domestication center for barley: biochemical and agromorphologicalevidence. Theor. Appl. Genet. 73:531–536.

131. Mon-forte, A.J., Tanksley, S.D. (2000). Development of a set of near isogenic and backcross recombinant inbred lines containing most of the Lycopersicon hirsutum genome in a L.esculentum genetic background: A tool for gene mapping and gene discovery. Genome 43:803–813.

132. Morgan, P.W. (1990). Effects of abiotic stresses on plant hormone systems, in: Stress Responses in plants: adaptation and acclimation mechanisms, Wiley-Liss, Inc., pp. 113–146

133. Mutters, R.G., Ferreira, L.G.R., Hall, A.E. (1989). Proline content of the anthers and pollen of heat-tolerant and heat sensitive cowpea subjected to different temperatures. Crop Sci 29: 1497–1500.

134. Nakashima, K., et al. (2009). Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2. 2, SRK2E/SnRK2. 6/OST1 and SRK2I/SnRK2. 3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant and Cell Physiology 50(7):1345-1363.

135. Nam, N,H., Y,S, Chauhan., C, Johansen. (2001). Effect of timing of drought stress on growth and grain yield of extra-short-duration pigeon pea lines, J. Agr. Sci. 136, 179–189.

136. Obando, J., fernández-T., Martínezja, A., Eduardoi, A. P., Monforte, A. J. (2008). Identification of melon fruit quality quantitative trait loci using near-

isogenic lines. Journal of the American Society for Horticultural Science 133: 139-151.

137. Ober, E.S., Setter, T.L., Madison, J.T., JThompson, J.F., Shapiro, P.S. (1991). Influence of water deficit on maize endosperm development: enzyme activities and RNA transcripts of starch and zein synthesis, abscisic acid, and cell division, Plant Physiol. 97, 154–164.

138. Okcu, G., Kaya, M.D., Atak, M. (2005). Effects of salt and drought stresses on germination and seedling growth of pea (Pisum sativumL.),Turk. J. Agr. For. 29, 237–242.

139. Öncel, I., Keles, Y., Ustun, A.S. (2000). Interactive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. Environ. Pollut., 107, 315-320.

140. Ouda, S.A., Noreldin, T., Amer, A. (2016). Rain Fed Areas in Egypt: Obstacles and Opportunities. In Management of Climate Induced Drought and Water Scarcity in Egypt (pp. 27-46). Springer International Publishing

141. Ovcharenko, I., et al. (2005) Mulan: multiple-sequence local alignment and visualization for studying function and evolution. Genome research 15(1):184-194.

142. Parida, A.K., Dagaonkar, V.S., Phalak, M.S., Aurangabadkar, L.P. (2008). Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. Acta Physiologiae Plantarum 30: 619–627.

143. Park, W., Scheffler, B.E., Bauer, P.J., Campbell, B.T. (2010). Identification of the family of aquaporin genes and their expression in upland cotton (Gossypium hirsutum L.). BMC Plant Biol 10: 142.

144. Passioura, J. (2007). The drought environment: physical, biological and agricultural perspectives, *Journal of Experimental Botany*, Volume 58, Issue 2, , Pages 113–117, https://doi.org/10.1093/jxb/erl212

145. Pei, Z-M., Ghassemian, M., Kwak, C.M., McCourt, P., Schroeder, J.I. (1998). Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science 282(5387):287-290.

146. Peighambari, S.A., Yazdi Samadi, B., Nabipour, A., Charmet, G., Sarrafi, A. (2005). QTL analysis for agronomic traits in barley doubled haploids population grown in Iran. Plant Science 169, 1008-1013.

147. Pennisi, E. (2008). Plant genetics. The blue revolution, drop by drop, gene by gene. Science (New York, N.Y.) 320, 171–173.

148. Pestsova, E.G., Börner, A., Röder, M.S. (2006). Development and QTL assessment of Triticum aestivum–Aegilops tauschii introgression lines. Theor Appl Genet 112:634–647.

149. Pettigrew, W.T. (2004). Physiological consequences of moisture deficit stress in cotton, Crop Sci. 44, 1265–1272.

150. Pillen, K., Zacharias, A., and Léon, J. (2004). Comparative AB-QTL analysis in barley using a single exotic donor of Hordeum vulgare ssp spontaneum. Theoretical and Applied Genetics ,108,1591-1601.

151. Pillen, K., Zacharias, A., Léon, J. (2003). Advanced backcross QTL analysis in barley (Hordeum vulgare L.). Theor Appl Genet;107:340–52.

152. Pinheiro, C., Chaves, M.M. (2011). Photosynthesis and drought: can we make metabolic connections from available data? Journal of Experimental Botany 62, 869–882. doi:10.1093/jxb/erq340.

153. Quigley, F., Rosenberg, J.M., Shachar-Hill, Y., Bohnert, H.J. (2001). From genome to function: the Arabidopsis aquaporins. Genome Biol 3: 1–17.

154. Rascher, U., Liebig, M., Lüttge, U. (2000). Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. Plant, Cell & Environment 23(12):1397-1405.

155. Raza, M., Saleem, M., Shah, G., Khan, I., Raza, A. (2014). Exogenous application of glycinebetaine and potassium for improving water relations and grain yield of wheat under drought. Journal of soil science and plant nutrition 14(2):348-364.

156. Reddy, K.R., et al. (2013). Exogenous application of glycinebetaine facilitates maize (Zea mays L.) growth under water deficit conditions. American Journal of Experimental Agriculture 3(1):1.

157. Reinert, S., NAZ, A.A., BOSTANCI. C., SEPERI. B., , LEON, J., BÖTTGER, C., SÜDEKUM, KH., and FREI, M. (2017). Mining the global diversity for bioenergy traits of barley straw: genomewide association study under varying plant water status. GCB Bioenergy , doi: 10.1111/gcbb.12433.

158. Rentsch, D., Schmidt, S., Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants FEBS Lett., 58, pp. 2281-2289

159. Reuscher. S., Akiyama, M., Mori, C., Aoki. K., Shibata, D., Shiratake, K. (2013). Genome-wide identification and expression analysis of aquaporins in tomato. PLoS One 8: e79052.

160. Ribarits, A., Abdullaev, A., Tashpulatov, A., Richte,r A., Heberle-Bors, E., Touraev, A. (2007). Two tobacco proline dehydrogenases are differentially regulated and play a role in early plant development. Planta 225:1313–1324.

161. Riley, T., Sontag, E., Chen, P., Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nature reviews Molecular cell biology 9(5):402-412.

162. Rousseaux, M.C,. Jones, C.M., Adams, D. (2005). QTL analysis of fruit antioxidants in tomato using Lycopersicon pennellii introgression lines. Theor Appl Genet 111:1396–1408.

163. Ruiz, J.M., Sanchez, E., Garcia, P.C., Lopez-Lefebre, L.R., Rivero, R.M., Romero, L. (2002). Proline metabolism and NAD kinase activity in greenbean plants subjected to cold-shock. Phytochemistry, 59, 473–478.

164. Saal, B., M., von Korff, M., Lèon, J. and Pillen, K. (2010). Advanced-backcross QTL analysis in spring barley: IV Localization of QTL 3 nitrogen interaction effects for yield-related traits. Euphytica, 177 (2), 223-239.

165. Sakurai, J., Ishikawa, F., Yamaguchi, T., Uemura, M., Maeshima, M. (2005). Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant Cell Physiol 46: 1568–1577.

166. Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, S., Yanofsky, M.F., Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288: 1613–1616.DOI: 10.1126/science.288.5471.1613.

167. Samarah, N.H. (2005). Effects of drought stress on growth and yield of barley. Agronomy for Sustainable Development 25, 145–149.

168. Sato, K., Nankaku, N., and Takeda, K. (2009). A high-density transcript linkage map of barley derived from a single population. Heredity 103: 110–117.

169. Sax, K. (1923). The association of size difference with seed-coat pattern and pigmentation in Phaseolus vulgaris. Genetics, 8, 552-560.

170. Sayed, M. A., El-sadek, A.N., Bakry, B, A., Ali, M. B., Leon, J., and, Salem, E.M. (2017). QTL analysis in Barley Across Environments in Egypt. Egypt.J.Agron. Vol.39, No.1, pp.53- 70.

171. Sayed, M.A., Schumann, H., Pillen, K., Naz, A.A., Léon, J. (2012) AB-QTL analysis reveals new alleles associated to proline accumulation and leaf wilting under drought stress conditions in barley (Hordeum vulgare L.). BMC Genetics 13:61.

172. Schmalenbach, I., Körber, N., Pillen, K. (2008). Selecting a set of wild barley introgression lines and verification of QTL effects for resistance to powdery mildew and leaf rust. Theor Appl Genet, 117(7):1093-1106.

173. Schmalenbach, I., March, T.J., Bringezu, T., Waugh, R., Pillen, K. (2011). High-resolution genotyping of wild barley introgression lines and fine-mapping of the threshability locus thresh-1 using the Illumina GoldenGate assay. G3: Genes, Genomes, Genetics 552, 1(3):187-196.

174. Schulze-Lefert, P., Dangl, J.L., Becker-Andre, M., Hahlbrock, K., Schulz, W. (1989). Inducible in vivo DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. The EMBO journal 8(3):651.

175. Schwartz, S., Kent, W.J., Smit, A., Zhang, Z., Baertsch, R., Hardison, R.C., Haussler, D., Miller, W. (2003b). Human–mouse alignments with BLASTZ. Genome Res. 13: 103-107.

176. Seki, M., Narusaka, M., Ishida, J. (2002). Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant Journal 31, 279–292.

177. Serraj, R., Sinclair, T.R. (2002). Osmolyte accumulation: Can it really help increase crop yield under drought conditions? Plant, Cell and Environment 25, 333–341.

178. Sharma, S., Villamor, J.G., Verslues, P.E. (2011). Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant Physiol. 157, 292–304. doi: 10.1104/pp.111.183210.

179. Shen, Q., Zhang, P., Ho, T. (1996). Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. The Plant Cell 8(7):1107-1119.

180. Siangliw, L., Jongdee, B., Pantuwan, G., Toojinda, T. (2007). Developing KDML105 backcross introgression lines using marker-assisted selection for QTLs associated with drought tolerance in rice. Sci Asia 33:207–214.

181. Souza, R., Machado, E., Silva, J., Lagôa, A., Silveira, J. (2004). Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (Vigna unguiculata) during water stress and recovery. Environ Exp Bot 51(1):45-56.

182. Strizhov, N., et al. (1997). Differential expression of two P5CS genes controlling proline accumulation during salt stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. The Plant Journal 12(3) : 557-569.

183. Subbarao, G.V., Johansen, C., Slinkard, A.E., Rao, R.C.N., Saxena, N.P., Chauhan, Y.S. (1995). Strategies and scope for improving drought resistance in grain legumes, Crit. Rev. Plant Sci. 14, 469–523.

184. Szabados, L., Savoure, A. (2010). Proline: a multifunctional amino acid. Trends in plant science 15(2):89-97.

185. Szalma, S.J., Hostert, B.M., LeDeaux, J.R., Stuber, C.W., Holland, J.B. (2007). QTL mapping with near-isogenic lines in maize. Theor Appl Genet 114:1211–1228.

186. Taie, H., Abdelhamid, M., Dawood, M., Nassar, R. (2013). Pre-sowing seed treatment with proline improves some physiological, biochemical and

anatomical attributes of faba bean plants under sea water stress. J Appl Sci Res 9(4):2853-2867.

187. Taiz, L., Zeiger, E. (2006). Plant Physiology, 4th Ed., Sinauer Associates Inc. Publishers, Massachusetts.

188. Taiz, L., Zeiger, E. (1991). Plant Physiology. California: The Benjamin/cummings publishing company, Inc. p346-368.

189. Tan, L.B., Liu, F.X., Xue, W., Wang, G.J., Ye, S., Zhu, Z.F., Fu, Y.C., Wang, X.K., Sun, C.Q. (2007). Development of Oryza rufipogon and O. sativa introgression lines and assessment for yield-related quantitative trait loci. J Integr Plant Biol 49:871–884.

190. Thomas, W.T.B., Powell, W., Swanston, J.S. (1991). The effects of major genes on quantitatively varying characters in barley. 4. The GPert and denso loci and quality characters. Heredity 66:381–389.

191. Tian, F., Li, D.J., Fu, Q., Zhu, Z.F., Fu, Y.C., Wang, X.K., Sun, C.Q. (2006a). Construction of introgression lines carrying wild rice (Oryza rufipogon Griff.) segments in cultivated rice (Oryza sativa L.) background and characterization of introgressed segments associated with yield-related traits. Theor Appl Genet 112:570–580.

192. Toniatti, C., Demartis, A., Monaci, P., Nicosia, A., Ciliberto, G. (1990). Synergistic trans-activation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites. The EMBO Journal 9(13):4467.

193. Turner, N.C., Wright, G.C., Siddique, K.H.M. (2001). Adaptation of grain legumes (pulses) to water-limited environments. Advances in Agronomy 71, 193–231.

194. Tuteja N., Baluska F., Mancuso S. (2009). Integrated calcium signaling in plants. Signaling in Plants I. Springer; Heidelberg, Germany: pp. 29–49.

195. Uno, Y., et al. (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences 97(21):11632-11637.

196. Varshney, R.K., Bansal, K.C., Aggarwal, P.K., Datta, S.K., Craufurd, P.Q. (2011). Agricultural biotechnology for crop improvement in a variable climate: hope or hype? Trends in Plant Science 16, 363–371. doi:10.1016/j.tplants.2011.03.004.

197. Varshney, R.K., Nayak, S.N., May, G.D., Jackson, S.A. (2009). Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends Biotechnol. 27, 522–530.

198. Verbruggen, N., Hermans, C. (2008). Proline accumulation in plants: a review. Amino Acids 35: 753 759.

199. Verma, V., Ravindran, P., Kumar, P.P. et al. (2016). Plant hormone-mediated regulation of stress responses. BMC Plant Biology 16, 86.

200. Vignal, A., et al. (2002). A review on SNP and other types of molecular markers and their use in animal genetics. Genetics Selection Evolution 34, 275–305.

201. Vinod, M.S., Sharma, N., Manjunatha, K., Kanbar, A., Prakash, N.B. and Shashidhar, H.E. (2006). Candidate genes for drought tolerance and improved productivity in rice (Oryza sativa L.). J. Biosci. 31:69-74.

202. von Korff M., Grando S., Greco A.D., This, D., Baum, M., Ceccarelli, S. (2008) Quantitative trait loci associated with adaptation to Mediterranean dryland conditions in barley. Theor Appl Genet 117:653-669

203. von Korff, M., Wang, H,. Léon, J. and Pillen, K. (2006). AB-QTL analysis in spring barley:II Detection of favourable exotic alleles for agronomic traits introgressed from wild barley (H vulgare ssp spontaneum). Theoretical and Applied Genetics, 112,1221-1231.

204. Wang, J., Sun, G., Ren, X., Li, C., Liu, L., Wang, Q., Du, B. and Sun, D. (2016). QTL underlying some agronomic traits in barley detected by SNP markers. BMC Genetics, 17,103-126.

205. Wang, X., et al. (2016). Genetic variation in ZmVPP1 contributes to drought tolerance in maize seedlings. Nat Genet.

Water Scarcity in Egypt (pp. 27-46).

206. Wehner, G., Balko, C., Enders, M., Humbeck, K., Ordon, F. (2015). Identification of genomic regions involved in tolerance to drought stress and drought stress induced leaf senescence in juvenile barley. BMC Plant Biol. 15.

207. Wu, Y., Cosgrove, D-J. (2000). Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins, Journal of Experimental Botany, 51, 1543-1553.

208. Xue, D.W., Chen, M.C., Zhou, M.X., Chen, S., Mao, Y., and Zhang, G.P. 2008. QTL analysis of flag leaf in barley (Hordeum vulgare L.) for morphological traits and chlorophyll content. J. Zhejiang Univ. Sci. B. 9: 938-943.

209. Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi, K., Shinozaki, K., Yoshiba, Y.(2005). Effects of free proline accumulation in petunias under drought stress. J Exp Bot, 56:1975-1981.

210. Yang, J., Zhang, J., Wang, Z., Zhu, Q., Wang, W. (2001). Remobilization of carbon reserves in response to water deficit during grain filling of rice, Field Crop. Res. 71, 47–55.

211. Yang, J., Zhang, J. (2006). Grain filling of cereals under soil drying. New Phytologist 169, 223–236. doi:10.1111/j.1469-8137.2005.01597.

212. Yin, X., Stam, P., Dourleijn, C.J., Kropff, M.J. (1999). AFLP mapping of quantitative trait loci for yield determining physiological characters in spring barley. Theor Appl Genet 99(1–2):244–253.

213. Yuan, X., Yang, Z., Li, Y., Liu, Q., Han, W. (2016). Effects of different levels of water stress on leaf photosynthetic characteristics and antioxidant enzyme activities of greenhouse tomato. Photosynthetica 54(1):28-39.

214. Yue, B., Xiong, L., Xue W., Xing, Y., Luo, L., Xu, C. (2005). Genetic analysis for drought resistance of rice at reproductive stage in field with different types of soil. Theor Appl Genet 111:1127-1136.

215. Zamir, D. (2001). Improving plant breeding with exotic genetic libraries. Nat. Rev. Genet. 2:983–989.

216. Zeid, I.M., Shedeed, Z.A. (2006). Response of alfalfa to putrescine treatment under drought stress, Biol. Plant. 50, 635–640.

217. Zhang, X., Zhou, S.X., Fu, Y.C., Su, Z., Wang, X.K., Sun, C.Q. (2006). Identification of a drought tolerant introgression line derived from dongxiang common wild rice (O. rufipogon griff.). Plant Mol Biol 62:247–259.

218. Zhao, J., Sun, H., Dai, H., Zhang, G., Wu, F. (2010). Difference in response to drought stress among tibet wild barley genotypes. Euphytica. 172, 395–403.

219. Zhou, S.X., Tian, F., Zhu, Z.F., Fu, Y.C., Wang, X.K., Sun, C.Q. (2006). Identification of quantitative trait loci controlling drought tolerance at seedling stage in Chinese Dongxiang common wild rice (Oryza rufipogon Griff.). Acta Genetica Sinica 33:551–558.

220. Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247-273

221. Zohary, D., Hopf, M. (1993). Domestication of plants in the Old World: The Origin and Spread of Cultivated Plants in West Africa, Europe, and the Nile Valley. 2nd edn. Oxford, 278.

222. Zohary, D., Hopf, M., & Weiss, E. (2012). Domestication of Plants in the Old World: The origin and spread of domesticated plants in Southwest Asia, Europe, and the Mediterranean Basin. Oxford University Press on Demand.

Appendix

Table S1. List of primers used to walk through positional cloning of major proline QTL in cultivated barley.

Primers	Primer sequence (5’ to 3’)	Purpose	Product size (bp)
HvP5CS1_GF	AAAGGGCAAATTGTGAATGG	Genotyping S42IL-143HR population	504
HvP5CS1_GR	TGTGGTTTTGCTTGCTCTTG	Genotyping S42IL-143HR population	504
HvP5CS1: P-F	TGAAGACTCCAGAACTTGATGACA	sqRT-PCR and RT analysis	411
HvP5CS1: P-R	CTTGACTACGCGATGGCTCT	sqRT-PCR and RT analysis	411
MLOC-40-F	CCGTGATGTGTTCATACTTCG	Positional cloning	502
MLOC-40-R	TGTGTGGGTTCTGTTGCAGT	Positional cloning	502
MLOC-45-F	AAAGGGCAAATTGTGAATGG	Positional cloning	504
MLOC-45-R	TGTGGTTTTGCTTGCTCTTG	Positional cloning	504
PC-F	AGTGACCCCGGTTGGAAACT	Positional cloning	1001
PC-R	GTGTGATGACGCATTCCTCT	Positional cloning	1001
HvP5CS1-F1	TCCTCTCTCTCTGACCTCCC	cDNA sequencing	909
HvP5CS1-R1	TGTGCATCTCAGAGCCTTGT	cDNA sequencing	909
HvP5CS1-F2	GGAGACAAGTCCCGTGTTG	cDNA sequencing	900
HvP5CS1-R2	CAGCAGACATGGATATGGCA	cDNA sequencing	900
HvP5CS1-F3	ATTCCTGTTCTTGGCCATGC	cDNA sequencing	952
HvP5CS1-R3	GTGCAGTGTAACGGTTGCTT	cDNA sequencing	952
Promoter_F	GATGCCGTAATGGATGTTCG	Promoter sequencing	956
Promoter_R	GACGGGATAATCGGTCAAAC	Promoter sequencing	956
Promoter_F1	AGTGACCCCGGTTGGAAACT	Promoter sequencing	1001
Promoter_R1	GTGTGATGACGCATTCCTCT	Promoter sequencing	1001
b-tubulin-F	ATGTTCAGGCGCAAGGCTT	Equalizing control	101
b-tubulin-R	TCTGCAACCGGGTCATTCAT	Equalizing control	101
Ef1-alpha-F	CGAGGAGGACAAGAAAGCAG	Equalizing control	375
Ef1-alpha-R	ACCTGTTGCTGCTGGATTCT	Equalizing control	375