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Identification of quantitative trait loci (QTL) for drought tolerance and leaf senescence in juvenile barley

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SUMMARY

Drought stress as a trait with increasing importance in the background of climate change is an important factor limiting barley yield. Induced by drought, leaf senescence may occur prematurely, leading to a stop of photosynthesis and to an early translocation of stored assimilates into grains. For barley breeding, the identification of quantitative trait loci (QTL) involved in drought stress and leaf senescence may be an advantage as reliable phenotyping for drought stress is difficult to achieve. Therefore, the aim of the present thesis was to identify markers associated to drought stress response and drought stress induced leaf senescence in juvenile barley through genome wide association studies (GWAS), which will facilitate efficient marker based selection procedures. In a first step, a screening method was developed for analysing drought stress response and early leaf senescence in juvenile barley. Next, in semi controlled greenhouse pot experiments 156 winter barley genotypes were analysed in early developmental stages under control and drought stress treatment. Drought application started at the primary leaf stage and continued for a four weeks stress period. These experiments were used for phenotyping six physiological parameters (biomass yield, leaf colour, electron transport rate at photosystem II, osmolality, content of free proline and total content of soluble sugars), as well as for gene expression analysis of genes involved in drought stress and leaf senescence. Significant genotypic and treatment effects were detected for all phenotypic traits and gene expression data. Based on these data and on 3,212 SNP markers of the Illumina 9k iSelect Chip, GWAS were conducted to detect QTL and expression QTL (eQTL). In total, 47 significant QTL were identified for the traits analysed under drought stress conditions and 15 significant eQTL were found for the relative expression of the 14 genes involved in these traits. Under drought stress conditions, two major QTL regions overlapping for different traits such as biomass yield and leaf colour were detected on chromosome 2H at 50 cM and on chromosome 5H at 45 cM. In these QTL, genes coding for proteins involved in drought stress or leaf senescence were identified. Four of these genes showed a differential expression and thus, eQTL were detected. One eQTL for TRIUR3 coincides with the phenotypic QTL on chromosome 5H. After validation respective markers BOPA1_9766-787 and SCRI_RS_102075 may be used in future barley breeding programmes for improving tolerance to drought stress and leaf senescence.

Keywords: Barley, Drought stress, Leaf senescence, QTL, eQTL

ZUSAMMENFASSUNG

Im Rahmen des Klimawandels kommt der Toleranz gegenüber Trockenstress eine steigende Bedeutung zu. Durch die durch Trockenstress ausgelöste frühzeitige Blattseneszenz kommt es zu einem Abbruch der Photosynthese und frühzeitig zu Umlagerungsprozessen von gespeicherten Assimilaten in das Korn. Da eine Phänotypisierung auf Trockenstress und Seneszenz in den Züchtungsprozess der Gerste nur schwer zu integrieren ist, sind markergestützte Selektionsverfahren von Vorteil. Ziel dieser Arbeit war es daher, mittels genomweiter Assoziationsstudien (GWAS) Marker zu identifizieren, die mit Trockenstress-, oder Blattseneszenzparametern in juveniler Gerste assoziiert sind. Für die Phänotypisierung hinsichtlich der Reaktion auf Trockenstress und dadurch induzierter Blattseneszenz, wurde ein Screening- Verfahren entwickelt, welches eine verlässliche Erfassung dieser Merkmale erlaubt. 156 Wintergerstengenotypen wurden in frühen Entwicklungsstadien im Gewächshaus unter Kontroll- und Stressbedingungen analysiert. Die Trockenstressapplikation erfolgte vom Primärblattstadium für 4 Wochen. In diesen Versuchen wurden sechs physiologische Merkmale erfasst (Biomasse, Blattfarbe, Elektronentransportrate am Photosystem II, Osmolalität, Gehalt an freien Prolin und der Gesamtgehalt an löslichen Zuckern), sowie Expressionsstudien für Gene welche in die Trockenstressreaktion bzw. die Seneszenz involviert sind, durchgeführt. Für diese Merkmale und die Expression der 14 ausgewählten Gene konnten signifikante Genotyp- und Behandlungseffekte nachgewiesen werden. Anhand dieser Daten und 3.212 SNP Markern des Illumina 9k iSelect Chips wurden GWAS durchgeführt, um Genomregionen (QTL und eQTL) zu lokalisieren. Insgesamt konnten für die physiologischen Merkmale 47 QTL für die Reaktion auf Trockenstress, und weiterhin 15 eQTL identifiziert werden. Dabei wurden zwei Haupt- QTL-Regionen auf Chromosom 2H bei 50 cM und 5H bei 45 cM lokalisiert, in denen einige QTL für unterschiedliche Parameter, wie Biomasse und Blattfarbe nachgewiesen wurden. In diesen QTL-Regionen wurden Proteine identifiziert, die im Zusammenhang mit Trockenstress und Blattseneszenz stehen. Vier der regulierenden Gene zeigten eine differentielle Expression und es wurden entsprechend eQTL identifiziert. Ein eQTL für TRIUR3 stimmte mit dem, mittels phänotypischer Daten identifizierten QTL auf Chromosom 5H überein. Die assoziierten Marker BOPA1_9766-787 und SCRI_RS_102075 können nach Validierung geeignete Marker für eine Selektion auf Trockenstresstoleranz und Blattseneszenz in der Gerstenzüchtung darstellen. **Schlagwörter:** Gerste, Trockenstress, Blattseneszenz, QTL, eQTL

1 GENERAL INTRODUCTION

The present thesis is concerned with the identification of quantitative trait loci and candidate genes involved in leaf senescence and drought stress tolerance in juvenile barley (*Hordeum vulgare* L.) through genome wide association studies (GWAS).

1.1 BARLEY

About 10,000 years ago, barley was domesticated and used as a crop plant. The origin of barley cultivation and domestication was evidenced by archaeological findings of barley seeds in the Fertile Crescent, which still is the centre of diversity (Harlan and Zohary 1966). It was proven that domesticated barley (*Hordeum vulgare* ssp. *vulgare* L.) traces back to *Hordeum vulgare* ssp. *spontaneum* C. Koch. In comparison to wild barley, cultivated barley has larger seeds, a non-brittle rachis, plants are shorter with larger flag leaves and ear width, endosperm grooves are less pronounced and in contrast to *H. spontaneum,* six rowed ears are known in cultivated barley, which arose independently by a loss of function mutations of *Vrs1* (Badr et al. 2000; Salamini et al. 2002; Sang 2009). Furthermore, barley genotypes with naked kernels are especially suited for human consumption caused by a mutation in the gene *Nud* (Taketa et al. 2008). A non-brittle rachis is inherited from the dominant, complementary genes *Btr1* and *Btr2* (Pourkheirandish et al. 2015; Senthil and Komatsuda 2005). However, studies on barley domestication are still ongoing. Recently, it was reported that plants with a non-brittle rachis were selected twice, spatially and temporally independent from each other (Pourkheirandish et al. 2015).

The genus *Hordeum* of the tribe *Triticeae* consists of about 30 species, which can be crossed to cultivated barley with a different success rate and are grouped in three gene pools based on crossability (von Bothmer et al. 2003). The primary gene pool consists of cultivated barley (*H. vulgare* ssp. *vulgare*) and its wild progenitor (*H. vulgare* ssp. *spontaneum*). Crosses within the primary gene pool can be conducted easily and unrestricted gene transfer is possible. The secondary gene pool of cultivated barley is exclusively represented by *H. bulbosum.* Crosses with *H. vulgare* are possible with difficulties only and often result in haploid plants. Therefore, *H. bulbosum* has been used to produce doubled haploids in barley for a long time (Devaux et al. 1993). All other

species of the genus *Hordeum* are included in the tertiary gene pool, in which crosses and gene transfer of valuable alleles to *H. vulgare* are almost impossible. For breeding the diversity present in *H. vulgare* ssp. *spontaneum* and *H. bulbosum* are a valuable source to improve resistance against pathogens or tolerance against abiotic stress (Forster et al. 2000). As an example, resistance genes *Rrs16*^{Hb} (scald i.e. *Rhynchopsorium secalis*) and *Rym16*^{Hb} (soil-borne viruses e.g. BaYMV and BaMMV) were transferred from *H. bulbosum* to *H. vulgare* (Pickering et al. 2006; Ruge-Wehling et al. 2006).

H. vulgare L. is a self fertilising species with an annual life cycle and a diploid genome (2n = 2x = 14) with seven chromosomes (1H – 7H). For barley, winter and spring types are known, differing in their vernalisation requirements (Roberts et al. 1988). Winter barley is mainly used for animal feed, whereas spring barley is applied for malting. To a small extent, barley is also used for direct human nutrition. Nevertheless, new barley cultivars were developed containing an increased β-glucan content and a desirable starch composition, which makes barley interesting as an alternative to wheat based foods (Ames and Rhymer 2008). Moreover, health claims were defined as β-glucan soluble fibre of barley reduces the plasma cholesterol level and thereby causes a risk of heart disease (Behall et al. 2004; Rowlands and Hoadley 2006). Because of this wide range of usage and its wide adaptation to diverse growing conditions, barley is the fourth most important cereal crop in the world next to wheat, maize and rice. In 2013, barley was grown on about 50 million hectare with a production of 143.9 million tonnes worldwide. Thereof, Europe produces 60% and Germany is the second largest producer in the world next to the Russian Federation, with a harvest amount of 10.3 million tonnes (FAOSTAT 2014).

Besides being a major crop plant, barley is to some extent a model species for monocots in genome research, due to its genome size of 5.1 Gbp, which is smaller than that of wheat (17 Gbp) and rye (8 Gbp). The first genetic map based on molecular markers was constructed applying restriction fragment length polymorphism (RFLP) markers (Graner et al. 1991). Based on polymerase chain reaction (PCR) marker technologies, which were developed in the 1990s, this reliable but expensive and time consuming marker development was replaced by amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) marker techniques (Qi et al. 1998; Russell et al. 1997; Waugh et al. 1997). Regarding the dominant nature of AFLP markers, simple sequence repeat (SSR) markers were established, which are codominant (Ramsay et al. 2000; Thiel et al. 2003; Varshney et al. 2007). Further on, diversity array technology

(DArT) markers (Wenzl et al. 2004) and single nucleotide polymorphism (SNP) markers (Comadran et al. 2012; Silvar et al. 2011b) were developed, which have a higher throughput. Based on these maps, a consensus map involving more than 6000 markers has been developed (Silvar et al. 2015). Often, a combination of different markers was used for the construction of barley genetic maps (Hearnden et al. 2007; Wenzl et al. 2006). Genomic data of barley is stored in open source databases such as the Barleymap tool (Cantalapiedra et al. 2015) including the barley POPSEQ map (Mascher et al. 2013a) and the barley physical map covering 4.98 Gbp of the genome. This first draft sequence of the barley genome, the barley gene space, was published by the international barley sequencing consortium (IBSC) in 2012 (Mayer et al. 2012). Besides this, genomic information known from other monocot species can be easily used in barley via the genome zipper (Mayer et al. 2011). These genomic resources provide a good basis for further basic and applied research on barley, e.g. by novel techniques such as exome capture (Mascher et al. 2013b).

1.2 DROUGHT STRESS

Global agriculture is and will have to deal with a growing world population and a simultaneous increase of drought periods (Tester and Langridge 2010). Drought, defined as limited and insufficient water availability, is the most important abiotic stress factor in crop production worldwide and will become even more important due to climate change (Coumou and Robinson 2013; El Hafid et al. 1998). Up to now, a lot of climate models were published on the impact of climate change on crop yield and agricultural production (White et al. 2011). Climate models were simulated for amounts of global warming ranging from <2°C, 2- 3°C, or >3°C (Pachauri and Meyer 2014; Scholze et al. 2006). Impacts of national ecosystems and risks associated with global warming depend on the amount of temperature increase (Adger et al. 2003). Despite losses of yield up to 8.1% caused by heat and 5.2% by drought worldwide (Lesk et al. 2016), cereal grain yields have to increase at least by 70% till 2050 to feed the earth´s growing population (Tweeten and Thompson 2009). Therefore, an important aim in recent breeding is to develop drought tolerant cultivars using most recent breeding techniques (Tuberosa and Salvi 2006). Especially barley is highly suitable for breeding for drought tolerance because of the large genetic diversity, which makes it widely adapted to different environmental conditions, e.g. abiotic stresses like drought (Amri et al. 2005; Dawson et

al. 2015; Nevo et al. 2012). This is further an advantage, as drought often occurs along with other abiotic stresses such as heat (Cattivelli et al. 2011).

In contrast to non crop plants, for crop plants it is most important to not only withstand drought periods but also to maintain high yields production (Araus et al. 2002; Richards 1996; Sinclair 2011). Generally, plants respond differentially to drought stress on the physiological, biochemical and molecular level (Reddy et al. 2004). They have developed strategies for adjustment comprising drought escape, avoidance and tolerance (Turner 1986; Verslues and Juenger 2011), which vary between species, cultivars (Rampino et al. 2006) and developmental stages (Szira et al. 2008). Over many generations, plants became adapted to drought by escape, i.e. fulfilling their life cycle before drought occurs. This is achieved through faster growth and maturity, as well as through early flowering (Franks et al. 2007) or a short grain filling period (Yang and Zhang 2006). In avoidance of drought stress, plants compensate water deficits for example through early vigour, as well as low biomass production (Blum 1996; Jamieson et al. 1995). Furthermore, acquisition of water is optimised by a large and deep root system and by early closure of stomata (González et al. 1999; Pace et al. 1999). Moreover, osmoprotectants are accumulated in the cells and transported in the vacuoles to maintain turgor and to increase osmotic potential for continuous water uptake (Blum 1989; Fricke et al. 1994; González et al. 2008). Osmotic adjustment is achieved, e.g. by the accumulation of amino acids such as proline or soluble sugars in the cells, as well as by inorganic ions like Ca^{2+} in vacuoles (Bajji et al. 2001; Sperdouli and Moustakas 2012). The content of osmoprotectants increases under drought stress conditions, e.g. in leaves (Sayed et al. 2012; Teulat et al. 2001). Drought tolerance or desiccation tolerance enables plants to survive drought periods by protecting cells against dehydration through reduced cell expansion and elasticity of the cells (Martínez et al. 2007). Also, maintenance of photosynthesis during drought periods is important to withstand dehydration (Chaves et al. 2009) and to minimise yield losses. Through a delay of chlorophyll degradation under drought, plants are photosythetically active for a longer time and can produce higher yields. A clearly positive correlation between chlorophyll content and chlorophyll fluorescence determined by non-invasive measurements (Mamnouie et al. 2010; Silva et al. 2007) and yield was observed (Kassahun et al. 2009; Verma et al. 2004; Zivcak et al. 2008). For the evaluation of the complex drought stress response, the analysis of a range of physiological parameters, also in combination, is useful. Therefore, high throughput phenotyping facilities, e.g. of LemnaTec were developed in the last years (de Souza 2010). Actually, a combination of spectral reflectance, fluorescence and thermal

imaging are applied (Munns et al. 2010). New phenotyping methods like remote sensing and robotic assisted imaging platforms will be included in high throughput systems in the future (Fahlgren et al. 2015).

Abscisic acid (ABA) is of prime importance in the response of plants to drought stress. This phytohormone stands at the beginning of the regulation of a lot of drought stress response mechanisms (Bray 1997). It initiates root growth, inhibits tillering and promotes stomatal closure. Furthermore, it transfers the drought stress signal from stress perception to gene expression (Zhang et al. 2006). Besides gene expression, transcription factors are regulated by ABA like bZIP, MYB and zinc finger genes, too (Bhargava and Sawant 2013). Moreover, ABA regulates aquaporins, which are tunnel proteins influencing water balance in the cell. They are important for the trans membrane water potential gradient (Morillon and Chrispeels 2001). Another protein family are the late embryogenesis abundant (LEA) proteins, which are accumulated in response to dehydration or ABA treatment, including for example heat shock proteins or proteins encoded by the HvA1 gene (Xu et al. 1996). Dehydrines (Dhn) are LEA proteins, too. They protect other proteins and membranes against dehydration triggered changes in structure (Borovskii et al. 2002; Campbell and Close 1997). Moreover, protein modifications are discussed as drought tolerance mechanisms and this was recently verified by N-terminal acetylation induced by ABA in *Arabidopsis thaliana* (Linster et al. 2015). Molecular responses to drought can be regulated also independently of ABA, for example by WRKY transcription factors such as WRKY38 in barley (Marè et al. 2004).

In order to analyse drought stress response at the molecular level genes coding for proteins involved in drought stress response can be identified for example by gene expression analysis and tested for functional analysis in transgenic plants (Babu et al. 2004; Sivamani et al. 2000). In barley, a lot of expression profiles for the response to drought have been published (Guo et al. 2009; Hazen et al. 2003). Some were based on high throughput microarrays (Korenková et al. 2015; Talamè et al. 2007). Besides this, RNASeq, which is a cDNA sequencing technology is an efficient tool for expression profiling (Deyholos 2010; Wang et al. 2009). Most of the genes identified to be involved in drought stress are summarised in the database DroughtDB (Alter et al. 2015). In addition to gene identification of the DNA and cDNA level, proteins involved in drought stress can be identified by 2D PAGE gel electrophoresis (Ashoub et al. 2013; Wendelboe-Nelson and Morris 2012), followed by mass spectrometry (Østergaard et al. 2002). Drought related proteins are collected in databases, too, such as the plant stress

protein database (PSPDB) linked to the UniProt information platform (Kumar et al. 2014; UniProt 2008).

Due to the complex response of plants to drought stress, tolerance to drought stress follows a quantitative mode of inheritance and is therefore difficult to select for in plant breeding (Araus et al. 2002). With open source knowledge and high throughput techniques, information on drought stress mechanisms can be gained more rapidly in the future and the above mentioned techniques allow for the rapid development of molecular markers facilitating an efficient marker based selection procedure for drought stress tolerance (Forster et al. 2000; Nevo and Chen 2010).

1.3 LEAF SENESCENCE

Another quantitative trait important for development and reproduction of plants is senescence. As plants are sessile organisms, they developed mechanisms for adaptation to unfavourable environmental conditions, which affect the timing of senescence. In final stages of plant development, senescence processes occur as a kind of programmed cell death (PCD) (Lim et al. 2007; Thomas 2013). In contrast to PCD, senescence proceeds over a much longer time period and till the “point of no return” leaf senescence is completely reversible (Thomas et al. 2003). Senescence includes organised, genetically regulated degradation processes. These lead to a repression of photosynthetically functional gene products and therewith to a destruction of chlorophyll (Hörtensteiner 2006; Miersch et al. 2000), as well as a degradation of proteins, carbohydrates, lipids, ribonucleic acids (RNA) and cell membranes (Buchanan-Wollaston 1997). Redundant nitrogen is saved for recycling. A lot of the underlying molecular mechanisms for recycling and regulation are still unknown (Buchanan-Wollaston et al. 2003; Fischer 2012).

Senescence can be subdivided into whole plant and organ senescence (Leopold 1961). In perennial plants like trees senescence concerns only single organs such as the flowers, fruits or leaves and the whole plant survives (organ senescence). Annual and biannual plants like *Arabidopsis thaliana* or *Hordeum vulgare* display senescence on the whole plant, resulting in the dieback of the whole plant (whole plant senescence). All resources are translocated to the seeds for reproduction. Whole plant senescence occurs at the leaves firstly and is subclassified into three steps (Munne-Bosch and Alegre 2004; Yoshida 2003). The first phase is the initiation phase, in which

developmental processes are stopped or delayed and a shift in gene expression begins. In the second reorganisation phase degradation processes are induced, e.g. through the reduction of photosynthesis. Nutrients and metabolites are translocated to “sink” organs and a lot of senescence associated genes are expressed. In the last terminal phase, a disruption of organelles as well as cell death, similar to apoptosis, occurs. For crop plants like barley, the second phase is the most important step determining the quality and quantity of yield (Gregersen et al. 2013). Only through a regulated rearrangement of nutrients and metabolites at a defined developmental stage, yield development can proceed correctly (Munne-Bosch and Alegre 2004). Hence, leaf senescence is an important trait for crop improvement (Gan 2014).

During the whole complex process of leaf senescence a lot of regulatory mechanisms occur at the molecular level (Buchanan-Wollaston 1997; Yoshida 2003). Up to 6,000 genes are differentially expressed under leaf senescence (Breeze et al. 2011). These senescence associated genes (SAG) are regulated spatially and temporally, mostly by transcription factors. A lot of transcription factors are activated through a specific binding at *cis* DNA elements of the promoter of target genes, or through the remodelling of chromatin structures (Humbeck 2013). Transcription factors are subclassified into up to 20 families, such as WRKY, NAC, zinc finger, MYB and AP2-EREBP (Balazadeh et al. 2008; Buchanan-Wollaston et al. 2005). Thereof, until now only a few of these functions are known, whereas WRKY transcription factors are well studied (Rushton et al. 2010; Ülker and Somssich 2004). Moreover, phytohormones like ABA, cytokinins, or salicylic acid are active in early senescence and interact together in networks (Gepstein and Glick 2013; Jibran et al. 2013). Also, micro RNAs can influence and regulate leaf senescence by cleavage or translation suppression (Naqvi et al. 2011; Sarwat et al. 2013).

Senescence processes are initiated by different developmental factors, such as reproductive stage and age. Furthermore, environmental factors such as pathogens, or abiotic factors like drought, light and nutrient deficiency can influence senescence (Buchanan-Wollaston et al. 2005; Lim et al. 2007). Leaf senescence often occurs as a consequence of drought stress (Munne-Bosch and Alegre 2004; Rivero et al. 2007). Furthermore, leaf senescence is mediated by endogenous factors like phytohormones or reactive oxygen species (ROS). Often, senescence is accelerated and prematurely introduced in response to abiotic stresses (Gepstein and Glick 2013). In this context, delayed senescence can be beneficial under abiotic stress conditions (Borrell et al. 2000). Though persistent photosynthetic activity, more time is available for carbohydrate gain and translocation processes, whereby plants can generate a higher yield.

Regulatory mechanisms for drought stress response and leaf senescence are overlapping or reacting in the same way. Transcription factors are described to be mostly similar for drought stress and leaf senescence genes (Nakashima et al. 2012; Simpson et al. 2003). Consequently, a few physiological traits are applied not only to determine drought stress tolerance, but also leaf senescence. These include stay green parameters such as leaf colour or chlorophyll fluorescence (Li et al. 2006; Netto et al. 2005; Thomas and Howarth 2000), as well as the total content of soluble sugars (Rosa et al. 2009; Wingler et al. 2006).

1.4 GENOME WIDE ASSOCIATION STUDIES (GWAS)

Conventional breeding by crossing of cultivars with desirable traits followed by long lasting selection processes for traits of interests is time consuming, in inbreeding species like barley. Today, this process can be abridged by using doubled haploid (DH) lines (Kuhlmann and Foroughi-Wehr 1989) and marker assisted selection (MAS) (Collard et al. 2005; Friedt et al. 2012). In MAS, molecular markers linked to traits of interest are used instead of phenotypic evaluation in the field. The advantage of this technique is that breeders can directly select genotypes which have the favourable alleles in early developmental stages, e.g. for resistances, independently from the occurrence of respective pathogens in the field.

Since the 1990s, markers have been developed and used for genotyping of barley lines and for construction of genetic maps (Agarwal et al. 2008). Today, SNP assays are commonly used in barley research enabling comparison of the results of different studies. SNP can be identified in high throughput array technologies like the Infinium iSelect SNP assay by Illumina (Comadran et al. 2012), the Axiom technology by Affymetrix (Thomson 2014) or genotyping by sequencing (GBS) (Elshire et al. 2011; Poland et al. 2012). For generating these chips, sequence information is required which can be easily generated today through next generation sequencing (NGS) (Ganal et al. 2012; Varshney et al. 2009). Through SNP markers, several genetic maps were constructed, e.g. by Comadran et al. (2012) or Muñoz-Amatriaín et al. (2014) and recently a consensus map was published (Silvar et al. 2015).

To connect markers with phenotypic information, segregation analyses or recent genome wide association studies (GWAS) are utilised. In both approaches, loci influencing the trait of interest are identified in the genome. Whereas the identification of quantitative

trait loci (QTL) was limited in former times to segregating bi-parental populations such as doubled haploid (DH) populations, recombinant inbreed lines (RILs), backcrossing populations, or F_2-populations (Collard et al. 2005), GWAS originally derived from human genetics and based on non related genotypes has been introduced into plant science. Advantages of GWAS in comparison to QTL analysis in bi-parental populations are a saving of time, as no segregating populations are required, and a more representative genetic background of a large set of diverse genotypes (Korte and Farlow 2013). For both approaches, studies were conducted in barley. On the one hand, genomic regions were identified by QTL analysis (Galal et al. 2014; Pillen et al. 2003; Saal et al. 2011; Sayed et al. 2012; von Korff et al. 2005; Yin et al. 2005) and on the other hand by GWAS, for example for agronomic traits (Kraakman et al. 2004; Lex et al. 2014; Pasam et al. 2012; Rode et al. 2012), abiotic stress tolerance (Ingvordsen et al. 2015; Long et al. 2013; Rostoks et al. 2005) and resistance to biotic stress (Massman et al. 2011; Roy et al. 2010). Recently, combined approaches were developed: firstly, the nested association mapping (NAM), which analyses associations for multiple families, mostly consisting of RILs (Yu et al. 2008) and secondly, the multi parent advanced generation inter cross (MAGIC) method, in which a population consisting of multiple crossings of diverse parents is utilised (Cavanagh et al. 2008). These approaches are applied de novo in barley, too (Maurer et al. 2015; Maurer et al. 2016; Sannemann et al. 2015).

Nowadays, genome wide association studies are commonly applied in genomics to detect and localise QTL. With this approach, multiple quantitative traits can be analysed in huge sets of diverse and non-related genotypes. Among others, yield, nutrient use efficiency and drought stress tolerance are quantitative traits. Favourable alleles and their impact can be quantified with GWAS, combining phenotypic data in form of variation between genotypes (phenotype) and marker based genetic maps based on sequence polymorphisms (genotype). Statistical methods such as general linear models (GLM) or mixed linear models (MLM) are applied to detect associations between a phenotype and a genotype, as well as QTL effects. In addition to the genetic map, genotypic and phenotypic data, the population structure and kinship are included as cofactors (Zhu et al. 2008) in order to avoid the detection of false positive associations. The population structure can be analysed by genomic control (GC) with markers independent of the trait of interest (Devlin et al. 2001), by structure association (SA) subdividing genotypes into subpopulations with a defined probability for each genotype (q matrix) (Pritchard et al. 2000) and by principal component analysis (PCA), in which genotypes are clustered statistically along principal components (Price et al. 2006). In dependence on population

structure, a further characterisation of the association panel should be conducted by an analysis of the degree of linkage disequilibrium (LD) (Breseghello and Sorrells 2006; Mackay and Powell 2007). LD provides a value for non-random associations of alleles at different loci and the level of LD e.g. depends on the species, allele frequency and the loci investigated (Slatkin 2008).

GWAS can be conducted not only with phenotypic data, also QTL associated to gene expression (eQTL), proteins (pQTL), or metabolites (mQTL) can be identified by genetical genomics approaches using current high throughput molecular profiling technologies (Jansen et al. 2009; Langridge and Fleury 2011). This implies a lot of advantages and profits for gene network analysis and for the identification and validation of candidate genes (Wayne and McIntyre 2002; Westra and Franke 2014). An analysis to identify QTL for phenotypic traits may be conducted firstly, followed by a more detailed investigation of regulatory mechanisms through eQTL, pQTL or mQTL (Chitwood and Sinha 2013). An advantage of organisms with open source genomic information such as barley is the comparability with reference genomes, gene ontology, or protein networks. This way, e.g. eQTL can be subdivided into *cis* and *trans* regulated eQTL (Druka et al. 2010; Li et al. 2010; Michaelson et al. 2009). In barley, several eQTL (Chen et al. 2010; Jia et al. 2011; Wise et al. 2014) and pQTL (Iimure et al. 2015; Witzel et al. 2011) studies were conducted, whereas no mQTL were published so far.

It has been demonstrated that GWAS is particularly suitable for complex quantitative traits like drought stress tolerance. Many QTL based on physiological data (Fan et al. 2015; Forster et al. 2004; Honsdorf et al. 2014; Varshney et al. 2012) and on gene expression data (Hübner et al. 2015; Potokina et al. 2006) were identified in barley. As senescence is a quantitative trait, too, few QTL were published also on leaf senescence in barley (Emebiri 2013; Guo et al. 2008; Mickelson et al. 2003).

1.5 Hypothesis and Objectives

In order to cope with drought periods expected to increase in number, intensity and duration due to climate change, the aim of this thesis was to get a better understanding of drought tolerance and senescence mechanisms in juvenile barley and to identify markers suited to be used in marker based selection procedures.

The thesis was built on the hypotheses that (i) genetic variation with respect to drought stress tolerance and leaf senescence in juvenile stages is present in winter barley, that (ii) QTL and candidate genes involved in these traits can be identified by genome wide association studies (GWAS), and (iii) that genes involved are differentially expressed between drought stress and well-watered treatment as well as between tolerant and non-tolerant genotypes.

In order to get detailed information thereon, (i) a reliable method to screen for differences in drought stress and leaf senescence was developed, (ii) genome wide association studies (GWAS) based on these data and genotypic data derived from the 9k iSelect Chip were conducted and (iii) expression analysis and eQTL analyses were performed.

2 ORIGINAL PAPERS

This thesis includes three publications concerning the identification of QTL and candidate genes involved in drought stress tolerance and leaf senescence in juvenile barley through genome wide association studies. The first one introduces the methods of phenotyping while the other two papers deal with QTL and eQTL detection.

2.1 EXPERIMENTAL DESIGN TO DETERMINE DROUGHT STRESS RESPONSE AND EARLY LEAF SENESCENCE IN BARLEY (*HORDEUM VULGARE* L.)

This publication presents the methodical approach to receive reliable phenotypic data on drought stress response and leaf senescence in juvenile barley, which is a prerequisite to identify QTL and candidate genes involved in these traits. S. 15 ff.

2.2 IDENTIFICATION OF GENOMIC REGIONS INVOLVED IN TOLERANCE TO DROUGHT STRESS AND DROUGHT STRESS INDUCED LEAF SENESCENCE IN JUVENILE BARLEY

This is one of only a few papers published on drought stress in early developmental stages of barley. By analysing six traits for drought stress and leaf senescence as described in Chapter 2.1, marker trait associations were detected and QTL as well as proteins involved were identified. S. 31 ff.

2.3 EXPRESSION PROFILING OF GENES INVOLVED IN DROUGHT STRESS AND LEAF SENESCENCE IN JUVENILE BARLEY

The third publication is a genetical genomics approach based on the same association panel described in Chapter 2.2. Through gene expression analyses, *cis* and *trans* eQTL involved in drought stress response and early leaf senescence were detected.

S. 46 ff.

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Experimental Design to Determine Drought Stress Response and Early Leaf Senescence in Barley (*Hordeum vulgare* L.)

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[Abstract] Premature leaf senescence induced by drought stress is a main factor for yield losses in barley. Research in drought stress tolerance has become more important as due to climate change the number of drought periods will increase and tolerance to drought stress has become a goal of high interest in barley breeding. However, reliable screening for drought stress tolerance is still a difficult task. This protocol describes the experimental design for the phenotyping for drought stress tolerance and early leaf senescence in the juvenile stage of barley (A) and the determination of six physiological parameters involved in drought tolerance and leaf senescence (B to G) according to Wehner *et al.,* (2015).

A. Experimental design

Materials and Reagents

1. Sticks for labels (Hermann Meyer KG, catalog number: 180230)
2. Plastic sticks (Hermann Meyer KG, catalog number: 180206)
3. Rubber binder (Hermann Meyer KG, catalog number: 321234)
4. Barley seeds
5. 70% white peat
6. 30% clay
7. N (nitrogen power)
8. P_2O_5
9. K_2O
10. Mixed clay soil ED73 (H. Nitsch & Sohn GmbH & Co. KG) (see Recipes)

Equipment

1. Greenhouse facility
2. Movable greenhouse benches (80 x 100 cm)
3. Square pots (16 x 16 x 16 cm) (Hermann Meyer KG, catalog number: 720016)
4. Beaker for watering (VWR International, catalog number: 213-3402)

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5. Labels (Baumann Industries, catalog number: 2.508.003)
6. Weighing scale (KERN & SOHN GmbH)
7. Compartment dryer (Heratherm oven) (Thermo Fisher Scientific)

Procedure

1. Adjust greenhouse by heating, ventilation and lighting for long day conditions in a temperature range from 20 to 22 °C at day (16 h) and 17 to 19 °C at night (8 h). If natural radiation is below 20 klx, additional light should be supplied from 6 am to 10 pm and above 30 klx shading should be used (light intensity of additional illumination at pot height is ~10 klx).
2. Homogenize content of different bags of ED 73 soil to optimize comparability between the pots.
3. In correspondence to respective humidity fill square pots with a defined weight of the soil (in our case 1,500 *x g* at 40% dry substance of the soil).
4. Label pots and sow ten seeds of each barley genotype per pot (in our case three replications for control and drought stress treatment for 156 genotypes). For better visibility labels could be pinned on label stakes (Figure 1).
5. Put 20 pots ordered by the number of genotypes at each greenhouse bench according to the experimental design chosen (in our case a split plot design). Control and stress treatment are allocated to separate benches (Figure 1).
6. Water all pots up to 70% of the maximal soil water capacity (WC). The weight of added water is calculated out of the saturated soil weight and dry weight according to DIN ISO 11465 1996-12 (Paech and Simonis, 1952) at three exemplary pots as follows:
   a. Put gauze at the bottom, fill three pots with soil and add water till saturation.
   b. Wait for 6 h till water is drained by gravity and weigh.
   c. Dry the soil (two days in compartment dryer) at 105 °C and weigh.
   d. 100% WC is calculated out of the weight of saturation minus the dry weight.
   e. The weight to which the pot is watered corresponds to 70% (control) or 20% (drought stress) WC plus the dry weight and the pot weight.
7. Water every day to 70% WC by weighing.
8. After germination, reduce seedlings to seven plants per pot.
9. To minimize effects of plant position, movable benches should be moved every day.
10. Drought stress starts at the primary leaf stage [BBCH 10, according to Stauss (1994)] seven days after sowing (das). At this time primary leaves of the juvenile barley plants are fully expanded. Stop watering of the stress variant till the pot weight reaches 20% WC. Check this by weighing ten exemplary stressed pots over the time of the experiment.
11. Then keep this level by weighing each pot and re-water to the weight of 20% WC daily.

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12. Water control plants daily to 70% WC (weight of the plants is neglected).

Figure 1. Pot experiment in greenhouse (10 days after sowing)

13. At 36 das (BBCH 33) a four weeks stress period is reached and physiological parameters are determined as well as biomass is harvested according to protocol B to G below. For analysing physiological traits primary leaves should be used because at these oldest leaves drought stress induced leaf senescence occurs first (Figure 2).

Figure 2. Primary leaves differentiating in drought stress response regarding leaf senescence

Notes

In our pot experiments it turned out, that it is necessary to measure parameters for leaf senescence in a separate pot experiment, because only in this way it was possible to separate the effects of drought stress induced leaf senescence and age-related leaf

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senescence from light deficiency induced leaf senescence. To achieve this, the shadowing effects of other leaves on the primary leaf are minimized by tying up all barley leaves (with rubber binder on stakes) about 14 das, except the primary leaf (Figure 3A). General settings are the same as described above, but only four plants are grown in smaller pots (12 x 12 x 12 cm with 550 g soil) and control and stress treated pots are mixed on the greenhouse benches in rows (Figure 3B).

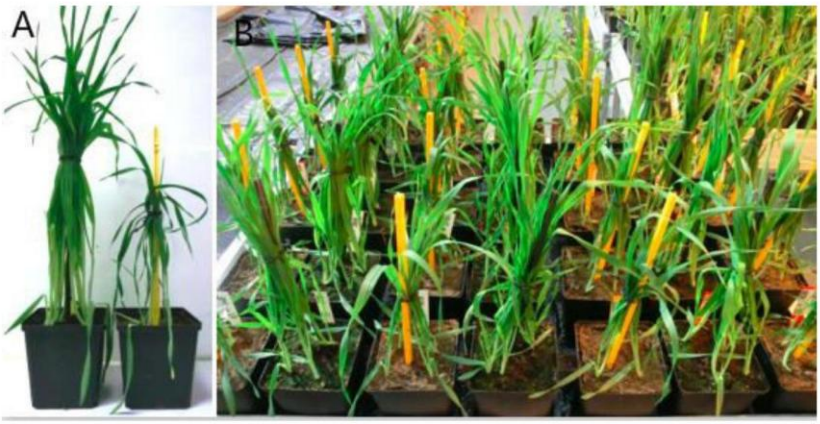

Figure 3. Barley plants with tied up leaves. A. Single plants at 36 days after sowing under control (left) and drought stress treatment (right). B. One greenhouse bench at 20 days after sowing with four rows stress treatment (yellow sticks) and three rows control treatment (black sticks).

Recipes

1. Mixed clay soil ED73
   70% white peat
   30% clay with pH ~6 and 1 kg/m³
   Mineral nutrients
   a. 14 % N
   b. 16 % P_2O_5
   c. 18 % K_2O
   2 kg/m³ long term fertilizer
   a. 20 % N
   b. 10 % P_2O_5
   c. 15 % K_2O

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B. Leaf senescence: Leaf colour

Materials and reagents

1. Barley plants grown as described above

Equipment

1. Chlorophyll Meter SPAD-502 Plus (Konica Minolta)

Procedure

1. Measure leaf colour directly in the greenhouse on the primary leaves at 33-34 days after sowing (das).
2. With Minolta SPAD readings leaf colour representing the status of leaf senescence is measured non destructive.
3. After calibration by an empty clip take five evenly distributed readings on each of three primary leaves (three barley plants) per pot which are averaged by the SPAD-Meter.
4. At each primary leaf, measurement should be done at the upper side of the leaf and in the middle of the leaf avoiding to clip the middle leaf-vein (Figure 4).

Figure 4. Positions for measurement of leaf colour on three primary leaves

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C. Leaf senescence and drought tolerance: Electron transport rate at PSII

Materials and reagents

1. Barley plants grown as described above

Equipment

1. OS1p-Chlorophyll fluorometer (Opti-Sciences)

Procedure

Determine the electron transport rate (ETR) at photosystem II (PSII) as a parameter for leaf senescence at 34-35 das. With the portable OS1p (Figure 5) chlorophyll fluorescence is measured non destructive on light adapted plants. Because this analysis is very light sensitive, measurement should be conducted exactly at the position where the pot is located on the greenhouse bench and the shading of the greenhouse should be used to ensure continuous lighting conditions during all measurements. Besides, measurement should be done not before 10 am and not after 3 pm (around noon) receiving the optimum of daily photosynthesis.

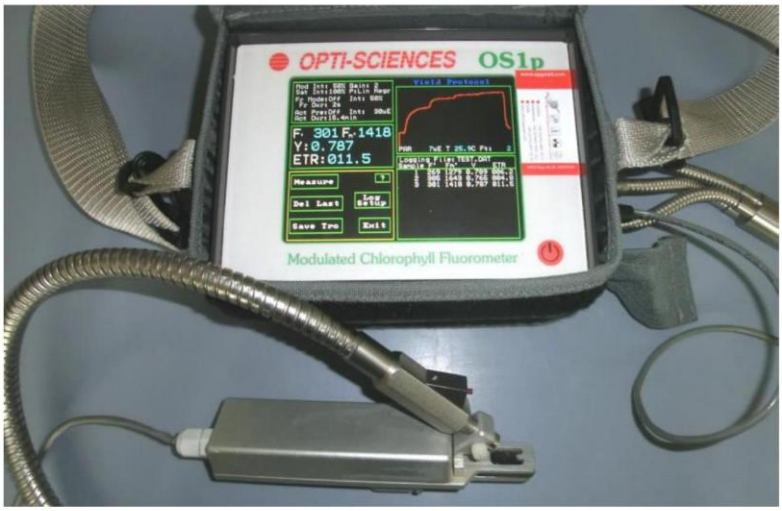

Figure 5. OS1p-Chlorophyll fluorometer. The display shows three exemplary measurements down right and general settings top left. Besides this, the leaf clip for measurement is shown consisting out of the clip with the trigger button and the sensor for detecting the photosynthetic active radiation (PAR).

1. For measurement with the fluorometer first select the photosynthetic yield menu Y(II).

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2. Adjust modulation intensity at 50%, saturation intensity at 100% and for the ETR calculation include the formula $ETR = Y(II) * PAR * 0{,}84 * 0.5$. Input values for constants representing light which is absorbed by the leaf (0.84) and light which is equally absorbed by PSI and PSII (0.5) in addition to the measured photosynthetic active radiation (PAR) and the quantum photosynthetic yield of PSII [Y(II)] (Krall and Edwards, 1992).
3. Take three measurements for each pot in the middle of three primary leaves at the upper side of the leaves. Put the leaves in the clip with the leaf margin at the bottom of the clip. Then wait till the light impulses are finished indicated by a peep.
4. Data dumps are stored in the memory of the fluorometer and can be read out later by USB transfer and averaged for each pot.

Notes

The Fluorometer gives also values for Y(II), but here the ETR (µmol/ m²s) is used because the PAR is included to compensate potential lighting variations.

D. Drought tolerance: Total content of soluble sugars

Materials and reagents

1. Pipette (1,000 µl/10 ml) (Eppendorf/Finnpipette Labsystems)
2. Tubes (15 ml) (VWR International, catalog number: 525-0308)
3. Tubes (2 ml) (VWR International, catalog number: 211-2120)
4. Test tubes (glass) (VWR International, catalog number: 4.902.002.75)
5. Barley plants grown as described above
6. Liquid nitrogen
7. D-Glucose (VWR International, catalog number: 1.08337.0250)
8. Polyvinylpolypyrrolidone (PVPP) (VWR International, catalog number: 1.07302.0100)
9. Distilled water
10. Sulfuric acid 100 % (VWR International, catalog number: 1.00713.1000)
11. Borosilicate glass bottle (brown) (VWR International, catalog number: 215-2328)
12. Anthrone (VWR International, catalog number: 1.01468.0010)
13. Thiourea (VWR International, catalog number: 1.07979.0250)
14. Sulfuric acid 70% (see Recipes)
15. Anthrone reagent (see Recipes)

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Equipment

1. Weighing scale (KERN & SOHN GmbH)
2. Scissors
3. Styropor box
4. Freeze dryer (Christ Alpha 1-4 LD plus)
5. Water bath (GFL)
6. Shaker (Vortex Genie 2) (Scientific industries)
7. Test tube racks (Geyer GmbH & Co, catalog number: 9.905 970)
8. Glass marbles (toy store, diameter of 15 mm)
9. Centrifuge (23,000 *x g*) (Tuttlingen Germany, model: Hettich Universal 16R)
   Note: Currently, it is "Gemini BV Laboratory, model: Hettich Universal 16R".
10. Cuvette (VWR International, catalog number: 634-9014)
11. Spectrophotometer (Thermo Fisher Scientific, model: Genesys 10S UV VIS)
12. Beaker (VWR International, catalog number: 213-1131)

Procedure

First, prepare a standard curve for glucose content which should be repeated for every anthrone reagent.

1. Prepare five different dilutions of D-Glucose (each 500 µl) in five test tubes: 0 µM, 50 µM, 100 µM, 150 µM and 200 µM.
2. Slowly add for each test tube 2.5 ml anthrone reagent by vortexing till a clear solution is present.
3. Continue with steps 12-14 together with the samples.
4. After measuring draw the standard curve for the glucose content (Figure 6).

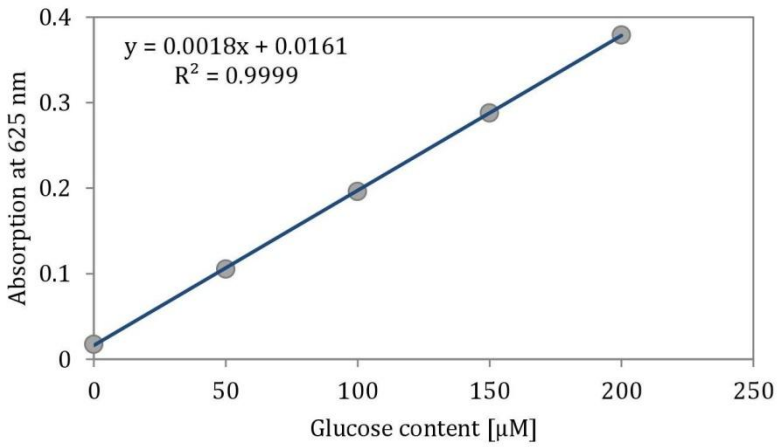

Figure 6. Standard curve for glucose content

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At 36 das samples for analysis of the total content of soluble sugars are taken and analysed according to Yemm and Willis (1954).

5. Cut 1 cm pieces from the middle of five primary leaves per pot (100 -200 mg).
6. Weigh these (fresh weight is only used to calculate dry weight content for osmolality), fill in tubes (15 ml) as pooled samples per pot and freeze in liquid nitrogen using a styropor box.
7. Samples are then freeze dried and reweighed (dry weight).
8. Add 4 ml distilled water (depends on the amount of leaf tissue sampled) and boil the samples in the water bath for 30 min by 100 °C (use the test tube racks for water baths).
9. After cooling down, add 1,000 µl of the sample solution to tubes (2 ml) filled with 0.05 g PVPP and vortex.
10. Centrifuge the tubes for 5 min at 23,000 *x g* (5,000 rpm).
11. Transfer 200 µl of the supernatant solution to test tubes and add 300 µl distilled water.
12. By vortexing slowly add 2.5 ml of the anthrone reagent till a clear solution is present.
13. Put on each glass tube a glass marble (Figure 7) and boil the solutions in the water bath for 15 min at 100 °C (use the test tube racks for water baths).
14. Cool down in a cold water bath (20 °C) for 10 min.
15. Samples (Figure 7) are measured directly (maximum in 1 h time) by a spectrophotometer at 625 nm using a cuvette. Zero calibration should be done with distilled water.

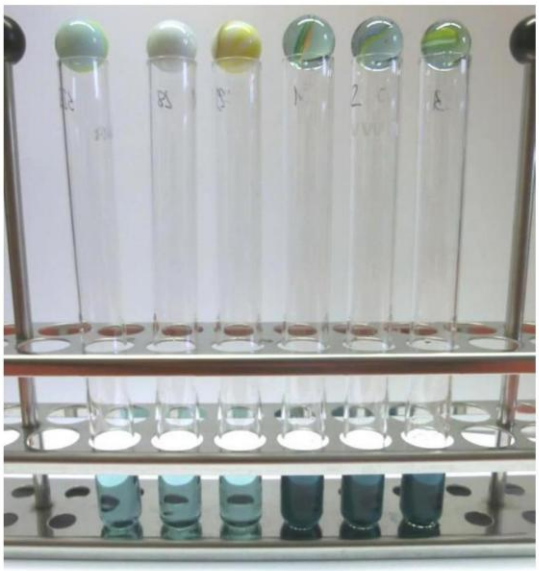

Figure 7. Test tubes ready for measurement of total content of soluble sugars. Exemplarily, three test tubes with samples for control (light blue, left) and three test tubes with samples for drought stress (dark blue, right) treatment are shown.

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16. Total content of soluble sugars is calculated using the standard curve and set in relation to dry weight (µmol/g).

Notes

The sample volume of 200 µl taken in step D11 is varying for different developmental stages of barley and also for different crops and depends on the amount of leaf tissue sampled. Important is, that the final volume in step D11 is 500 µl. Volume should be defined for the optimum range of the standard curve.

Recipes

1. 70% sulfuric acid (mix in a brown bottle and incubate for 12 h in dark)
   70 ml sulphuric acid (100%)
   30 ml distilled water
2. Anthrone reagent (stable for one day)
   Sulphuric acid (70%)
   920 mg/l anthrone
   920 mg/l thiourea

E. Drought tolerance: Content of free proline

Materials and reagents

1. Aluminium foil
2. Tubes (15 ml) VWR International, catalog number: 525-0308)
3. Stopper (VWR International, catalog number: 217-0511)
4. Pipette (1,000 µl/10 ml) (Eppendorf/Finnpipette Labsystems)
5. Barley plants grown as described above
6. Liquid nitrogen
7. L-Proline (VWR International, catalog number: 1.07434.0010)
8. Toluol (VWR International, catalog number: 1.08325.1000)
9. Distilled water
10. Ninhydrin (VWR International, catalog number: 1.06762.0100)
11. Glacial acetic acid (AppliChem, catalog number: A0820.2500)
12. Ninhydrin reagent (see Recipes)

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Equipment

1. Weighing scale (KERN & SOHN GmbH)
2. Scissors
3. Styropor box
4. Freeze dryer (Christ Alpha 1-4 LD plus)
5. Water bath (GFL)
6. Test tubes (glass) (VWR International, catalog number: 4.902.002.75)
7. Test tube racks (Geyer GmbH & Co, catalog number: 9.905 970)
8. Glass marbles (toy store, diameter of 15 mm)
9. Shaker (Scientific industries, model: Vortex Genie 2)
10. Magnetic mixer (RCT basic) (IKA Labortechnik)
11. Fortuna® Optifix® Safety Dispenser (Sigma-Aldrich, catalog number: Z260207)
12. Fume hood
13. Cuvette (VWR International, catalog number: 634-9014)
14. Spectrophotometer (Thermo Fisher Scientific, model: Genesys 10S UV VIS)
15. Beaker (VWR International, catalog number: 213-1131)

Procedure

First, prepare a standard curve for proline content which should be repeated for every ninhydrin reagent.

1. Prepare five different dilutions of L-Proline (each 500 µl) in five test tubes: 0 µM, 25 µM, 50 µM, 75 µM and 100 µM.
2. Slowly add for each test tube 2 ml ninhydrin reagent by vortexing till a clear solution is present.
3. Continue with steps D11-16 together with the samples.
4. After measuring draw the standard curve for the proline content (Figure 8).

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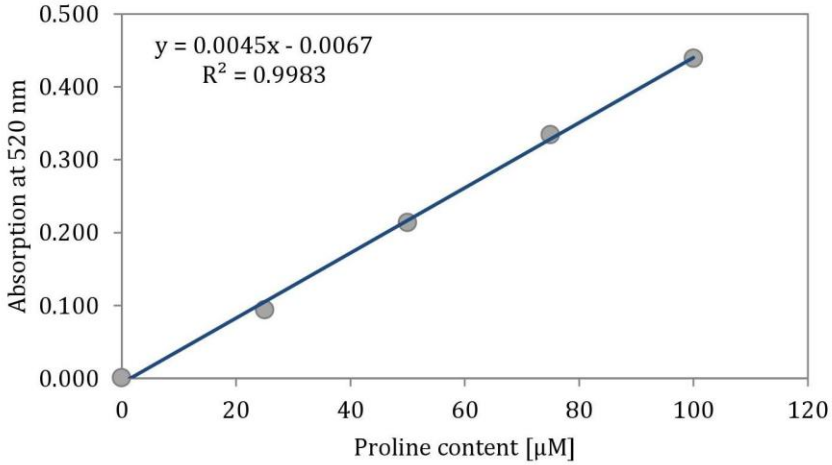

Figure 8. Standard curve for proline content

At 36 das samples for analysis of the content of free proline are taken and analysed according to Bates *et al.* (1973). For this analysis the same sample solution can be used as for the total content of soluble sugars because steps 5-8 are the same.

5. Cut 1 cm pieces from the middle of five primary leaves per pot (100 -200 mg).
6. Weigh these (fresh weight is only used to calculate dry weight content for osmolality), fill in tubes (15 ml) as pooled samples per pot and freeze in liquid nitrogen using a styropor box.
7. Samples are then freeze dried and reweighed (dry weight).
8. Add 4 ml distilled water (depends on the amount of leaf tissue sampled) and boil the samples in the water bath for 30 min at 100 °C (use test tube racks for water baths).
9. After cooling down, 1,000 µl of the sample solution are filled in a test tube, each.
10. By vortexing slowly add 2 ml of the ninhydrin reagent till a clear solution is present.
11. Put on each test tube a glass marble (see Figure 7) and boil the solutions in the water bath for 15 min at 100 °C (use test tube racks for water baths).
12. Cool down in a cold water bath (20 °C) for 10 min.

Use a laboratory fume hood for the next steps:

13. Remove the glass marble and add 5 ml toluol (*e.g.* with a dispenser).
14. Close the test tube with a stopper and vortex for 15 sec.
15. Incubate the samples for the next 90 min in darkness (up to 4 h are possible) at room temperature.
16. Two phases occur (Figure 9) from which the upper one is now transferred in a cuvette for measurement with a spectrophotometer at 520 nm. Zero calibration should be done with toluol.

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Figure 9. Test tubes ready for measurement of content of free proline. Exemplarily, three test tubes with samples for control (light red, left) and three test tubes with samples for drought stress (dark red, right) treatment are shown.

17. Content of free proline is calculated using the standard curve and set in relation to the dry weight (µmol/g).

Notes

In step E9, 1,000 µl of the sample solution are used. This volume is varying for different developmental stages of barley and also for different crops and depends a lot on the amount of leaf tissue sampled. Volume should be defined for the optimum range of the standard curve.

Recipes

1. Ninhydrin reagent (stable for one day, mix in dark, cover a beaker with aluminium foil and dissolve on a magnetic mixer)
   0.5 g ninhydrin
   20 ml distilled water
   30 ml glacial acetic acid

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F. Drought tolerance: Osmolality

Materials and reagents

1. Tubes (2 ml) (VWR International, catalog number: 211-2120)
2. Pipette (100 µl/200 µl) (Eppendorf)
3. Barley plants grown as described above
4. Liquid nitrogen
5. Distilled water
6. Calibration standard for the osmomat (Gonotec GmbH, catalog number: 30.9.0020)

Equipment

1. Weighing scale (KERN & SOHN GmbH)
2. Scissors
3. Styropor box
4. Tweezer
5. Swing mill (Retsch, model: MM200)
6. Grinding balls (VWR International, catalog number: 412-0070)
7. Grinding jars (Retsch, catalog number: 22.008.0005)
8. Shaker (Scientific industries, model: Vortex Genie 2)
9. Centrifuge (21,000 *x g*) (Wehingen, model: Hermle Z233MK2)
   Note: Currently, it is "HERMLE Labortechnik GmbH, model: Hermle Z233MK2".
10. Osmomat (Gonotec GmbH, model: Osmomat 3000 Gonotec)
11. Tubes for the osmomat (Gonotec GmbH, catalog number: 30.9.0010)

Procedure

At 36 das the osmolality is determined by comparative measurements of the freezing points of distilled water and the cell sap.

1. Cut 1 cm pieces from the middle of five primary leaves per pot.
2. Weigh these, fill pooled samples per pot in tubes (2 ml) and freeze in liquid nitrogen using a styropor box.
3. Put also the grinding balls and grinding jars into the liquid nitrogen (into the styropor box).
4. Take each tube and fill in two grinding balls with a tweezer.
5. Take off the grinding jars and fill them with five tubes each.
6. Grind the leaves in the swing mill for 3 min (30 /sec).
7. Add 200 µl distilled water into the tubes and vortex them.
8. Take the grinding balls out of the tubes with the tweezer.

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9. Centrifuge the tubes for 15 min at 21,000 *x g* (15,000 rpm) by 4 °C.
10. Pipette the supernatant cell sap in new tubes (2 ml).

For measurement with the osmomat use the original tubes for the osmomat.

11. Calibrate the osmomat with 15 µl calibration standard till a value of exactly 0.3 is reached.
12. Adjust a zero value with 15 µl distilled water.
13. Measure 15 µl cell sap each.
14. Values (osmol/kg) can be corrected for the water content (difference of fresh and dry weight). Dry weight is calculated in relation to the dry weight content of the samples for the determination of content of free proline and the total content of soluble sugars (see above).

G. Drought tolerance: Biomass yield

Materials and reagents

1. Barley plants grown as described above

Equipment

1. Weighing scale (KERN & SOHN GmbH)
2. Crispack bags (Baumann, catalog number: 3.331.100)
3. Scissors
4. Compartment dryer (Heratherm oven) (Thermo Fisher Scientific)

Procedure

1. At 36 das cut the whole biomass above ground per pot.
2. Put biomass for each pot with the label in one crispack bag.
3. Close bags.
4. Dry the biomass within the bags in a compartment dryer at 105 °C until weight doesn't change (about two days with ventilation).
5. By weighing the dry weight, total biomass yield (g) is detected.

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Identification of genomic regions involved in tolerance to drought stress and drought stress induced leaf senescence in juvenile barley

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Abstract

Background: Premature leaf senescence induced by external stress conditions, e.g. drought stress, is a main factor for yield losses in barley. Research in drought stress tolerance has become more important as due to climate change the number of drought periods will increase and tolerance to drought stress has become a goal of high interest in barley breeding. Therefore, the aim is to identify quantitative trait loci (QTL) involved in drought stress induced leaf senescence and drought stress tolerance in early developmental stages of barley (*Hordeum vulgare* L.) by applying genome wide association studies (GWAS) on a set of 156 winter barley genotypes.

Results: After a four weeks stress period (BBCH 33) leaf colour as an indicator of leaf senescence, electron transport rate at photosystem II, content of free proline, content of soluble sugars, osmolality and the aboveground biomass indicative for drought stress response were determined in the control and stress variant in greenhouse pot experiments. Significant phenotypic variation was observed for all traits analysed. Heritabilities ranged between 0.27 for osmolality and 0.61 for leaf colour in stress treatment and significant effects of genotype, treatment and genotype x treatment were estimated for most traits analysed. Based on these phenotypic data and 3,212 polymorphic single nucleotide polymorphisms (SNP) with a minor allele frequency >5 % derived from the Illumina 9 k iSelect SNP Chip, 181 QTL were detected for all traits analysed. Major QTLs for drought stress and leaf senescence were located on chromosome 5H and 2H. BlastX search for associated marker sequences revealed that respective SNPs are in some cases located in proteins related to drought stress or leaf senescence, e.g. nucleotide pyrophosphatase (AVP1) or serine/ threonin protein kinase (SAPK9).

Conclusions: GWAS resulted in the identification of many QTLs involved in drought stress and leaf senescence of which two major QTLs for drought stress and leaf senescence were located on chromosome 5H and 2H. Results may be the basis to incorporate breeding for tolerance to drought stress or leaf senescence in barley breeding via marker based selection procedures.

Keywords: Barley, Leaf senescence, Drought stress, GWAS, QTL

Background

Barley (*Hordeum vulgare* L.) is one of the first cereals domesticated in the Fertile Crescent [1] and today it is the fourth most important crop species concerning acreage next to wheat, maize, and rice [2]. Worldwide, barley is mainly used for animal feed and malting and only a very small amount is used for direct human consumption and bakery. Average yield of barley on the worldwide level is 2.9 t/ha but in some European countries, e.g. Germany average yield is up to 6.5 t/ha [2]. Barley yield in many parts of the world is reduced by biotic stress but also by abiotic stress e.g. heat, salt, deficits in nitrogen nutrition and drought [3–6]. Especially, in the juvenile stages from sowing to tillering, drought can severely influence barley development already reducing the potential yield [7]. Research on drought stress tolerance has become more important worldwide as due to climate change the number of drought periods will increase in the future [8, 9]. Up to now, most studies

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conducted in barley focused on effects of terminal drought stress whereas drought in juvenile stages is less well documented [10].

Drought tolerance is a complex quantitative trait, that is controlled by various mechanisms [11, 12]. Abscisic acid (ABA) is a key phytohormone involved in adaption to environmental stresses and regulation of plant development. It promotes the closure of stomata under drought stress conditions initiated by a loss of turgor [13]. Furthermore, it increases the hydraulic conductivity of water, promotes chlorophyll breakdown and leads to leaf senescence [14]. Another relevant protein is ubiquitin which regulates the degradation of proteins [15, 16]. Moreover, late embryo abundant (LEA) proteins and heat shock proteins that are involved in the protection of functional proteins are induced in response to various abiotic stresses [17–19]. For example protein kinases and protein phosphatases which activate or deactivate proteins by phosphorylation and dephosphorilation [20], as well as the LEA protein dehydrin which is described to have different functions in different stresses [21] is often present along with drought stress [22]. A lot of parameters indicative for drought stress influenced by these and additional genes were analysed in different crops [23, 24]. For example, biomass production [25], yield [26], photosynthesis rate [27], as well as the content of free proline [28], total content of soluble sugars [29], or osmolality [30] are parameters which are affected by drought stress in barley.

Another factor relevant for yield improvement is leaf senescence [31], which is a natural degradation process at the final stage of the development of organs and plants. This process is divided into three steps and starts with reprogramming of gene expression to turn on senescence activating genes. Before programmed cell death in the terminal phase occurs, the second step in which nutrients and metabolites are transported from source (e.g. roots, leaves) to sink (e.g. fruits, seed) is important for yield and quality of the seeds harvested [32]. During this reorganization phase, degradation of chlorophyll and a decrease in photosynthesis is observed [33, 34]. Because of degradation of chlorophyll, yellowing of the leaves is a symptom of leaf senescence [35], which in many studies is rated visually [36, 37], but can be more precisely determined by Soil Plant Analysis Development (SPAD) readings which estimate leaf greenness [38]. Degradation of chlorophyll is regulated by chlorophyllase, pheophorbide α oxygenase and red chlorophyll catabolite reductase among others [34], but so far for regulation of leaf senescence only a few genes are known [39, 40]. Leaf senescence is a process which is influenced by a lot of external stress conditions e.g. drought stress [24, 41]. Stress often results in premature induction of leaf senescence and therefore leads to an inefficient recycling of resources and a massive yield loss [42–44]. In contrast, plants showing delayed leaf senescence under stress, represented by a "stay green effect", minimize yield loss [45].

Genome wide association studies (GWAS) are a powerful tool to subdivide such complex pathways as drought stress and leaf senescence by the detection of quantitative trait loci (QTL) out of the regression analysis of genotypic and phenotypic data [19, 46–48]. Up to now, some QTLs involved in drought stress response were published in barley [49–52], whereas for leaf senescence only few QTLs are known [42, 53]. Besides this, QTLs which are involved in the response to other abiotic stresses, e.g. salt stress were identified in barley [6, 54]. Molecular markers, such as single nucleotide polymorphisms (SNP) flanking QTLs having a significant influence on the respective trait can be used for efficient marker assisted selection and smart breeding procedures [55].

The aim of the present study is therefore the identification of QTLs for drought stress and drought stress induced leaf senescence in early developmental stages of barley suited to be used in future barley breeding programs using GWAS followed by the identification of the function of these QTLs associated to respective traits.

Material & methods

Plant material and experimental setup

A set of 156 winter barley genotypes (Additional file 1) consisting of 113 German winter barley cultivars [49 two-rowed and 64 six-rowed, [56] and 43 accessions of the Spanish barley core collection (SBCC) [57] were used to investigate drought stress induced leaf senescence in juvenile barley plants. Drought stress was applied in greenhouses of the Julius Kühn-Institut in Groß Lüsewitz, Germany according to Honsdorf *et al.* [51]. Trials were conducted in a split plot design with three replications per genotype and variant (control, drought stress). Ten seeds of each accession were sown per plastic pot (16x16x16 cm) containing 1,500 g of a mixed clay soil ED73 (H. Nitsch & Sohn GmbH & Co. KG, Dorsten Germany). After germination, seedlings were reduced to seven plants per pot. Plants were grown under semi-controlled long day conditions in a temperature range from 20 to 22 °C at day (16 h) and 17 to 19 °C at night (8 h). If natural radiation was below 20 klx, additional light was applied from 6 a.m. to 10 p.m. Drought stress started at the primary leaf stage (BBCH 10) seven days after sowing (das). At this time watering of the stress variant was stopped till the soil reached 20 % of the maximal soil water capacity, and then this level was kept by weighting each pot and re-watering. Control plants were continuously watered to 70 % of the maximal soil water capacity. Water capacity was calculated of the saturated soil weight and drought weight according to DIN ISO 11465 1996–12 [58]. At the end of a four weeks stress period (BBCH 33) physiological traits were determined

and above ground biomass was harvested (experimental setup A). Experimental setup A was repeated in three years. A modified experimental setup was conducted to optimize lightening conditions of the primary leaves for the measurement of leaf colour (SPAD) and electron transport rate at photosystem II (ETR) (experimental setup B). General settings were the same as in setup A, but only four plants were grown in smaller pots (12x12x12 cm) to allow wider spacing of pots and all leaves except the primary leaves were tied up to reduce shading. Experimental setup B was repeated in two years.

Physiological parameters determined

Six physiological traits, i.e. leaf colour (SPAD), electron transport rate at photosystem II (ETR), content of free proline (CFP), content of soluble sugars (CSS), osmolality (OA) and the aboveground biomass yield (BY) were determined in the control and stress treatment. Measurement and sampling respectively were conducted on primary leaves.

Chlorophyll content which was used as the main indicator for drought stress induced leaf senescence was measured 33–34 das by Minolta SPAD readings (Konica Minolta Chlorophyll Meter SPAD-502 Plus, Osaka Japan), which gives a value for leaf colour. Three primary leaves of three plants for each pot were measured at five positions per leaf. These SPAD readings turned out to be correlated to the chlorophyll content analysed photometrically [59, 60]. Because of this relation, the chlorophyll content can be indirectly measured by SPAD [61].

At 34–35 das chlorophyll fluorescence was measured in all genotypes using light adapted plants with the OS1P-Chlorophyll Fluorometer (OPTI-SCIENCE, Hudson USA) in the middle of three primary leaves per pot at one position per leaf. The relative electron transport rate at photosystem II (PSII) ($ETR = Y(II) * PAR * 0,84 * 0,5$) was calculated including the photosynthetically active radiation (PAR), as well as the quantum photosynthetic yield of PSII (Y(II)) and constants representing light which is absorbed by the leaf (0.84) and light which is equally absorbed by PSI and PSII (0.5) [62].

At 36 das five primary leaves per pot were harvested and cut in pieces of 1 cm length for the analysis of CFP, CSS and OA. These samples were frozen in liquid nitrogen immediately and samples for CFP and CSS measurement were freeze dried. For CFP measurement the ninhydrin method [63] was applied, and for CSS measurement the anthron method [64] was used. Both traits were measured photometrical using a spectrophotometer. The concentration of these ingredients was determined with a standard curve calculated on a dry weight basis. To assess OA, frozen leaf samples were grinded in a swing mill (30/s for 3 min), filled up with 200 μl water and centrifuged at 15,000 rpm for 15 min to get cell sap for the measurement of osmolality with a freezing-point osmometer (Osmomat O-30 Gonotec, Berlin Germany). Osmolality was corrected for the water content of fresh and dry weight. Above ground biomass was harvested 36 das, too. Leaf material was dried in a compartment dryer at 105 °C and weighted.

For all traits an outlier test was calculated to exclude extreme deviations [65]. To get information on the stability of all analysed traits in response to drought stress compared to the control, the drought susceptibility index (DSI) was calculated [66] for each trait and across treatments according to the formula:

$$DSI = (1 - \text{LSMean Trait Genotype, Stress} / \text{LSMean Trait Genotype, Control}) / D, \textit{ with}$$

$$D = 1 - (\text{LSMean Trait Assortment, Stress} / \text{LSMean Trait Assortment, Control})$$

DSI is a relative value estimated for each genotype and trait. According to the formula genotypes revealing a DSI close to one are highly susceptible to drought and those close to zero or showing a negative value are tolerant.

Statistical analysis of phenotypic data

Statistical analyses were performed with SAS 9.3 [67]. Least square means (LSMeans) were calculated with GLM procedure for the replications of each genotype in the respective years and for both control and drought conditions. Descriptive statistics was calculated out of LSMeans by PROC UNIVARIATE. Analysis of variance (ANOVA) was calculated using PROC MIXED with genotypes (G), drought stress treatment (T) and GxT as fixed effects. Replication (R), year (Y) and row type are chosen as random factors. The heritability (h^2) was calculated with SAS in two steps. First, the variance components for the genotypes (V_G), variance associated with the genotype by year interaction (V_{GY}) and V_E which is the error variance were calculated with PROC VARCOMP. Next, h^2 was calculated with the following formula: $VG/(VG + VGY/Y + VE/RY)$ for both well watered and drought stress conditions. Furthermore, the coefficient of correlation (PROC CORR) by Pearson was calculated with SAS based on LSMeans.

Genotyping and genome wide association study (GWAS)

For genotyping the whole set of genotypes was analysed with the barley Illumina 9 k iSelect SNP-chip [68]. Population structure was calculated with STRUCTURE 2.3.4 [69] based on 51 simple sequence repeat (SSR) markers covering the whole genome. The STRUCTURE programme was run 20 times for pre-defined k (the number of population groups) from 1 to 5 each. To get the number of calculated subpopulations (k) with highest likelihood the procedure of Evanno *et al.* [70] was applied. An

independent run with 500,000 iterations of a Monte Carlo Markov Chain with a length of the burn in period of 500,000 was conducted for the k with the highest likelihood to obtain the q-matrix. Kinship was calculated with SPAGeDi 1.3d [71] based on 51 SSRs and allele size correlation coefficient [72] with 5,000 permutations.

Out of 3,886 genetically mapped SNP markers (398 at 1H, 690 at 2H, 583 at 3H, 342 at 4H, 781 at 5H, 546 at 6H and 546 at 7H) [68], 3,212 polymorphic markers with minor allele frequencies higher than 5 % were taken into account. Based on these data and respective phenotypic data (LSMeans) GWAS was conducted applying a mixed linear model (MLM) using TASSEL 3.0 [73]. All results with p values <0.001 were considered as significant marker trait associations. Linkage disequilibrium (LD) was calculated on mapped polymorphic SNPs with R [74] by an estimate of the average decay [75] over all barley chromosomes.

Sequences of significantly associated SNP-markers (p <0.001) were downloaded from the James Hutton Institute [http://bioinf.hutton.ac.uk/iselect/app] and respective sequences were compared against the plant proteome in the UniProtKB/Swiss-Prot protein database by BlastX (Basic Local Alignment Search Tool, p $<10^{-5}$ or query cover of minimum 80 % in NCBI [https://www.ncbi.nlm.nih.gov] accessed Oct 2014) to get information on the proteins coded by these sequences [76]. Using UniProt [77] the involvement of respective proteins in drought stress and leaf senescence processes was analysed. In a last step a genetic map with all significantly associated SNPs in genes coding for proteins known to be involved in drought stress tolerance was generated using MapChart 2.2 [78].

Results

Phenotyping

The experiments revealed variability for genotype and treatment in all analysed traits as shown in Table 1. For the traits biomass yield (BY), leaf colour (SPAD) and the electron transport rate (ETR) the mean values for the stress treatment were lower than in the well watered variant. An exception are some genotypes of the SBCC (SBCC 3, 12, 14, 76, 80, 138 and 140) showing no decrease in SPAD and ETR or even an increase, represented by negative values across treatments (DSI). In contrast to the above mentioned traits, osmolality (OA), content of free proline (CFP) and total content of soluble sugars (CSS) increased under drought stress.

The coefficient of variation (CV) was comparable for control and drought stress treatment (Table 1) for all six traits. Heritabilities (h^2) estimated ranged between 0 for OA to 0.80 for BY in the control treatment and 0.27 for OA and 0.61 for SPAD in the stress treatment. Generally, h^2 was higher for the stress treatment except for BY

Table 1 Descriptive statistics, heritability (h^2) and number of significant (p <0.001) quantitative trait loci (QTL)

Trait[a]	Description	Treat.[b]	Unit	Min[c]	Max[c]	Mean[c]	SD[c]	CV[c]	LSD[c]	h^2	No. QTL (SNPs)
BY	Total above ground biomass yield	Control	g	4.47	18.18	10.70	2.68	25.08	4.12	0.80	72 (179)
		Stress	g	2.73	9.98	5.57	1.83	32.80	1.45	0.58	19 (32)
		DSI	unit free	0.65	1.28	0.98					50 (87)
SPAD	Leaf colour	Control	unit free	4.70	48.80	35.76	8.33	23.30	23.39	0.64	8 (12)
		Stress	unit free	7.83	46.77	34.16	6.56	19.20	21.41	0.61	3 (6)
		DSI	unit free	−7.70	9.41	0.93					2 (2)
ETR	Relative electron transport rate at PSII	Control	Y(II) x PAR x 0,84 x 0,5	6.12	32.26	15.73	4.63	29.46	19.47	0.08	
		Stress	Y(II) x PAR x 0,84 x 0,5	2.00	30.70	13.41	4.54	33.85	16.38	0.50	2 (2)
		DSI	unit free	−4.09	4.82	0.90					
OA	Osmolality	Control	osmol kg^{-1}	0.23	0.78	0.46	0.05	11.57	0.19	0.00	
		Stress	osmol kg^{-1}	0.40	1.16	0.67	0.08	11.48	0.23	0.27	22 (29)
		DSI	unit free	0.44	2.00	1.02	0.26				1 (1)
CFP	Content of free proline	Control	µmol g^{-1}	0.00	22.36	3.66	3.11	84.89	7.33	0.13	
		Stress	µmol g^{-1}	1.02	78.57	23.94	16.86	70.43	44.30	0.29	
		DSI	unit free	0.00	3.46	1.15					1 (2)
CSS	Total content of soluble sugars	Control	µmol g^{-1}	86.07	568.07	231.40	85.51	36.95	240.72	0.13	
		Stress	µmol g^{-1}	164.61	981.40	419.64	164.03	39.09	411.52	0.30	1 (1)
		DSI	unit free	0.08	2.95	1.07					

[a]BY: biomass yield, SPAD: leaf colour, ETR: electron transport rate at PSII, OA: osmolality, CFP: content of free proline, CSS: total content of soluble sugars
[b]Control and drought stress treatment, as well as DSI: drought susceptibility index across treatments by Fischer & Maurer (1978)
[c]Minimum, maximum, mean, standard deviation in % (SD), coefficient of variation (CV) (standard deviation divided by mean) and least significant difference (LSD)

and SPAD. Analysis of variance (ANOVA) revealed significant ($p < 0.001$) genotype and treatment effects for all investigated traits and genotype x treatment interactions for BY, CFP and CSS (Table 2).

To get information on the influence of the physiological parameters estimated on biomass yield as the indicator for drought stress and SPAD as the indicator for drought stress induced leaf senescence, correlations to these traits were calculated (Table 3). For control and stress treatment BY is significantly correlated to SPAD with $r = 0.39$ and $r = 0.36$, respectively. A significant correlation was also determined for CSS to SPAD with $r = 0.42$ and for CFP with $r = 0.42$ in the drought stress treatment whereas for the control treatment significantly negative correlations were found. Low but nevertheless significant correlations to SPAD were also detected for ETR and OA in the control treatment. Similar correlations were detected for BY. High and significant correlations were found between BY and CSS ($r = 0.36$) and CFP ($r = 0.31$) for the drought stress treatment. Under control conditions the SBCC being a sub-population of its own, influences the correlation by producing less BY which results in reduced shading of the primary leaves and a negative correlation especially to CFP and CSS. By correlating only the German cultivars, these effects are excluded and no correlations ($r = -0.16$ for CFP and $r = -0.03$ for CSS) were observed. ETR and OA were not significantly correlated to BY.

Table 2 Analysis of variance (ANOVA) of analysed traits showing F and p values

Trait[a]	Effect[b]	F value	P value
BY	Genotype	7.61	<.0001
	Treatment	10878.9	<.0001
	G x T	4.16	<.0001
SPAD	Genotype	8.81	<.0001
	Treatment	30.74	<.0001
	G x T	0.83	0.9348
ETR	Genotype	2.08	<.0001
	Treatment	43.69	<.0001
	G x T	0.97	0.6007
OA	Genotype	1.45	0.0004
	Treatment	3737.86	<.0001
	G x T	1.16	0.0962
CFP	Genotype	2.59	<.0001
	Treatment	2544.91	<.0001
	G x T	2.89	<.0001
CSS	Genotype	2.85	<.0001
	Treatment	1984.1	<.0001
	G x T	2.42	<.0001

[a]BY: biomass yield, SPAD: leaf colour, ETR: electron transport rate at PSII, OA: osmolality, CFP: content of free proline, CSS: total content of soluble sugars
[b]Genotype, Treatment and GxT: genotype x treatment interaction effect

Table 3 Coefficient of correlation (PEARSON) for control and drought stress treatment

	Treatment	SPAD	ETR	OA	CFP	CSS
BY	Control	0.395***	0.091	−0.127	−0.328***	−0.220**
	Stress	0.361***	−0.087	−0.124	0.307***	0.367***
SPAD	Control		0.160*	−0.185*	−0.239**	−0.192*
	Stress		−0.105	0.034	0.425***	0.418***

[a]BY: biomass yield, SPAD: leaf colour, ETR: electron transport rate at PSII, OA: osmolality, CFP: content of free proline, CSS: total content of soluble sugars
r is significant with *p <0.05, **p <0.01 and ***p <0.001

Genotyping

The set of genotypes was analysed with the 9 k iSelect SNP-chip available for barley. In summary 6,807 SNPs turned out to be polymorphic. Out of these, 3,212 SNPs are mapped on the seven barley chromosomes [68], showing a minor allele frequency (MAF) >5 %. This set of SNPs was used for the calculation of the linkage disequilibrium decay (LD), which turned out be on average 2.52 cM for this set of genotypes. The number of subpopulations was estimated at $k = 4$ (Fig. 1).

Genome wide association study (GWAS)

Results of GWAS are shown in detail in Additional file 2 and summarized in Tables 4 and 5. 191 SNPs significantly ($p < 0.001$) associated to traits estimated in the control variant, 70 significantly associated SNPs in the stress treatment and 92 significantly associated SNPs across treatments (DSI), were detected using the MLM analysis in TASSEL. Significant associations were found on all barley chromosomes. Most of the significant

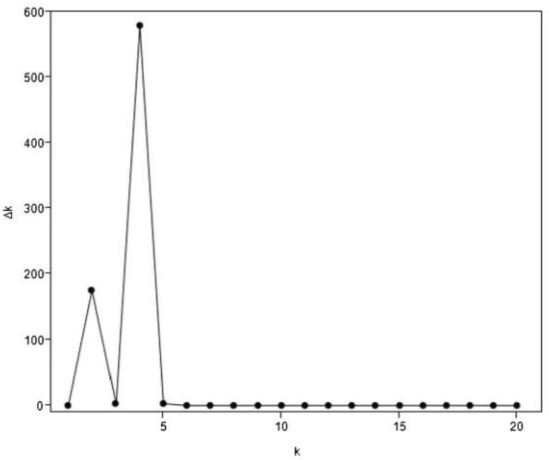

Fig. 1 Optimal k of the population structure. The number of subpopulations within the set of barley genotypes was estimated at $k = 4$ by calculation described in Evanno *et al.* [70]

Table 4 Significant markers traits associations detected under drought stress conditions at a significance of p <0.001

Trait[a]	Number of genomic regions associated with the traits on the seven linkage groups (barley chromosomes)[b,c]							
	1H	2H	3H	4H	5H	6H	7H	Total QTL
BY	81.7 cM (3 SNP)	2 cM (3 SNP)	76.2 cM (1 SNP)	99.1 cM (1 SNP)	46.7 cM (8 SNP)		48.3 cM (1 SNP)	**19 (32 SNPs)**
	92.2 cM (1 SNP)	5.5 cM (1 SNP)	135.5 cM (1 SNP)		59.7 cM (1 SNP)		70.2 cM (1 SNP)	
		12.1 cM (1 SNP)			80.3 cM (1 SNP)		133.9 cM (1 SNP)	
		90.2 cM (3 SNP)			110.1 cM (1 SNP)			
					139.1 cM (1 SNP)			
					152.4 cM (1 SNP)			
					167.7 cM (1 SNP)			
SPAD		49.2 cM (1 SNP)			44.2 cM (4 SNP)		128.3 cM (1 SNP)	**3 (6 SNPs)**
ETR						59.4 cM (1 SNP)	2.1 cM (1 SNP)	**2 (2 SNPs)**
OA	116.8 cM (1 SNP)	51.8 cM (1 SNP)	2.4 cM (1 SNP)	52.3 cM (1 SNP)	46.5 cM (1 SNP)	10.3 cM (1 SNP)	106.5 cM (1 SNP)	**22 (29 SNPs)**
		60.8 cM (2 SNP)	36.8 cM (2 SNP)	110.2 cM (1 SNP)	55.7 cM (1 SNP)	47.5 cM (1 SNP)		
		81.5 cM (4 SNP)	51.8 cM (1 SNP)		95 cM (1 SNP)	51 cM (2 SNP)		
		135.8 cM (1 SNP)	61.9 cM (1 SNP)		137.9 cM (1 SNP)			
		146.5 cM (1 SNP)	89.4 cM (1 SNP)					
			100.7 cM (2 SNP)					
CSS	95.8 cM (1 SNP)							**1 (1 SNP)**
Total QTL	**4 (6 SNPs)**	**10 (18 SNPs)**	**8 (10 SNPs)**	**3 (3 SNPs)**	**12 (22 SNPs)**	**4 (5 SNPs)**	**6 (6 SNPs)**	**47 (70 SNPs)**

[a]BY: biomass yield, SPAD: leaf colour, ETR: electron transport rate at PSII, OA: osmolality, CSS: total content of soluble sugars
[b]One genomic region up to 2.6 cM (LD); the chromosomal position in cM was taken from the respective SNP with the highest R^2
[c]Chromosome positions are based on Comadran *et al.* (2012)

marker trait associations were located on barley chromosome 2H and 5H. A large number of SNPs on chromosome 5H around 45 cM turned out to be associated to SPAD and BY in the stress treatment (Fig. 2). Most significant associations for these traits were detected on chromosome 5H. The highest number of marker trait associations was detected for BY. No associations were observed for CFP in control and stress treatment, whereas across treatments one significant association was found. For ETR two significant associations and for CSS one significant association in the stress treatment explaining 5.5 % (ETR) and 1.6 % (CSS) of the phenotypic variance respectively were detected. The strongest association was observed on chromosome 1H for BY across treatments with a (–log p) value of 7.57 explaining 7.1 % of the phenotypic variance. For OA 29 significant associations were detected in the stress treatment located over all barley chromosomes, whereas in the control treatment no associations and across treatments only one association was found on chromosome 4H.

For SPAD as an indicator of leaf senescence, twelve significant (p <0.001) marker trait associations in the control treatment, six under stress conditions and two across treatments were detected. For the drought stress indicator biomass yield 179 significant marker trait associations were found in the control treatment, 32 in the stress treatment and 87 across treatments. Significant marker trait associations for BY were evenly distributed over all chromosomes. Out of these, eight were identical in the stress treatment, control treatment and across treatments and a high number of 65 marker trait associations were identical in control treatment and across treatments.

In total the 191 significant associations estimated in the control treatment account for 80 genomic regions (LD = 2.52 cM), the 70 significant associations determined in the stress treatment represent 47 genomic regions and the 92 significant associations across treatments (DSI) account for 54 genomic regions. By comparing the localisation of marker trait associations detected, it turned out that significant associations were found for different traits at same positions, e.g. on chromosome 2H at 50 cM for SPAD and OA in the stress treatment and at 120 cM for BY and SPAD in the control treatment. Furthermore, on chromosome 5H at 45 cM significant marker trait

Table 5 Significant blasted proteins related to drought stress or leaf senescence

Protein (Top BlastX hit with p $<10^{-5}$ or query cover >80 %)	Protein abbr.	Accession	Function[a]	Marker[b]	Chr.[b]	Pos. in cM[b]	Trait[c]	Treat.[d]
Protease Do-like	DEGP2	[Swiss-Prot:O82261.2]	ls	BOPA1_8166-525	1H	47.5	BY	C, DSI
Cullin-1	CUL1	[Swiss-Prot:Q94AH6.1]	ls	SCRI_RS_85918	1H	47.7	BY	C
Serine/threonine-protein phosphatase PP1 isozyme 3	TOPP1	[Swiss-Prot:P48483.1]	ds	SCRI_RS_17924	1H	47.7	BY	C, DSI
Electron transfer flavoprotein-ubiquinone oxidoreductase	ETFQO	[Swiss-Prot:O22854.1]	ls	SCRI_RS_132604	1H	48.4	BY	C, DSI
ATP-dependent zinc metalloprotease	FTSH3	[Swiss-Prot:Q84WU8.1]	ls	BOPA1_2881-935	1H	81.7	BY	S
ABC transporter G family member 43	ABCG43	[Swiss-Prot:Q7PC81.1]	ds	BOPA2_12_31319	1H	92.4	SPAD	C, DSI
Probable pectinesterase 49	PME49	[Swiss-Prot:Q9LY18.1]	ds	SCRI_RS_235724	1H	95.8	CSS	S
Sucrose synthase 4	SUS4	[Swiss-Prot:Q9M111.1]	ls	SCRI_RS_239231	2H	49.2	SPAD	S
Metal-nicotianamine transporter YSL	YSL2	[Swiss-Prot:Q6H3Z6.2]	ds	SCRI_RS_221886	2H	80.9	BY	C
Glutamate dehydrogenase 2	GDH2	[Swiss-Prot:Q38946.1]	ls	BOPA1_3469-1152	2H	81.5	BY	C
Probable glutamate carboxypeptidase 2	AMP1	[Swiss-Prot:Q9M1S8.3]	ds	SCRI_RS_156090	2H	81.5	BY, OA	C, S
Probable phospholipid hydroperoxide glutathione peroxidase	GPX1	[Swiss-Prot:O23968.1]	ds	BOPA1_1635-691	2H	89.8	BY	S
Ethylene receptor 1	ETR1	[Swiss-Prot:Q9SSY6.1]	ls	SCRI_RS_185665	2H	114.9	BY	C, DSI
Cullin-3A 3B	CUL3A CUL3B	[Swiss-Prot:Q9ZVH4.1] [Swiss-Prot:Q9C9L0.1]	ls	BOPA1_3608-2133	2H	129.7	BY	C
Senescence-induced receptor-like serine/threonine-protein kinase	SIRK	[Swiss-Prot:O64483.1]	ls	SCRI_RS_8420	2H	139.9	BY	C
Putative F-box/LRR-repeat protein 21	FBL21	[Swiss-Prot:Q9M0U8.1]	ds	SCRI_RS_115423	3H	36.3	OA	S
1-aminocyclopropane-1-carboxylate oxidase	ACO1	[Swiss-Prot:Q9ZQZ1.1]	ds	SCRI_RS_167825	3H	100.3	BY, OA	C, S, DSI
Dehydrin	DHN 3	[Swiss-Prot:P12948.1]	ds	BOPA1_ABC13753-1-2-167	3H	105.3	BY	C
ABC transporter D family member 1	ABCA1	[Swiss-Prot:Q94FB9.1]	ds	SCRI_RS_142818	3H	148.2	BY	C
Abscisic acid receptor	PYL5	[Swiss-Prot:Q9FLB1.1]	ds	SCRI_RS_157396	4H	52.3	OA	S
Ethylene-responsive transcription factor	ERF011	[Swiss-Prot:Q9SNE1.1]	ls	SCRI_RS_9164	4H	113.7	BY	C, DSI
Nucleotide pyrophosphatase/phosphodiesterase	AVP1	[Swiss-Prot:Q687E1.2]	ds	BOPA1_9766-787	5H	44	BY	S
Abscisic acid-inducible protein kinase	TRIUR3	[Swiss-Prot:Q02066.1]	ds	SCRI_RS_102075	5H	44	SPAD	S
Serine/threonine-protein kinase	ATM	[Swiss-Prot:Q75H77.1]	ds	BOPA1_ABC08327-1-1-353	5H	44	SPAD	S
Serine/threonine-protein kinase	SAPK9	[Swiss-Prot:Q75V57.1]	ds	SCRI_RS_102075	5H	44	SPAD	S
Anthocyanin regulatory R-S protein	R-S	[Swiss-Prot:P13027.1]	ds	BOPA1_12045-83	5H	46.7	BY	S
Fasciclin-like arabinogalactan protein 2	FLA2	[Swiss-Prot:Q9SU13.1]	ds	BOPA1_5004-375	5H	83.5	BY	C
Serine/threonine-protein kinase	ATM	[Swiss-Prot:Q9M3G7.1]	ds	BOPA1_6315-914	5H	94.7	SPAD	C
Dehydration-responsive element-binding protein 1A	DREB1A	[Swiss-Prot:Q64MA1.1]	ds	BOPA2_12_30852	5H	95	BY, OA	C, S

Table 5 Significant blasted proteins related to drought stress or leaf senescence *(Continued)*

Probable zinc metalloprotease EGY1	EGY1	[Swiss-Prot:Q852K0.3]	ls	SCRI_RS_208686	5H	137.4	BY	C, DSI
Cation/H(+) antiporter 2	CHX	[Swiss-Prot:Q9SAK8.1]	ds	SCRI_RS_160297	7H	2.1	ETR	S
Ethylene-responsive transcription factor	ERF062	[Swiss-Prot:Q9SVQ0.1]	ls	SCRI_RS_150783	7H	48.3	BY	S

[a]Function of the proteins related to drought stress (ds) or leaf senescence (ls), given by UniProt
[b]Markers and chromosome positions are based on Comadran *et al.* (2012)
[c]BY: biomass yield, CSS: total content of soluble sugars, ETR: electron transport rate at PSII, CFP: content of free proline, OA: osmolality, SPAD: leaf colour
[d]C: control treatment, S: stress treatment, DSI: drought susceptibility index across treatments by Fischer & Maurer (1978)

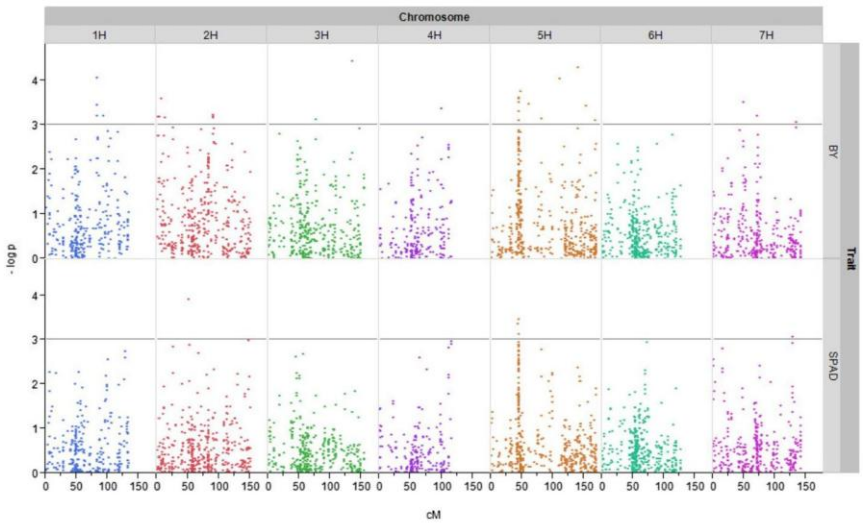

Fig. 2 Manhattan plots. Showing –log p of association between Illumina SNPs and the analysed traits biomass yield (BY) and leaf colour (SPAD) against the position of the markers on all seven linkage groups (barley chromosomes) for stress treatment. Threshold indicates a significance level of $p < 0.001$

associations for BY, SPAD and OA were detected in the stress treatment, and at 95 cM significant associations for BY and SPAD in the control treatment.

Summarizing, overlapping of QTL was found across treatments and for different traits, especially for BY and SPAD, which are also significantly correlated (Table 3). One interesting QTL was observed on chromosome 5H at 45 cM where a significant association to BY and SPAD in the drought stress treatment was found, which is also within the LD of a significant association to OA (Table 4). Therefore, at this position a putative major QTL for drought stress and leaf senescence may be located.

From the 353 significantly associated SNPs detected in the control treatment, stress treatment and across treatments (DSI), 127 proteins were identified by an NCBI Blast of the marker sequences. Out of these 19 proteins turned out to be related to drought stress, 10 proteins related to leaf senescence and 98 proteins turned out to be not related to drought stress or leaf senescence. Out of the 29 proteins for drought stress and leaf senescence (Table 5), 16 revealed associations under drought stress conditions. These were in a next step assigned to the barley chromosomes by the known genetic localization of respective SNPs (Fig. 3). Most of these were located at barley chromosome 2H and 5H, none were mapped on chromosome 6H.

Discussion

Using the experimental design described above a clear cut influence of drought stress on biomass yield and physiological parameters was observed (Table 1). This effect of reduced biomass under drought conditions in juvenile stages was also reported in Honsdorf *et al.* [51] with an even higher reduction due to drought in early developmental stages of barley by Jamieson *et al.* [25]. In experiments on terminal drought stress application in barley this effect was not so pronounced [25, 79, 80] giving hint that barley is most susceptible to drought stress in early developmental stages. Furthermore, a reduction of the chlorophyll content under drought stress conditions in barley has been observed [80, 81], but there are also reports on adverse effects, e.g. for rapeseed or potato [82, 83], which may be due to a reduced leaf growth under drought stress conditions resulting in a reduced cell expansion leading to a relatively higher chlorophyll density in the leaves. In the present study measurement was done on primary leaves, which were fully expanded at the initiation of drought stress, so that this effect was excluded. For biomass yield a significant correlation to the leaf senescence parameter chlorophyll content was observed (Table 3). This correlation may be based on a true genetic relationship between these parameters, as it is also reported in drought stress field studies on wheat [59, 84] and in glasshouse experiments [85].

The electron transport rate at PS II as a parameter for the chlorophyll fluorescence decreased under drought stress indicating the degradation of chlorophyll during drought stress induced leaf senescence (Table 1), as already shown by Fang *et al.* [86], Li *et al.* [27], Netto *et al.* [87] and Silva *et al.* [88].

As expected, osmolality increased under drought stress thereby protecting cells against a turgor loss [30]. This is also reported in other drought stress studies on barley [29], but is more often detected in barley under salt

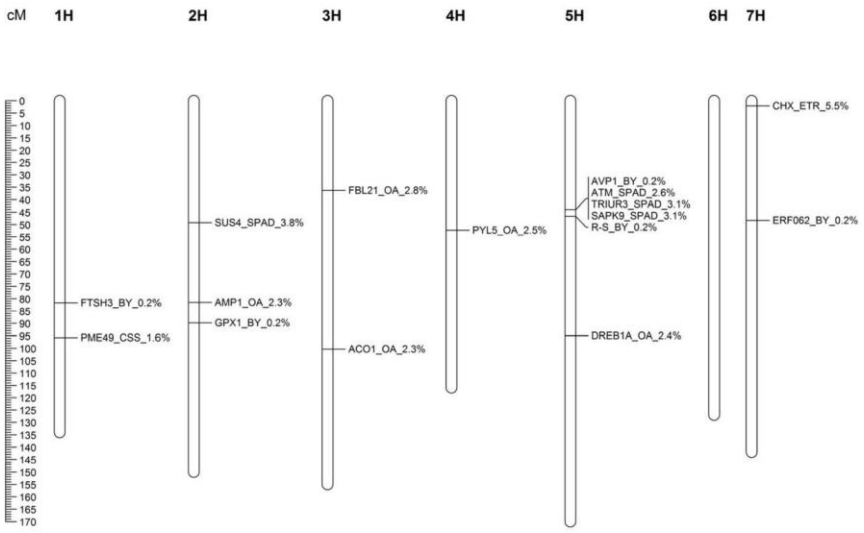

Fig. 3 Genetic map. Shows QTL including the significant associated SNP marker positions for significant blasted proteins (BlastX) linked to drought stress or leaf senescence, related traits for drought stress treatment and percentage of phenotypic variance (explained R^2 in %) of the SNPs for all linkage groups (barley chromosomes)

stress conditions [89, 90]. For OA no correlation was found to BY, as also shown in a drought stress study on spring barley [91].

The amino acid proline has been described as an osmo-protectant [92] and is accumulated along with several abiotic stresses, such as drought stress, as seen in the present study (Table 1). This effect was also found in other drought stress studies, for example on barley in pot experiments [28], in greenhouse [93] and on *Arabidopsis thaliana* in climate chamber experiments [94]. The role of proline accumulation is still controversially discussed as it is described to function as a radical scavenger, antioxidant and is involved in the regulation of apoptosis and in seed development [95, 96, 97]. High correlations were detected for CFP to SPAD and BY giving hint that this trait is involved in drought stress tolerance and leaf senescence (Table 3). The correlation of the proline content to SPAD was also found in winter survival studies of barley [98] and in studies on salt stress tolerance in *Trigonella foenum-graecum* [99]. Up to now no correlations of CFP to BY under drought stress have been described for barley, but positive correlations to yield in wheat were observed under drought stress [100].

Soluble sugars are acting also as osmo-protectants and consequently like in our study an increase was detected in several drought stress experiments on barley [29], wheat [101], potato [102] and also pea [103]. Furthermore, studies showed that an increase of soluble sugars occurs along with leaf senescence [104] and that CSS was correlated to leaf senescence and biomass production [105, 106]. Interactions between sugar and ABA signalling may be responsible for the induction of senescence during drought stress [107].

Quite high values for the heritability of respective traits estimated under drought stress conditions (Table 1) give hint that such an experimental design is suited together with a set of diverse genotypes and the respective number of SNP-markers to detect QTLs using a genome wide association approach (GWAS). Like in other studies [49, 108, 109], the highest number of associations was detected for the traits with the highest heritability. In the present study these were SPAD and BY. Most associations were found on barley chromosomes 2H and 5H on which QTLs were located at 50 cM and at 45 cM, respectively. Also in other GWAS studies of barley significant QTLs for SPAD and BY were located on these chromosomes. Close to the QTL for SPAD located on chromosome 2H (50 cM) a QTL for SPAD under drought stress was also mapped by Li *et al.* [50]. Moreover, on chromosome 2H at 115 cM a QTL for SPAD was identified in a pot experiment with post-flowering drought stress [53]. QTLs for SPAD were also located on chromosome 2H at 102.7 cM and on chromosome 5H at 165.2 cM in Mediterranean dry land experiments (110), but no significant marker trait associations were detected at these positions in our experiments. The same holds true for a QTL for SPAD on chromosome 5H at 139 cM [49]. Varshney *et al.* [49] also detected a QTL for biomass yield on chromosome 5H at 95 cM and 156 cM, which is near to associations, which were found

in our study on chromosome 5H at 152 cM and 167 cM. In addition, in the present study a QTL for SPAD and BY under drought stress treatment was detected on chromosome 5H at 45 cM, which has not been described before. Furthermore, a lot of significant marker trait associations were observed for osmolality under stress treatment distributed over all barley chromosomes. This was also reported for barley based on growth chamber drought experiments [29].

Proteins involved in drought stress and leaf senescence were detected by a blast of SNP marker sequences and it turned out that they are distributed over all barley chromosomes, except 6H with a focus on chromosomes 2H and 5H (Table 5). Most interesting proteins detected in the drought stress treatment (Fig. 3) are discussed in detail.

On chromosome 1H an ATP-dependent zinc metalloprotease (FTSH3) which is a regulator of heat shock proteins turned out to be associated to BY under drought stress. This protein is involved in the thylakoid formation and in the removal of damaged D1 in the photosystem II, preventing cell death under high-intensity light conditions. In interaction with heat shock proteins it reduces chlorophyll a/b ratios in heat tolerance regulation in *Arabidopsis thaliana* [111]. Heat stress often occurs simultaneously with drought and also leads to leaf senescence. Besides this, on chromosome 1H the pectin methylesterase 49 (PME49) was found by sequence alignment to be associated to CSS. This protein acts in the modification of cell walls via demethylesterification of pectin and turned out to be up-regulated by drought stress in rice [112]. It influences the mechanical stability of cell walls and thereby also of leaves.

On chromosome 2H a sucrose synthase 4 (SUS4) turned out to be associated with SPAD. This is a sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways and is involved in nucleic acid break down during leaf senescence as revealed by expression analysis e.g. in cucumber and rapeseed [113]. The SNP marker with the homolog sequences to this protein was associated to the leaf senescence parameter SPAD at 49.2 cM. So there may be a direct relationship between the SPAD values and the activation of this enzyme, especially because this SNP marker explains 3.8 % of the phenotypic variance. Furthermore, a probable glutamate carboxypeptidase (AMP) revealed an association to OA which plays an important role in shoot apical meristem development and phytohormone homeostasis. By microarray analysis it turned out that AMP mediates ABA production and is involved in abiotic stress response such as drought stress in *Arabidopsis thaliana* [114]. Moreover, a marker with a sequence homologue to a phospholipid hydroperoxide glutathione peroxidase (GPX1) associated to BY was found which protects cells and enzymes from oxidative damage. Photometrical analyses of protein quantity and activity showed that the expression of GPX1 and GPX3 is reduced under drought stress and restored after recovery in winter wheat [115].

On chromosome 3H an F-box protein was detected (FBL21) associated to OA. These proteins are ubiquitin related and negatively regulate ABA mediated drought stress response in *Arabidopsis thaliana* [116]. Furthermore, an association of OA to 1-aminocyclopropane-1-carboxylate oxidase (ACO1) was detected which limits leaf growth by inhibiting the ethylene biosynthesis and so leads to drought tolerance. This was figured out in barley by expression analyses of protein related genes [117]. Surprisingly, in the control treatment associated to BY, a well known drought stress related protein, i.e. dehydrin (DHN) [118] was found which was also in another study located on chromosome 3H [119]. This protein belongs to the family of late embryogenesis abundant (LEA) proteins and is reported to be up-regulated in the protection mechanisms activated by plants in response to drought stress in wheat [120].

On chromosome 4H an ABA receptor (PYL5) was located and associated to OA that activates ABA signalling and ABA-mediated responses such as stomatal closure and germination inhibition. Immuno-detection experiments of protein extracts revealed that ABA signalling is involved in several stresses for example drought stress in *Arabidopsis thaliana* [121].

On chromosome 5H nucleotide pyrophosphatase/phosphodiesterase (AVP1) was found associated to BY which facilitates auxin transport by modulating apoplastic pH and regulating auxin-mediated developmental processes. Increased expression of protein related genes in transgenic barley confers tolerance to NaCl and to drought by increasing ion retention [122]. Furthermore, three protein kinases associated to SPAD were located on this chromosome regulating protein activity by phosphorylation. First a serine/threonine-protein kinase (ATM) which leads to stress induced programmed cell death, shown in *Arabidopsis thaliana* by expression profiles of protein related genes [123], second a serine/threonine-protein kinase (SAPK9) which is activated by hyperosmotic stress in rice [124] and third the abscisic acid-inducible protein kinase (TRIUR3) observed in wheat, which is also involved in dehydration stress response [125]. Moreover, an anthocyanin regulatory protein (R-S) was detected associated to BY. Anthocyanin is often accumulated in abiotic stress response, among others in drought stress with a photoprotective function as shown in *Arabidopsis thaliana* [94, 126]. All of these proteins showed homologies to sequences of SNPs at chromosome 5H around 45 cM and were associated significantly to BY or SPAD, representing an interesting candidate QTL for drought stress and leaf senescence. In addition, a dehydration-responsive element-binding

protein (DREB1A) was found associated to OA, which delays water stress symptoms and promotes expression of drought tolerance genes in transgenic wheat [127].

On chromosome 7H another ethylene responsive protein was found to be associated to BY under drought stress. The transcription factor ERF062 is involved in the regulation of gene expression by stress factors (transcriptional repressors) and progression of leaf senescence in *Arabidopsis thaliana* [128]. Besides, on chromosome 7H the cation/H(+) antiporter 2 (CHX) was detected associated to ETR, which is important for pH gradients in the cell. This protein plays a vital role in maintaining both cellular and intercellular ionic balances under stresses such as drought stress as observed in *Arabidopsis thaliana* [129].

In summary the blast of the associated SNPs to protein data bases revealed many proteins which are known to be involved in drought stress response or leaf senescence, respectively giving hint that the GWAS approach is well suited for the genetic dissection of these traits in barley. Out of the QTL detected, the ones on chromosome 2H at 50 cM and chromosome 5H at 45 cM are of prime importance and may be involved in breeding barley for drought tolerance in the future due to the quite high amount of phenotypic variance explained.

Conclusions

By GWAS marker trait associations for above ground biomass and physiological traits involved in drought stress tolerance and leaf senescence in early developmental stages of barley were detected. Major QTL for BY and SPAD under drought stress were located at chromosome 2H at 50 cM and chromosome 5H at 45 cM, giving hint that in these regions putative major QTLs for drought stress and leaf senescence are located. With respect to the QTL on chromosome 2H, QTLs for drought stress and leaf senescence were located at comparable positions in other GWAS studies while the one on chromosome 5H was detected for the first time. By BlastX of respective SNP carrying sequences, 29 proteins were identified being involved in drought stress or leaf senescence, respectively. Respective QTLs may be the starting point for marker based selection in barley for drought stress tolerance in the juvenile stage.

Additional files

Additional file 1: Overview of the 156 analysed genotypes. [a]SBCC: Spanish Barley Core Collection.

Additional file 2: Significant associations (p <0.001) of the genome wide association study and blasted proteins out of the marker sequences. [a]BY: biomass yield, CSS: total content of soluble sugars, ETR: electron transport rate at PSII, CFP: content of free proline, OA: osmolality, SPAD: leaf colour. [b]C: control treatment, S: stress treatment, DSI: drought susceptibility index across treatments by Fischer & Maurer (1978). [c]Markers and chromosome positions are based on Comadran et al. (2012).

Abbreviations

e.g.: for example; i.e.: id est; GWAS: Genome wide association study; QTL: Quantitative trait locus; SNP: Single nucleotide polymorphism; ABA: Abscisic acid; LEA: Late embryogenesis abundant protein; SPAD: Soil Plant Analysis Development; measurement of chlorophyll content by colour; SBCC: Spanish Barley Core Collection; das: Days after sowing; ETR: Electron transport rate; CFP: Content of free proline; CSS: Total content of soluble sugars; OA: Osmolality; BY: Biomass yield; PSII: Photosystem two; PAR: Photosynthetic active radiation; DSI: Drought susceptibility index; value across treatments; LSMeans: Last Square Means; h^2: Heritability; CV: Coefficient of variation; SSR: Simple sequence repeat; MAF: Minor allele frequency; k: number of subpopulations; MLM: Mixed linear model; LD: Linkage disequilibrium; Blast: Basic Local Alignment Search Tool.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GW conducted all experiments, including statistical and bioinformatics analyses, as well as the sequence alignment and mainly wrote the manuscript. ME participated in the GWAS and LD calculation. CB, KH and FO designed the research, supervised the experimental design, data analysis and participated in writing the manuscript. All authors approved the final manuscript.

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RESEARCH ARTICLE Open Access

Expression profiling of genes involved in drought stress and leaf senescence in juvenile barley

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Abstract

Background: Drought stress in juvenile stages of crop development and premature leaf senescence induced by drought stress have an impact on biomass production and yield formation of barley (*Hordeum vulgare* L.). Therefore, in order to get information of regulatory processes involved in the adaptation to drought stress and leaf senescence expression analyses of candidate genes were conducted on a set of 156 barley genotypes in early developmental stages, and expression quantitative trait loci (eQTL) were identified by a genome wide association study.

Results: Significant effects of genotype and treatment were detected for leaf colour measured at BBCH 25 as an indicator of leaf senescence and for the expression level of the genes analysed. Furthermore, significant correlations were detected within the group of genes involved in drought stress ($r = 0.84$) and those acting in leaf senescence ($r = 0.64$), as well as between leaf senescence genes and the leaf colour ($r = 0.34$). Based on these expression data and 3,212 polymorphic single nucleotide polymorphisms (SNP) with a minor allele frequency >5 % derived from the Illumina 9 k iSelect SNP Chip, eight *cis* eQTL and seven *trans* eQTL were found. Out of these an eQTL located on chromosome 3H at 142.1 cM is of special interest harbouring two drought stress genes (GAD3 and P5CS2) and one leaf senescence gene (Contig7437), as well as an eQTL on chromosome 5H at 44.5 cM in which two genes (TRIUR3 and AVP1) were identified to be associated to drought stress tolerance in a previous study.

Conclusion: With respect to the expression of genes involved in drought stress and early leaf senescence, genotypic differences exist in barley. Major eQTL for the expression of these genes are located on barley chromosome 3H and 5H. Respective markers may be used in future barley breeding programmes for improving tolerance to drought stress and leaf senescence.

Keywords: Barley, Leaf senescence, Drought stress, High-throughput qPCR, Gene expression, eQTL

Background

In order to analyse genetic networks and stress response, real time polymerase chain reaction (PCR) is an important tool [1]. For several years high-throughput instruments e.g. the BioMark System from Fluidigm have enabled large scale quantitative PCR studies [2]. Because of this and the possibility to analyse a large number of genotypes easily on expression chips [2] a range of genome wide association studies (GWAS) using expression data were conducted in the last years [3–5]. Expression quantitative trait loci (eQTL) were detected first in medicinal studies in humans and later also in plants [6–10]. In plants most eQTL studies were performed for complex pathways and aimed at a better understanding of the molecular networks [11]. Whereas in biotic stress the resistance is often controlled by a single gene, responses to abiotic stresses such as drought stress are controlled by many genes [12–14] and so these processes are particularly suitable for high throughput expression analyses and genetical genomics approaches [15]. Even in early developmental stages drought stress and drought stress induced premature leaf senescence have major influences on yield formation [16]. Therefore, it is of prime importance

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to understand regulatory processes of drought stress [17] and leaf senescence [18].

In plants drought stress is initiated by water deficit in soil resulting in osmotic and oxidative stress and cellular damage [19]. This leads to defined drought stress responses for instance regarding the maintenance of turgor by an increase of osmoprotective molecules as soluble sugars [20–22], as well as measurable lower water content and decreased growth in the stressed plants compared to a control [23, 24]. Stress perception is assigned by special receptors, such as abscisic acid (ABA) receptors, hexokinases, or ion channel linked receptors [25]. The stress signal is then transducted for example via serine-threonine kinases, serin-threonine phosphatases, calcium dependent protein kinases, or phospholipases [25]. Finally, the gene expression is regulated by effector genes coding for late embryo abundant (LEA) proteins, dehydrin, or reactive oxygen species (ROS) and transcription factors, such as MYB, WRKY, NAC, AP2/ERF, DREB2, or bZIP to activate stress responsive mechanisms, re-establish homeostasis and protect and repair damaged proteins and membranes [13, 19, 25, 26]. Besides the above mentioned genes, drought stress associated metabolites such as osmoprotectants, polyamines and proteins involved in carbon metabolism and apoptosis are part of drought stress tolerance [12, 27]. Disturbing the regulatory processes in drought stress response results in irreversible changes of cellular homeostasis and the destruction of functional and structural proteins and membranes, leading to cell death [19] and decreased yield formation [28]. A huge transcriptome analysis for drought stress associated genes was done for example in barley [29] and wheat [30] showing differential response of genes involved in drought stress tolerance.

Initiated by external signals e.g. various stresses such as drought, as well as by internal factors for example phytohormones leaf senescence often occurs as a natural degradation process at the final stage of plant development [31]. Drought stress induced leaf senescence proceeds in three steps. Perception of drought stress is the initiation phase in which senescence signals are transferred via senescence associated genes (SAG) [32]. These are regulatory genes which often encode transcription factors regulating gene expression by binding to distinct *cis*-elements of target genes [33]. In the following reorganisation phase resources are transported from source (e.g. roots, leaves) to sink (e.g. fruits, seed) organs being important for yield formation [34]. With this translocation chlorophyll, proteins, lipids and other macromolecules are degraded and the content of antioxidants, ABA and ROS increases induced by a change in gene expression [35, 36]. Differentially expressed genes and their regulation during leaf senescence were identified by transcriptome analysis using microarrays in *Arabidopsis thaliana* [37, 38]. While the genes for photosynthesis and chloroplast development are down-regulated, the genes for the degradation of macromolecules and recycling of resources are up-regulated [39]. For example, expressed genes for chlorophyll degradation are *PA42*, *Lhcb4* and *psbA* [40] and genes for N mobilization and transport are transcription factors WRKY [41] and NAC [42] as well as glutamine synthetase [38]. Genes differentially expressed can be grouped to those accelerating leaf senescence and genes delaying leaf senescence [43]. The latter possibly resulting in a "stay green" effect and improved drought tolerance [34, 44]. The reorganisation phase is the crucial step for reversibility, after which senescence is irreversible and leads to the final step where leaves and cells often die [45].

In barley (*Hordeum vulgare* L.), a crop plant of worldwide importance, most mechanisms for leaf senescence are still not well understood [18, 34]. The response to drought in juvenile stages is less well documented, as only few studies are focused on early developmental stages [20, 24, 46, 47] whereas a lot of studies were conducted for drought stress in the generative stage [48]. Nevertheless, barley is to some extent a model organism for research at a genome wide level. The barley gene space has been published [49] and with this information gene positions can be compared to these data. Comparing the position of the analysed genes in the Morex genome with positions of the detected eQTL, resulted in the co-localization of eQTL and genes involved in drought stress [11, 50]. Therefore, the present study aimed at the identification of eQTL in barley for genes involved in drought stress in the juvenile phase and early leaf senescence (Table 1) based on a genome wide association study.

Results

Leaf senescence

Leaf colour (SPAD, soil plant analysis development) measured at 20 days after drought stress induction (BBCH 25, according to Stauss [51]) being indicative for leaf senescence revealed significant differences between treatments and genotypes but no significant interaction of genotype and treatment was observed at this stage (Fig. 1 and Table 2) giving hint to physiological changes and changes in gene expression.

Relative expression of candidate genes

At the same developmental stage (BBCH 25) expression analyses were conducted for the whole set of 156 genotypes analysing 14 genes (Table 1). The relative expression (-ΔΔCt) ranges from −8.5 to 14.9 (Fig. 2, Additional file 1). In most genotypes all five drought stress related genes (A1, Dhn1, GAD3, NADP_ME and P5CS2) showed a higher expression under stress treatment relative to the control whereas for genes involved in leaf senescence

Table 1 Primer pairs for the selected genes and the reference gene

	Gene	Functional annotation	Acc. No.	Primer (FOR and REV)	Ampl.
Drought stress genes	A1	ABA inducible gene	GenBank:X78205.1	ACACGGCGCAGTACACCAAGGAGTCCCACCACGGCGTTCACCAC	100 bp
	Dhn1	Dehydrin 1	GenBank:AF181451	GCAACAGATCAGCACACTTCCAGCTGACCCTGGTACTCCATTGT	141 bp
	GAD3	Glutamate decarboxylase 3	GenBank:AY187941	ATGGAGAACTGCCACGAGAAGGAGATCTCGAACTCGTCGT	147 bp
	NADP_ME	NADP-dependent malic enzyme-like	GenBank:XM_003569737	ATGGCGGGAAGATCAGGGATCCCTCAGCAGGGAATGC	165 bp
	P5CS2	Delta 1-pyrroline-5-carboxylate synthase 2	GenBank:AK249154.1	GTATACATGCACGTGGACCCCAGAGGGTTTTCGCCGAATC	164 bp
Leaf senescence genes	Contig7437	SAG senescence associated gene	GenBank:KF190467.1	GCTGAACGGCTGCCACTCCCGAAACCATCGCGCCTGTGGTG	78 bp
	GSII	Glutamine synthetase 2	GenBank:X53580.1	ACGAGCGGAGGTTGACAGCGCCCCACACGAATAGAG	94 bp
	hv_36467	SAG senescence associated gene	GenBank:AK367894.1	CAGTCCTTTTGCGCAGTTTTCCCAAGCGAGAATGCCTTGTAA	152 bp
	LHC1b20	Light-harvesting complex I	GenBank:S68729.1	CTGACCAAGGCGGGGCTGATGAACTCGTGGGGCGGGAGGCTGTAG	200 bp
	pHvNF-Y5α	SAG senescence associated gene	GenBank:AK370570	CATGAAGCGAGCTCGTGGAACAGGTGCGAAGGTGGGACTACTCTGA	126 bp
Genes out of GWAS[a]	AVP1	Vacuolar proton-inorganic pyrophosphatase	GenBank:AY255181.1	GACCCTCTCAAGGACACCTCTCCCAACCGGCAAAACTAGA	160 bp
	ETFQO	Electron transfer flavoprotein-ubiquinone oxidoreductase	GenBank:BT000373.1	CCACAACCCTTTCTTGAATCCGGATCTAAGGGCGTGGTGAATTT	160 bp
	SAPK9	Serine/threonine protein	GenBank:AB125310.1	TCATGCAAGACTGTTTCTTGGGTTTCTTCTTGGCACAAAGCATATT	149 bp
	TRIUR3	Protein kinase			
			GenBank:M94726	ACATTGACGTTGAGAGCAGCGCTACAGAGAATTTGTGACCCA	151 bp
	HvGAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GenBank:DQ196027.1	CAATGCTAGCTGCACCACCAACTGCTAGCAGCCCTTCCACCTCTCCA	165 bp

[a]Genes coding for proteins identified by BlastX of significant marker sequences out of a previous genome wide association study (GWAS) by Wehner et al. [20]

opposite effects were detected for all genes (GSII, hv_36467, LHC1b20 and pHvNF-Y5α) except Contig7437. The genes out of the GWAS [20], i.e. AVP1 and TRIUR3 which are drought stress related genes, were up-regulated, whereas SAPK9 and ETFQO showed a lower expression relative to the control. In total, eight genes were up and six genes were down-regulated relative to the control but not all genotypes responded in the same way.

The mean quality score for all amplifications was 0.954. Because ΔCt and ΔΔCt values were not normally distributed (data not shown) further statistical analysis was done with logarithmic values ($\log_2$). Analysis of variance (ANOVA) revealed significant (p <0.001) effects for genotype and treatment for the 14 genes except Contig7437 (Table 2).

Highest significant correlations for differences in gene expression were identified within groups, i.e. within the group of drought stress genes, leaf senescence genes and genes out of GWAS (Table 3). The highest correlation was observed for the group of drought stress genes between relative expression of GAD3 and P5CS2 (r = 0.84), for the group of leaf senescence genes for GSII and pHvNF-Y5a (r = 0.64), and for the genes out of GWAS between AVP1 and TRIUR3 (r = 0.54). For no gene the differential expression was significantly correlated to the expression differences of all other genes, but ETFQO was correlated to all except Dhn1, and GAD3 and Contig7437 were correlated to all except GSII and AVP1, and SAPK9 and NADP_ME, respectively. Significant correlations were also detected between the relative SPAD values for change in leaf colour and all leaf senescence genes except hv_36467 with the highest coefficients of correlation for GSII (r = 0.24) and pHvNF-Y5a (r = 0.34). Moreover, significant correlations were observed for relative SPAD

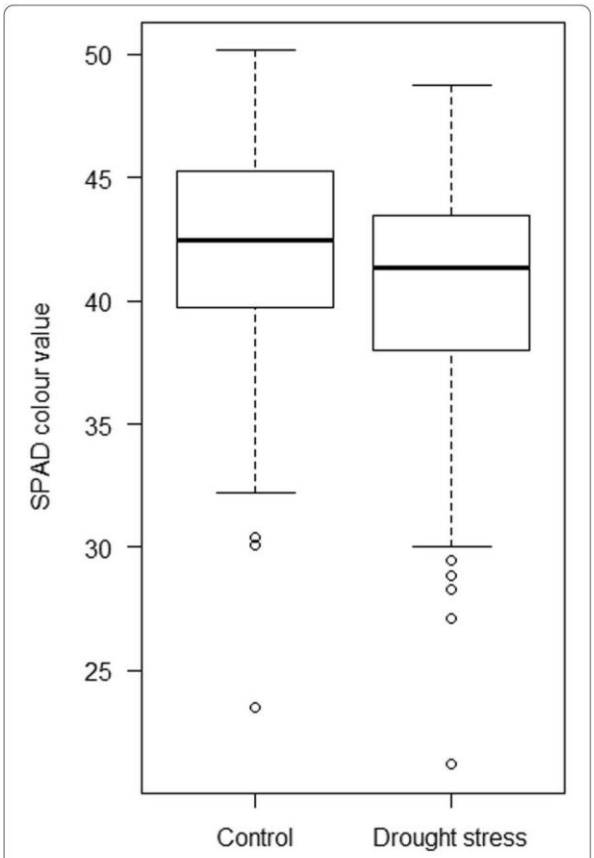

Fig. 1 Box whisker plots for status of leaf senescence. Leaf colour (SPAD) for control and drought stress treatment at 27 days after sowing (das) including all 156 analysed barley genotypes

values to two genes out of GWAS (r = 0.16 for AVP1 and r = 0.15 for TRIUR3).

Genome wide association study

Significant (p <0.001) marker gene expression associations were detected on all barley chromosomes except 4H with the highest number on chromosome 5H (8 single nucleotide polymorphisms, SNP) (Table 4). The largest transcriptional variance was explained by the marker SCRI_RS_181376 associated to the expression of ETFQO (R^2 = 11.55 %) and the highest likelihood of odds (LOD) was observed for the marker SCRI_RS_161614 associated to the expression of TRIUR3 (LOD = 3.82) on barley chromosome 5H. Five SNP were significantly associated to the relative expression of the genes for drought stress, six to those for leaf senescence and seven to the genes out of the previous GWAS. Within the group of drought stress genes, expression differences of three genes (A1, GAD3 and P5CS2) and within the group of leaf senescence genes expression differences of four genes (Contig7437, GSII, hv_36467 and pHvNF-Y5α) were associated

Table 2 Analysis of variance for leaf colour (SPAD) and the expression of the selected genes

	Trait/Gene	Effect of treatment		Effect of genotype	
		F value	*p* value	*F* value	*p* value
	SPAD	11.2	0.0009	6.6	<2E-16
Drought stress genes	A1	50.1	4.88E-12	8.8	<2E-16
	Dhn1	138.4	<2E-16	23.5	<2E-16
	GAD3	81.8	<2E-16	96.7	<2E-16
	NADP_ME	315.5	<2E-16	4.1	4.63E-09
	P5CS2	229.6	<2E-16	335.4	<2E-16
Leaf senescence genes	Contig7437	0.9	0.342	128.7	<2E-16
	GSII	175.4	<2E-16	65.1	<2E-16
	hv_36467	160.2	<2E-16	46.9	<2E-16
	LHC1b20	102.4	<2E-16	156.7	<2E-16
	pHvNF-Y5α	76.5	<2E-16	196.4	<2E-16
Genes out of GWAS[a]	AVP1	51.4	2.06E-12	37.9	<2E-16
	ETFQO	16.3	5.98E-05	41.3	<2E-16
	SAPK9	9.0	0.00312	5.8	2.88E-07
	TRIUR3	96.5	<2E-16	38.1	<2E-16

[a]Genes coding for proteins identified by BlastX of significant marker sequences out of a previous genome wide association study (GWAS) by Wehner et al. [20]

to markers. Out of these, three were located on chromosome 3H at 142.1 cM. This eQTL was detected for the relative expression of two drought stress genes (GAD3 and P5CS2) and one leaf senescence gene (Contig7437) which were also highly and significantly correlated (Table 3). Furthermore, an eQTL was observed for the relative expression of A1 on chromosome 5H at 149.9 cM associated to two markers. Associations for the relative expression of three genes (AVP1, ETFQO and TRIUR3) out of the four GWAS genes were detected on barley chromosomes 3H and 5H. For the expression of TRIUR3 three markers were found on 5H at 44.5 cM, and the expression of AVP1 was associated to a marker on chromosome 5H at 62.5 cM.

The five SNP significantly associated to the relative expression of drought stress genes and the seven markers associated to genes out of GWAS all marked *cis* eQTL, while two *trans* eQTL were detected for P5CS2 and AVP1 (Table 5). In contrast, for the six markers significantly associated to leaf senescence genes only one *cis* eQTL was observed for pHvNF-Y5α. In summary, seven *trans* eQTL were detected and eight *cis* eQTL for which the Morex contigs showed a high identity to the gene analysed. Furthermore, *cis* eQTL explained a higher transcriptional variance (R^2) than those in *trans* (Table 4 and Table 5).

Discussion

Drought stress and leaf senescence genes

As shown by the significantly decreased SPAD values at 27 days after sowing (das, BBCH 25), drought stress had

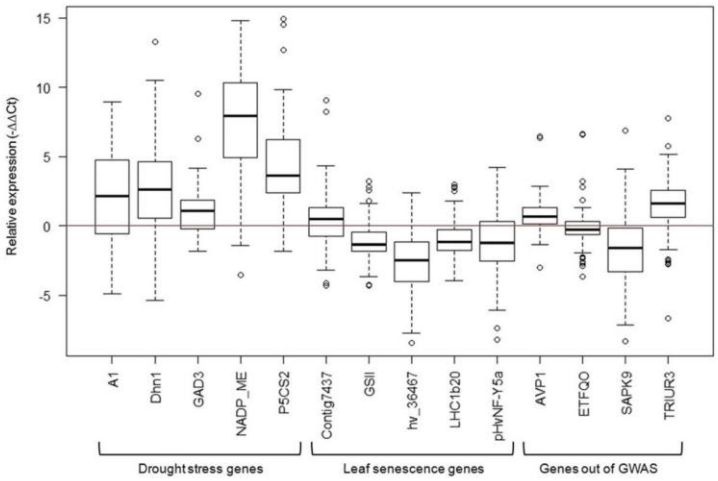

Fig. 2 Expression profile for drought stress and leaf senescence genes. Relative Expression (-ΔΔCt) for the selected genes at 26 days after sowing (das) shown in box whisker plots including all 156 analysed barley genotypes

an accelerating influence on natural leaf senescence in barley (Fig. 1 and Table 2). Furthermore, the drought stress answer in this juvenile stage was observed by differential expression of 14 genes induced by drought stress or leaf senescence (Table 1, Fig. 2).

A1 is a gene which is induced by ABA or abiotic stresses like drought, cold and heat [19, 52, 53]. In the present study expression under drought stress was higher than in the well watered treatment (Fig. 2). This was also shown by several studies first in barley [53] and other species including transgenics [54–57]. Dehydrins (Dhn) are well known to be expressed under dehydration stress [58]. For instance Dhn1 is described to be up-regulated under drought stress in barley [59, 60] which was also found in this study (Fig. 2). The glutamate decarboxylase gene (GAD3) is regulated by calcium and the protein encoded by this gene catalyzes the reaction of glutamate to γ-aminobutyric acid (GABA) [61, 62]. GABA may be involved in drought stress [63] by up-regulation of genes encoding a GABA receptor [29] which was also shown in the present study (Fig. 2). The NADP-dependent malic enzyme-like (NADP_ME) is involved in lignin biosynthesis, and regulates cytosolic pH through balancing the synthesis and degradation of malate [64]. As described in a drought stress study on barley, this effect is used for control of stomatal closure during the day under water-deficit conditions [29]. Comparable to the present study (Fig. 2) the gene for NADP_ME turned out to be higher expressed under drought stress [29]. The delta 1-pyrroline-5-carboxylate synthase 2 gene (P5CS2) is included in proline synthesis [65]. Content of proline is still controversially discussed as an indicator for drought tolerance [66], but it was shown in a previous study that the proline content increased under drought stress [20]. For approving its role, this gene was selected and showed up-regulation under drought stress (Fig. 2). Up-regulation under drought stress was also observed in tobacco [67] and transgenic rice [68].

The Contig7437 is a senescence associated gene (SAG) which is up-regulated under drought stress, as also shown by Guo et al. [29] in barley for drought stress during the reproductive stage. Other analysed SAGs are hv_36467 and pHvNF-Y5α, which were down-regulated in most genotypes under drought stress in our study (Fig. 2) whereas in literature reverse effects are described. The gene hv_36467 is a SAG12 like gene which is a senescence associated cystein protease and turned out to be up-regulated during natural leaf senescence in barley [69] and during dark induced senescence in tobacco [70]. In *Arabidopsis thaliana* the gene NFYA5 similar to pHvNF-Y5α was analysed by microarrays showing that the expression of this gene was induced by drought stress and ABA treatments [71], as well as under nitrogen stress [72]. Our data indicate a specific regulation of these two genes under different conditions. The protein encoded by the glutamine synthetase 2 (GSII) gene was found in photosynthetic tissues where its main role is the re-assimilation of photorespiratory ammonia [73, 74]. During senescence, the activity of GSII decreased representing down-regulation of associated genes in rice [73], barley and wheat [75] which was confirmed in the present study (Fig. 2). With chlorophyll degradation during leaf senescence the light harvesting complexes (LHC) of PSI and PSII remain stable, but synthesis rates of apoproteins of LHC decrease early in senescence [76]. In the present study LHC1b20 was down-regulated for most genotypes during drought stress

Table 3 Coefficients of correlation for relative expression of the selected genes and the relative SPAD values

		A1	Dhn1	GAD3	NADP_ME	P5CS2	Contig7437	GSII	hv_36467	LHC1b20	pHvNF-Y5a	AVP1	ETFQO	SAPK9	TRIUR3
	Rel. SPAD	0.09	0.02	−0.10	0.01	0	−0.16*	0.24**	−0.13	0.19*	0.34***	0.16*	0.09	−0.15	0.15*
Drought stress genes	A1		0.68***	0.68***	0.44***	0.76***	0.38***	0.15	0.10	−0.16	−0.12	0.14	0.18*	0.37**	−0.11
	Dhn1			0.73***	0.35**	0.72***	0.64***	0.08	0.26**	−0.17*	−0.11	0.12	0.15	0.30*	−0.18*
	GAD3				0.43***	0.84***	0.65***	0	0.17*	−0.31***	−0.28***	0.09	0.20*	0.34**	−0.34***
	NADP_ME					0.49***	0.15	0.29*	0.15	−0.01	0.10	0.27*	0.24*	0.22	0.25*
	P5CS2						0.50***	0.17*	0.13	−0.19*	−0.09	0.10	0.18*	0.40**	−0.18*
Leaf senescence genes	Contig7437							−0.17*	0.45***	−0.24**	−0.35***	0.18*	0.16*	0.21	−0.25**
	GSII								0.09	0.55***	0.64***	0.47***	0.53***	0.18	0.44***
	hv_36467									0.19*	−0.09	0.15	0.30***	0.03	0.01
	LHC1b20										0.49***	0.38***	0.39***	0.10	0.39*
	pHvNF-Y5a											0.42***	0.28***	−0.26*	0.41***
Genes out of GWAS[a]	AVP1												0.46***	0.22	0.54***
	ETFQO													0.17*	0.35*
	SAPK9														0.06

r is significant with *p <0.05, **p <0.01 and ***p <0.001

[a]Genes coding for proteins identified by BlastX of significant marker sequences out of a previous genome wide association study (GWAS) by Wehner et al. [20]

Table 4 Significant marker gene expression associations (p <0.001) with positions of eQTL

	Gene (log ΔΔCt)	Marker[b]	Chr.[b]	Pos. in cM[b]	*F* value	*p* value	-log p (LOD)	R^2 in %
Drought stress genes	A1	SCRI_RS_134358	5H	149.9	7.45	8.86E-04	3.05	9.5
	A1	SCRI_RS_165400	5H	150.1	7.45	8.86E-04	3.05	9.5
	GAD3	BOPA2_12_31177	1H	38.0	7.81	6.03E-04	3.22	8.9
	GAD3	BOPA1_4403-885	3H	142.1	12.09	6.67E-04	3.18	6.9
	P5CS2	BOPA1_4403-885	3H	142.1	11.31	9.84E-04	3.40	7.5
Leaf senescence genes	Contig7437	BOPA1_4403-885	3H	142.1	7.36	9.05E-04	3.01	7.1
	GSII	BOPA2_12_30065	7H	40.4	11.36	9.60E-04	3.04	9.5
	hv_36467	BOPA1_6547-1363	1H	111.8	8.11	4.58E-04	3.02	7.9
	hv_36467	BOPA2_12_31461	2H	131.9	13.14	4.00E-04	3.34	11.2
	pHvNF-Y5a	SCRI_RS_152393	6H	64.4	11.48	9.09E-04	3.04	7.8
	pHvNF-Y5a	SCRI_RS_194841	7H	81.5	12.91	4.49E-04	3.35	8.7
Genes out of GWAS[a]	AVP1	SCRI_RS_140294	5H	62.5	13.46	3.42E-04	3.47	9.1
	ETFQO	BOPA1_10126-999	3H	53.3	7.44	8.37E-04	3.08	10.1
	ETFQO	SCRI_RS_181376	5H	143.1	8.34	3.86E-04	3.41	11.5
	TRIUR3	BOPA1_4392-450	5H	44.5	7.64	7.07E-04	3.15	9.9
	TRIUR3	BOPA2_12_30717	5H	44.5	7.64	7.07E-04	3.15	9.9
	TRIUR3	SCRI_RS_41519	5H	44.5	7.64	7.07E-04	3.15	9.9
	TRIUR3	SCRI_RS_161614	5H	139.7	15.17	1.51E-04	3.82	9.8

[a]Genes coding for proteins identified by BlastX of significant marker sequences out of a previous genome wide association study (GWAS) by Wehner et al. [20]
[b]Marker positions are based on Comadran et al. [101]

Table 5 Positions of the selected genes based on the barley Morex-contigs and their mode of action

	Gene	POPSEQ[b,c]	Chr.[b]	cM[b]	Identity in %[c]	eQTL[d]
Drought stress genes	A1	morex_contig_38178	5H	156.9	76	*cis*
	GAD3	morex_contig_790741	1H	42.0	81	*cis*
	GAD3	morex_contig_135241	3H	147.0	75	*cis*
	P5CS2	morex_contig_2549060	3H	30.2	76	*trans*
Leaf senescence genes	Contig7437	morex_contig_47765	4H	54.3	94	*trans*
	GSII	morex_contig_274546	7H	70.8	92	*trans*
	hv_36467	morex_contig_138818	1H	132.4	91	*trans*
	hv_36467	morex_contig_458133	2H	58.0	81	*trans*
	pHvNF-Y5a	morex_contig_244610	6H	76.0	100	*trans*
	pHvNF-Y5a	morex_contig_60611	7H	70.8	95	*cis*
Genes out of GWAS[a]	AVP1	morex_contig_80803	5H	44.1	75	*trans*
	ETFQO	morex_contig_6218	3H	51.8	95	*cis*
	ETFQO	morex_contig_1570014	5H	152.4	100	*cis*
	TRIUR3	morex_contig_81592	5H	42.0	88	*cis*
	TRIUR3	morex_contig_160473	5H	129.9	71	*cis*

[a]Genes coding for proteins identified by BlastX of significant marker sequences out of a previous genome wide association study (GWAS) by Wehner et al. [20]
[b]Gene positions are based on POPSEQ map (ibsc 2012)
[c]Morex contigs and identity comes out Blastn of the gene sequences against the Morex genome (ibsc 2012)
[d]*cis* eQTL coincide with the location of the underlying gene (position <10 cM), whereas *trans* eQTL are located in other regions of the genome Druka et al. [11]

induced leaf senescence in juvenile barley (Fig. 2) which was also shown in rice [77] and barley [78, 79] for natural leaf senescence in the generative stage.

In this study, all five selected drought stress genes were up-regulated under drought stress (Fig. 2) according to literature which demonstrates a clear drought stress answer and a good experimental setup for detecting and analysing drought stress response. In contrast, four out of the five selected genes for leaf senescence were down-regulated (Fig. 2) because a few of these genes are involved in photosynthesis and chloroplast development. Results for three of these genes (Contig7437, GSII and LHC1b20) were in accordance with results known from literature, while this was not the case for two of them (hv_36467 and pHvNF-Y5α). However, for all of these genes the adverse effect was detected for some genotypes (Fig. 2). Results revealed that drought stress in early developmental stages of barley leads to premature induced leaf senescence as already observed by physiological parameters [20] and by expression analysis of drought stress and leaf senescence related genes in this study.

Expression differences in three genes (GAD3, P5CS2 and Contig7437) were significantly associated to barley chromosome 3H at 142.1 cM (Table 4). At this position also quantitative trait loci (QTL) were found for drought stress [20, 80] as well as for leaf senescence [81]. These facts and the high correlation of these genes (Table 3) make this eQTL very interesting for marker assisted breeding in barley.

Genes out of GWAS

To verify the QTL identified for drought stress and drought stress induced leaf senescence by Wehner et al. [20] an expression profile and eQTL analysis was conducted with genes coding for proteins identified within respective QTL. The genes ETFQO, SAPK9, TRIUR3 and AVP1 were differentially expressed (Fig. 2).

The protein encoded by the electron transfer flavoprotein-ubiquinone oxidoreductase gene (ETFQO) is located in the mitochondria where it accepts electrons from ETF, transfers them to ubiquinone and acts downstream in the degradation of chlorophyll during leaf senescence [82, 83]. Expression studies showed that ETFQO is up-regulated under darkness induced leaf senescence [83, 84] whereas in this study on drought stress induced leaf senescence no clear direction was observed (Fig. 2). A gene coding for a serine/threonine-protein kinase (SAPK9) was analysed which can be activated by hyperosmotic stress and ABA in rice [85]. In the present study SAPK9 was down-regulated in most genotypes (Fig. 2). Furthermore, the abscisic acid-inducible protein kinase gene (TRIUR3) which is also involved in dehydration stress response [86] was differentially expressed. Until now, no relative expression analysis has been conducted for this gene, but a huge amount of ABA inducible genes are up-regulated under drought stress in rice [87]. In the present study TRIUR3 was also up-regulated under drought stress (Fig. 2). The nucleotide pyrophosphatase/phosphodiesterase gene (AVP1) is a gene which is up-regulated under drought stress [88] which was confirmed in the current study (Fig. 2). Expression of this gene was also observed in transgenics showing a higher drought stress tolerance [89–92].

Three of these genes (SAPK9, TRIUR3 and AVP1) were located within the QTL on barley chromosome 5H at 45 cM [20]. Furthermore, expression differences of two of them (TRIUR3 and AVP1) were again associated to markers on chromosome 5H around 45 cM (Table 4) and this position was also validated in the Morex genome (Table 5). A high and significant correlation between the relative expression data of both genes as well as to the relative SPAD values (Table 3) promotes this finding. At the same position on chromosome 5H two markers which turned out to be significantly associated to SPAD and biomass yield under drought stress treatment were identified [20]. So, these results [20] and those of this study give hint that the two SNP markers, i.e. BOPA1_9766-787 and SCRI_RS_102075 may be used in marker based selection procedures in barley breeding programmes aiming at the improvement of drought stress tolerance.

For the understanding of complex mechanisms, such as the process of drought stress tolerance and drought stress induced leaf senescence as a basis for future breeding activities it is of prime importance to understand how and when regulatory genes are activated and where they are located in the barley genome. Results of this study contribute to elucidate the regulation of drought stress induced leaf senescence during early developmental stages in barley. The present genetical genomics approach helps to localize and understand transcriptional regulation and gene interaction, both from *cis*-acting elements and *trans*-acting factors (Table 5). When analysing the expression regulation of the barley genome, *cis* eQTL were found for the genes A1, GAD3, pHvNF-Y5α, ETFQO and TRIUR3. Markers which were significantly associated to *cis* eQTL explained up to 11.55 % of the transcriptional variance (Table 4 and Table 5). Therefore, most of the strongest eQTL acted in *cis* which was also observed in previous eQTL studies [8, 93, 94].

Factors that act in *trans* regulating the expression levels of the genes of interest were mainly found for the group of leaf senescence genes. Some of these genes are described as SAGs (Contig7437, hv_36467 and pHvNF-Y5α), because up to now little is known about their function. Results of the present study give hint that these SAGs are regulated in *trans*.

Conclusion

With respect to the expression of genes involved in drought stress response and early leaf senescence

genotypic differences exist in barley. Major eQTL for the expression of these genes are located on barley chromosome 3H and 5H. The eQTL on chromosome 5H coincides with the QTL for drought stress induced leaf senescence identified in a previous GWAS [43]. Respective markers, i.e. BOPA1_9766-787 and SCRI_RS_102075 may be used in future barley breeding programmes for improving tolerance to drought stress and early leaf senescence, respectively.

Methods

Plant material and phenotypic characterisation

Phenotyping, genotyping and QTL analysis were conducted as described in Wehner et al. [20] on a set of 156 winter barley genotypes consisting of 113 German winter barley cultivars (49 two-rowed and 64 six-rowed, [95]) and 43 accessions of the spanish barley core collection (SBCC) [96]. The same set of genotypes as well as the same experimental design was used for expression- and eQTL analysis in the present study. In brief, trials were conducted in greenhouses of the Julius Kühn-Institut in Groß Lüsewitz, Germany and drought stress was applied in a split plot design with three replications per genotype and treatment (control, drought stress). In each pot four plants were sown and all leaves were tied up, except the primary leaf per plant. Drought stress was induced by a termination of watering at the primary leaf stage (BBCH 10, according to Stauss [51]) seven days after sowing (das). From this time drought stress developed slowly till 20 das when the final drought stress level was reached. The drought stress variant was kept at 20 % of the maximal soil water capacity and the control variant at 70 % by weighing the pots resulting in a relative water content (36 das) ranging between 88.8 % and 91.5 % in the control variant and 80.9 % and 86.1 % in the drought stress treatment. The experimental setup and growth conditions for these pot experiment are described in detail as design B in Wehner et al. [20].

At 26 das (BBCH 25) leaf material for RNA extraction was sampled by harvesting one primary leaf per pot taking the middle part for further analyses. Mixed samples out of the three leaf pieces (circa 100 mg) per genotype and treatment (312 samples) each were immediately frozen in liquid nitrogen and stored at −80 °C. Furthermore, to get information on the influence of drought stress on leaf senescence leaf colour (SPAD, Konica Minolta Chlorophyll Meter SPAD-502 Plus, Osaka Japan) was measured 27 das on three primary leaves per pot at five positions each.

RNA isolation and cDNA synthesis

The frozen primary leaves were homogenized with a tube pestle (Biozym) in liquid nitrogen. Total RNA from the primary leaves was isolated with the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular), using lysis solution RP and following the manufacturer's instructions. After incubation for 15 min at room temperature, an additional incubation for 3 min at 55 °C was conducted to get a higher RNA yield. Total RNA yield was measured by Qubit fluorometric quantification (Life technologies) and concentration was adjusted to 50 ng. RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. cDNA was stored at −20 °C.

Expression analysis using quantitative real-time PCR (qPCR)

A high throughput system (BioMark) was used for expression analysis in which four Fluidigm chips (96.96) were analysed for the 312 samples. Default space on these chips allows to analyse 48 genes in two technical replications. Out of these 48 analysed genes (23 genes involved in drought stress, 12 leaf senescence genes, 11 genes coding for proteins out of a previous GWAS [20] and two reference genes), 14 differentially expressed genes revealing clear differences between genotypes and showing a low number of missing values were selected for the present study. Five of these genes were involved in leaf senescence, five in drought stress response and four genes coding for proteins related to leaf senescence or drought stress out of the previous genome wide association study [20] were chosen. In addition, as a reference gene GAPDH was included (Table 1). To identify the gene for those proteins identified in the GWAS studies by Wehner et al. [20] the significant associated marker sequences were compared to the plant nucleotide collection by Blastn (Basic Local Alignment Search Tool, ncbi [www.ncbi.nlm.nih.gov] accessed June 2014) and the gene with the best hit was chosen for primer design.

Primers (Eurofins HPSF purified) were constructed using the primer designing tool of NCBI ([www.ncbi.nlm.nih.gov/tools/primer-blast] accessed June 2014) with a length of 20 bp, annealing temperature of 59 °C and product size of 100–200 bp (Table 1).

qPCR was performed using the high throughput platform BioMark HD System and the 96.96 Dynamic Array IFC (Fluidigm) following the manufacturer's instructions. 5 μl Fluidigm sample premix consisted of 1.25 μl preamplified cDNA, 0.25 μl of 20x DNA binding dye sample loading reagent (Fluidigm), 2.5 μl of SsoFast EvaGreen Supermix with low ROX (BioRad) and 1 μl of RNase/DNase-free water. Each 5 μl assay premix consisted of 2 μl of 100 μM primers, 2.5 μl assay loading reagent (Fluidigm) and 0.5 μl RNase/DNase-free water. Thermal conditions for qPCR were: 95 °C for 60 s, 30 cycles of 96 °C for 5 s, 60 °C for 20 s plus melting curve analysis. Data were processed using BioMark Real-Time PCR Analysis Software 3.0.2 (Fluidigm). The quality threshold was set at the

default setting of 0.65 and linear baseline correction and automatic cycle threshold method were used.

Data analysis

The analysis software (Fluidigm Real- Time PCR Analysis Software) gave cycle threshold (Ct) values and calculated ΔCt values, as well as a quality score for each amplification. Out of these ΔCt values calculated out of the Ct value of the gene of interest minus the Ct value of the housekeeping gene (GAPDH) for each genotype, treatment and replication, the relative expression (ΔΔCt) was calculated out of the ΔCt values for stress treatment minus the ΔCt values for control treatment for each genotype and replication [97]. ΔΔCt values without correction of PCR efficiency were used for calculation, because genes were tested and selected by their efficiency in preliminary experiments. A mean PCR efficiency (Quality Score of Fluidigm) was calculated for all amplifications.

Shapiro-Wilk test for normal distribution and analysis of variance (ANOVA) using a linear model were carried out using R 2.15.1 [98] to test effects of genotype (using ΔΔCt values) and treatment (using ΔCt values). Furthermore, coefficients of correlation (Spearman) were calculated in R between relative expression of the genes and the relative SPAD values [20, 99]. Moreover, for the SPAD values an ANOVA mixed linear model (MLM) was calculated (replication as random) in R to test effects of genotype, treatment and interaction of genotype and treatment. For relative expression as well as for the SPAD values box whisker plots were calculated in R.

Expression quantitative trait loci (eQTL) analysis

For the 14 selected genes a genome wide association study (GWAS) for eQTL detection was conducted on the 156 genotypes applying a mixed linear model (MLM) using TASSEL 3.0 [100]. For this purpose a genetic map with 3,212 polymorphic SNP markers with minor allele frequencies larger than 5 % [101], a population structure calculated with STRUCTURE 2.3.4 [102] based on 51 simple sequence repeat (SSR) markers covering the whole genome, a kinship calculated with SPAGeDi 1.3d [103] based on 51 SSRs and the relative expression data (means for replications) were used. For comparability the methods were the same as used for GWAS in Wehner et al. [20]. All results with p values <0.001 (likelihood of odds, LOD = 3) were considered as significant marker gene expression associations.

To compare genomic positions of the eQTL with those of the analysed genes, sequences of the genes were compared against high confidential genes (CDS sequences) of the barley Morex genome by Blastn (Basic Local Alignment Search Tool, IPK Barley Blast server [http://webblast.ipk-gatersleben.de/barley/viroblast.php] accessed May 2015) and the Morex contig with the highest identity on the associated linkage group (chromosome) was chosen. With this information eQTL were divided in *cis* and *trans* eQTL. *cis* eQTL coincide with the location of the underlying gene (position <10 cM), whereas *trans* eQTL are located in other regions of the genome [11].

Additional file

Additional file 1: Relative expression of the 14 genes with mean quality scores for each amplification. [a]SBCC: spanish barley core collection. [b]GWAS: genome wide association study. (XLSX 111 kb)

Abbreviations

ΔΔCt: relative expression; ABA: abscisic acid; Blast: Basic Local Alignment Search Tool; Ct: cycle threshold; das: days after sowing; e.g: for example; eQTL: expression quantitative trait locus/loci; GWAS: genome wide association study; i.e: id est; LEA: late embryogenesis abundant protein; LOD: likelihood of odds; MLM: mixed linear model; PCR: polymerase chain reaction; qPCR: quantitative real-time polymerase chain reaction; QTL: quantitative trait locus/loci; ROS: reactive oxygen species; SAG: senescence associated genes; SBCC: Spanish Barley Core Collection; SNP: single nucleotide polymorphism; SPAD: soil plant analysis development; measurement of chlorophyll content by colour; SSR: single sequence repeat.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GW conducted all experiments, including expression, statistical and bioinformatics analyses and mainly wrote the manuscript. EZ provided the Fluidigm BioMark System and supervised the gene expression experiments. CB, KH and FO designed the research, supervised the experimental design and participated in writing the manuscript. All authors approved the final manuscript.

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3 GENERAL DISCUSSION

The determined physiological traits turned out to be suitable to assess drought stress response and early leaf senescence in the experimental design described. Based on these, 47 QTL involved in drought stress response and 15 eQTL were identified by GWAS. Thereof, two interesting SNP markers, i.e. BOPA1_9766-787 and SCRI_RS_102075 were detected on the barley chromosome 5H at 45 cM, which may be used in barley breeding for marker based selection concerning drought stress.

The following chapter aims at discussing aspects of experiments which are not covered in detail in the published papers.

3.1 DROUGHT STRESS EXPERIMENTS

Depending on the research aim, different systems can be used for drought stress application, all differing in their possibilities to control the environment (Poorter et al. 2012). Growth chamber experiments allow for the replication of experiments independent of environmental conditions multiple times per year. The environment can be optimised and controlled, resulting in a high reproducibility of experiments (Poorter et al. 2012). In greenhouse facilities, conditions are only partially controlled and thus, they depend to some extent on environmental conditions. Whereas temperature and lighting can be regulated, day length and light intensity are factors depending on outside conditions and are influencing plant performance. Within both facilities, drought stress can be applied through a stop of watering in pot experiments or through the use of stress inducing solutions, e.g. polyethylene glycol (PEG) in hydro cultures (Blum 1989) or even in *in vitro* cultures. With both methods, a defined stress level can be achieved. However, it is easier to preserve a continuous stress level with hydroponics. While in a hydroponic system, it is easy to analyse roots, the disadvantage of this system is a lack of oxygen availability for roots even with ventilation (Mexal et al. 1975; Munns et al. 2010), which makes conditions incommensurable to the field. To fast phenotype for drought stress, single leaves or leaf pieces can be stressed as well, e.g. with PEG (Ibarra-Caballero et al. 1988; Trotel et al. 1996). Unfortunately, results of these analyses are often not comparable to the whole plant response (Anyia et al. 2007; Balvanera et al. 2006).

In general, watering is conducted manually in these experiments, whereas newly developed systems, e.g. Lemna tec allow to regulate watering automatically (Eberius and Lima-Guerra 2009) and plants are moving through the greenhouse on conveyor belts which optimises lighting. Another advantage of this system is the measurement of parameters during rotation in the greenhouse. For example, the whole plant leaf area as well as leaf colour and chlorophyll fluorescence can be measured automatically with camera systems over the course of time (Chen et al. 2014; Honsdorf et al. 2014; Neumann et al. 2015).

In the field, drought can be initiated under natural conditions in locations with low rainfall or by withholding water through rainout shelters (Yahdjian and Sala 2002). These are foil tunnels or roofs which protect field trails against precipitation. On the one hand, plants grow under nearly natural conditions with all abiotic and biotic factors except rainfall, but on the other hand, under stationary rainout shelters effects such as higher temperatures and lower radiation occur (Fay et al. 2000; Poorter et al. 2012; Yahdjian and Sala 2002), which influence plant development and yield. Therefore, movable shelters have been developed to minimise environmental effects (Chauhan et al. 1997; Dugas and Upchurch 1984).

For the present studies on barley, greenhouse experiments were chosen as a compromise between climate chambers and field. Thus, all experiments conducted for this thesis were accomplished in semi controlled greenhouse facilities. In contrast to field experiments, temperature, as well as lighting, can be controlled under these conditions to a limited extent. Nevertheless, annual and seasonal variation had an influence on the experiments and the growth of plants. To account for annual effects, experiments were replicated in several years. Seasonal variations were partially excluded by conducting the three years pot trails once a year under comparable conditions regarding day length. Pot experiments were replicated in three (autumn), or two years (spring) respectively, therefore. Autumn pot experiments (experimental design A) were conducted for the drought stress parameters biomass yield (BY), osmolality (OA), content of free proline (CFP) and total content of soluble sugars (CSS), whereas spring pot experiments (experimental design B) were adapted to determine the leaf senescence parameters leaf colour (SPAD), electron transport rate at PSII (ETR) (Chapter 2.1 and 2.2) and BY for comparison (Table 1). Whereas temperature was balanced relatively well between the experiments, differences in light intensity were higher especially in experiments conducted in spring (Table 1). For example, the spring pot experiment in 2013 showed high light intensity and consequently biomass production was higher in this experiment

compared to the other experiments (Table 1). Due to the fact that measurements and analyses of physiological parameters were done on primary leaves, lighting conditions within the plant stand is an important factor. Within greenhouse trials, guaranteeing equal light perception at the primary leaves is difficult, as plants on greenhouse benches are shading each other and the position of the plants on the benches have a large influence on continuous lighting. Moving around the benches, this effect was minimised in our experiments. However, differences in growth between well-watered and drought stressed plants strongly influenced lighting on the primary leaves. Thus, light deficiency induced leaf senescence in the control variant overlapped with drought stress induced leaf senescence on the primary leaf. Therefore, all leaves except the primary leaf per plant were tied up to reduce shading on the primary leaf at which the measurement of leaf senescence parameters in spring pot experiments was conducted (Chapter 2.1).

TABLE 1: Temperature and light intensity of the greenhouse trials and biomass yield in the control treatment.

Parameters		2011 Autumn	2012 Autumn	2013 Autumn	2013 Spring	2014 Spring
Outside temperature in °C	Mean	11.4	10.5	10	-0.6	7
	Min	-0.5	1.7	-1.2	-14.6	-3.4
	Max	22.4	20.9	18	9.4	18.5
Inside temperature in °C	Mean	21.7	21.2	21.3	20.5	21
	Min	17.6	17.8	17.9	17.6	18
	Max	31.1	27.8	30.7	26.7	26.1
Outside light intensity in klx	Mean	6.2	5.1	4.5	8.3	6.6
	Min	0	0	0	0	0
	Max	46.3	46.1	46.1	54.6	46.5
Biomass yield (dry weight) in g	Mean	13.6	9.2	9.3	21.4	10.7
	Min	9.5	4.9	4.5	19.0	7.3
	Max	18.2	12.6	13.6	25.8	13.7

For the drought application, watering was stopped until a defined stress level for the detection of drought stress response and drought stress induced leaf senescence was reached. Stress intensity was set to 20% of the maximum water capacity of the soil according to primary experiments and literature on severe drought conditions for barley (Guo et al. 2009; Ivandic et al. 2000; Samarah 2005). For a four weeks stress phase, the stress level was maintained through manual watering.

Results of this thesis concerning phenotyping and QTL detection are based on the experimental design in the greenhouse. Between different systems, such as field trails or climate chamber experiments results and especially QTL regions can differ strongly. Therefore, in order to get information on the transferability of these results a subset of the most tolerant and non-tolerant genotypes may be analysed in field trials in rainout shelters.

Additionally, different developmental stages can be analysed in each of the systems described above. Most of the barley yield is defined by the conditions during the generative stages and thus, many studies on drought stress are focused on stress during flowering and grain filling (Blum 2005; Guo et al. 2008; Samarah 2005; Varshney et al. 2012). However, drought periods can also occur in autumn and early spring during the juvenile stage and can lead to yield losses (El Hafid et al. 1998; Rosenzweig et al. 2001). Moreover, detection of molecular markers for drought stress response and stay green traits in early developmental stages is an advantage in the time consuming breeding procedures (Korzun 2002; Richards 1996). Therefore, early developmental stages were analysed in this study. Here, differences between drought stress reactions of different genotypes and different traits can be identified as the basis of the development of molecular markers (Table 1 and Table 2, Chapter 2.2). For comparison to other developmental stages, further experiments should be conducted till the generative stage.

3.2 PHYSIOLOGICAL PARAMETERS

Already in early developmental stages of barley, physiological parameters indicated a differential drought stress response for the set of genotypes analysed (Table 1 and Table 2, Chapter 2.2). Therefore, these parameters are suitable for phenotyping drought tolerance and early leaf senescence. By analysing osmo-protectants, i.e. soluble sugars (CSS) and free proline (CFP), as well as the overall osmolality (OA), the plant response was documented regarding cell metabolism and turgor maintenance under drought (Blum 1989; Delauney and Verma 1993; Teulat et al. 2001). Furthermore, limited growth under drought was determined by investigating the aboveground biomass yield (BY). In addition, a decrease in chlorophyll and photosynthesis activity under drought stress induced leaf senescence was observed through the measurement of leaf colour (SPAD) and the electron transport rate at PSII (ETR) (Table 1, Chapter 2.2). In this respect, the above ground biomass and the leaf colour turned out to be of special importance

because of the high heritability, the significant correlation of both traits and the number of QTL detected (Table 1, Table 3 and Figure 2, Chapter 2.2).

As in juvenile stages grain yield cannot be analysed, total biomass yield (BY) was measured at the end of the experiments instead. BY was regarded as the total biomass above ground in dry weight at 36 das (days after sowing) on juvenile barley plants (BBCH 36). Plants which were able to generate high biomass yield under drought stress periods may have metabolic advantages and may save resources for grain yield development. It has been observed that vegetative biomass is highly correlated to yield parameters in terminal stages in barley and wheat (Boukerrou and Rasmusson 1990; Saleem 2003). Nevertheless, biomass is influenced to a larger extent by drought stress in juvenile stages, whereas in terminal drought stress this effect is not so pronounced (Jamieson et al. 1995). Moreover, the less biomass is influenced by drought, the more the reduction is compensable by growth during re-watering (Skirycz and Inze 2010). Therefore, minimising biomass losses through drought stress may be a major goal in plant breeding to optimise grain yield development, and so biomass above ground is a trait of interest while analysing drought response (Saleem 2003; Salekdeh et al. 2009).

Another physiological parameter of prime importance is the leaf colour (SPAD), representing the status of leaf senescence by indirect measurement of the chlorophyll content. To prove the relation between SPAD values and chlorophyll content, the chlorophyll content was analysed according to Arnon (1949) with samples of one spring pot experiment (experimental design B) and of 28 selected genotypes differing in leaf senescence. The aim was to calculate a regression curve, which can be used for a chlorophyll estimation of the pot experiments conducted within this thesis and for future experiments. For chlorophyll content, a clear significant decrease under drought stress was measured, which significantly correlated to the SPAD leaf colour with r=0.86 in both treatments. The best fit was represented by a quadratic regression following the formula:

$$Chl = 1.58 + \sqrt{-0.07\,SPAD + 2.92}$$

Whereas some studies figured out linear relationships (Champbell et al. 1990; Rodriguez and Miller 2000), results obtained on wheat, which is related to barley confirmed the quadratic regression (Cartelat et al. 2005). The high correlation indicates that leaf colour is a highly suitable trait to indirectly measure the chlorophyll content and thus, crucial for analysing stay green effects. Moreover, the measured leaf colour values turned out to be significantly correlated to the relative expression of most of the analysed genes for leaf senescence (Table 3, Chapter 2.3). Therefore, it may be concluded that SPAD values were suitable to represent the status of drought stress induced leaf senescence.

3.3 TOLERANCE RANKING

Analysing 156 genotypes (Additional file 1, Appendix), including German elite cultivars and selected genotypes of the Spanish Barley Core Collection (SBCC) a huge variation between genotypes was identified concerning drought stress response and drought stress induced leaf senescence (Table 2, Chapter 2.2). Combining phenotypic data (Chapter 2.2) and relative expression data (Chapter 2.3), a tolerance ranking was done to identify genotypes displaying delayed drought stress induced leaf senescence or drought stress response and ultimately, an increased drought stress tolerance.

The tolerance ranking of the 156 analysed genotypes was conducted based on the relative drought susceptibility index (DSI, Table 1, Chapter 2.2) of all six physiological parameters. Two of these parameters, BY and SPAD DSI values are shown in Figure 1A and Figure 1B. Genotypes revealing a DSI close to one are highly susceptible to drought and those close to zero or showing a negative value are tolerant (Fischer and Maurer 1978). Looking at all physiological traits, several tolerant and non-tolerant genotypes were detected. The same genotypes with values close to zero for all traits were Cosima, Candesse and some of the SBCC (SBCC 65, 80, 119, 136), which were classified as drought tolerant (Figure 1A, 1B). In contrast, Alraune, Tafeno, Trixi and SBCC 89 were classified as drought sensitive genotypes (Figure 1A, 1B).

In addition to the tolerance ranking with DSI, a tolerance ranking with the relative expression of the 14 selected genes for each genotype (Table 1, Chapter 2.3) was conducted as well. Expression data for two of these genes, AVP1 and TRIUR3 are displayed in Figure 1C and Figure 1D. Tolerant genotypes are characterised by a high expression relative to the control treatment and in contrast, sensitive genotypes are characterised by a low expression relative to the control, e.g. for those genes which were described to be up-regulated under drought stress, and the other way around for genes which were down-regulated under drought stress (Degenkolbe et al. 2009; Ermolayev et al. 2003). Also, with this ranking the most tolerant and non-tolerant genotypes were identified, which were the same for more than 10 out of the 14 genes analysed. Utilising this approach, Candesse, Grete, Anastasia, Birgit, as well as some genotypes out of the SBCC (28, 119 and 136) turned out to be drought tolerant, whereas Babylone and Petra were classified as sensitive (Figure 1C, 1D).

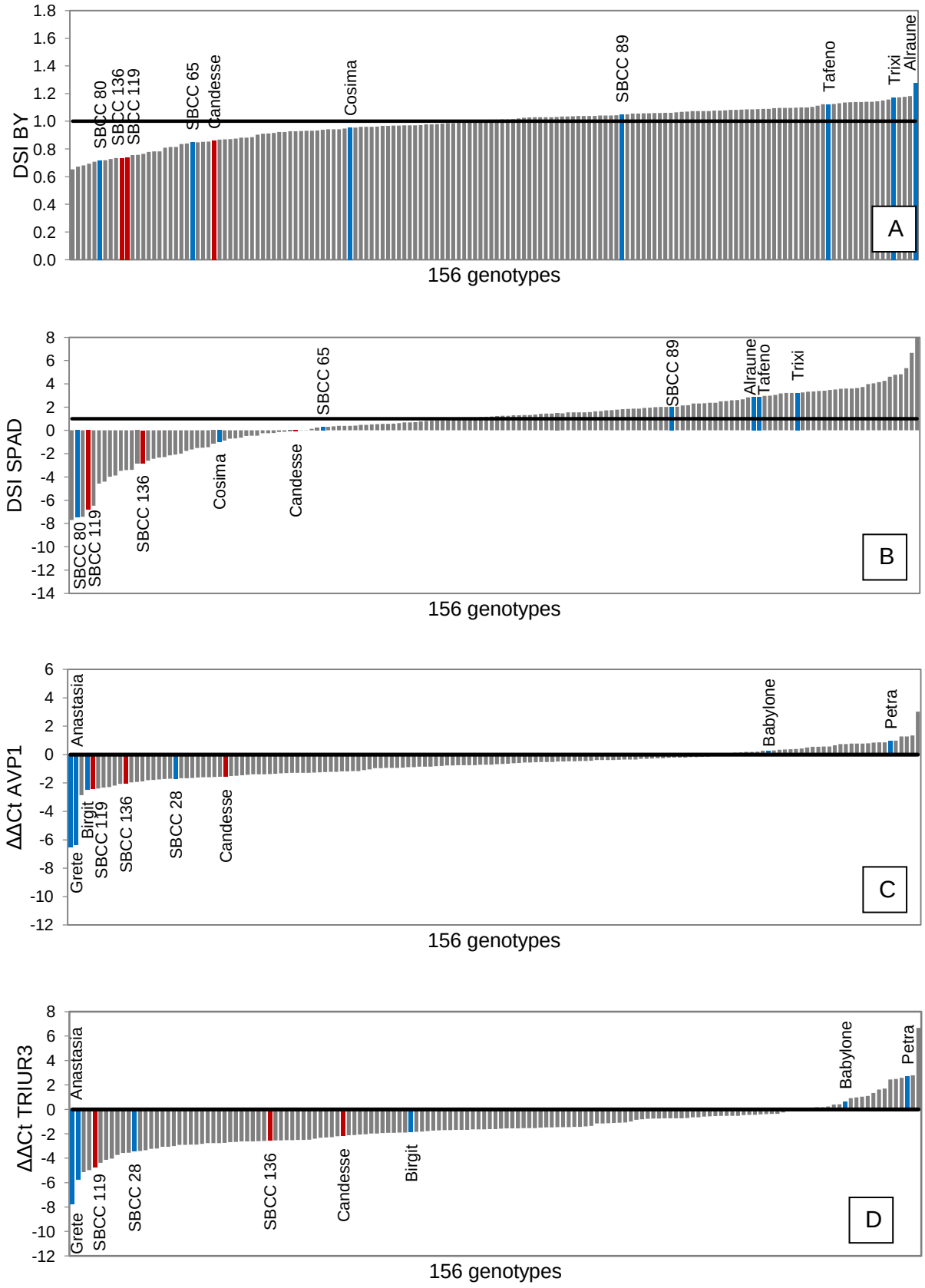

FIGURE 1: Tolerance ranking of phenotypic (exemplarily shown for DSI BY [A] and DSI SPAD [B]) and expression data (exemplarily shown for AVP1 [C] and TRIUR3 [D]). The drought tolerant and sensitive genotypes detected in one approach only are coloured blue, whereas genotypes highlighted red were found in both rankings.

Comparing the ranking with DSI, with this obtained on relative expression, three drought tolerant genotypes are the same in both rankings and were marked in red in Figure 1A and Figure 1B for the physiological ranking and in Figure 1C and Figure 1D for the ranking using the expression data (Figure 1). These genotypes are Candesse, SBCC 119 and SBCC 136 (Figure 1), which may be specially suited for future barley breeding concerning drought stress tolerance and leaf senescence. Thereof, Candesse is a six rowed German cultivar which is highly susceptible to net blotch (*Pyrenophtora teres f. teres*) (König et al. 2013). However, it is also described as high yielding in extreme weather conditions, e.g. heavy rainfall in autumn or heat and drought in spring and summer (Völkel 2003). The yield potential of the SBCC (28 - 35 dt/ha) is generally lower than that of the German cultivars (60 - 70 dt/ha) (DESTATIS 2015), but the two identified drought tolerant genotypes, SBCC 119 and SBCC 136 are described to reach a high yield (22.38 - 24.5 dt/ha) under drought stress conditions compared to other genotypes of the SBCC (Lasa 2008).

The best ranked genotypes showing over expression of drought tolerance genes may be used in further studies on the genetic basis of drought stress tolerance in barley. Furthermore, based on correlations of the relative leaf colour (DSI SPAD) representing changes in leaf senescence, to the relative expression of senescence associated genes (Table 3, Chapter 2.3) the best ranked genotypes may be used for further studies on leaf senescence in barley.

3.4 THE SPANISH BARLEY CORE COLLECTION (SBCC)

Before the emergence of modern breeding, diversity in barley with respect to tolerance to drought and other abiotic stresses used to be higher (Ullrich 2011; Zamir 2001). Therefore, this diversity may still exist in landraces derived from geographical regions, where these traits are of prime importance. Therefore, genotypes of the SBCC (Igartua et al. 1998) were included in the present study. For barley breeding purposes, the SBCC is of special interest as this germplasm collection consists of old barley accessions and landraces collected in Spain prior to the emergence of modern barley breeding. Thus, the genetic diversity is much higher and lost features such as abiotic stress tolerances or biotic stress resistances may have been preserved. During the 1980s, the SBCC was created containing 175 barley lines which were developed from 2000 barley accessions collected in Spain. This core collection was further on genotyped (Silvar et al. 2011b) and

phenotyped for vegetative plant parameters, plant diseases and yield as well as yield under abiotic stress (Lasa et al. 2001; Silvar et al. 2010; Silvar et al. 2011a). All information on the SBCC is summarised online at: http://161.111.227.80/EEAD/barley/index.php?lng=1.

In every pot experiment, differences of the SBCC to the German cultivars were detected. A huge amount of genotypes of the SBCC was classified as drought tolerant genotypes concerning the relative phenotypic (DSI) and expression data (Figure 1). In comparison to the German cultivars, differences in the drought stress reaction were detected especially for SPAD and ETR represented by negative relative values (Table 1, Chapter 2.2 and Figure 1A, 1B). For these traits, unexpected higher values were found in the drought stress treatment. In comparison to the control treatment, delayed leaf senescence could be observed, while primary leaves stayed green. This fact is supported by allelic differences, which were found in the highest significantly associated markers and markers explaining the highest phenotypic variance for respective traits between genotypes of the SBCC and the German cultivars (Additional file 2, Appendix). This can also be observed when taking into account all SNP in a principal component analysis (PCA) in which most of the SBCC represent an own subpopulation of the set of analysed genotypes separated from the German cultivars by the principal component 2 (Figure 2). Furthermore, the German cultivars were separated by the principal component 1 in two-rowed and six-rowed genotypes (Figure 2). Thus, the PCA subdivided the analysed set of winter barley genotypes in two-rowed and six-rowed genotypes as well as accessions of the SBCC. All genotypes of the SBCC are six–rowed, except for SBCC 148, which is grouped closer to the German two-rowed genotypes (Additional file 1, Appendix and Figure 2).

As other studies show (Hamblin et al. 2010; Rostoks et al. 2006), components of the population structure differentiate depending on origin, row number and growth habit (spring, winter). The linkage disequilibrium is highly dependent on population structure. While the geographic region explains the highest percentage of molecular diversity (Malysheva-Otto et al. 2006), the row number and diversity of the set influences the LD, too. The effect of the row number was analysed separately for two- (10.75 cM) and six-rowed barley (8.24 cM), each showing higher LD values compared to the whole set (7.35 cM) (Rode et al. 2012). A high LD was also calculated for winter barley genotypes (7.35 cM) (Rode et al. 2012) and spring barley genotypes (20 cM), which consists only of six-rowed barley genotypes (Kraakman et al. 2006). The diverse set in the present study, including six-rowed and two-rowed German cultivars in addition to genotypes of the

SBCC, revealed a lower LD of 2.52 cM (Chapter 2.2), which does not only result in a higher resolution, but also the need for a higher marker density in comparison to the study of Rode et al. (2012), which was based on 1536 markers.

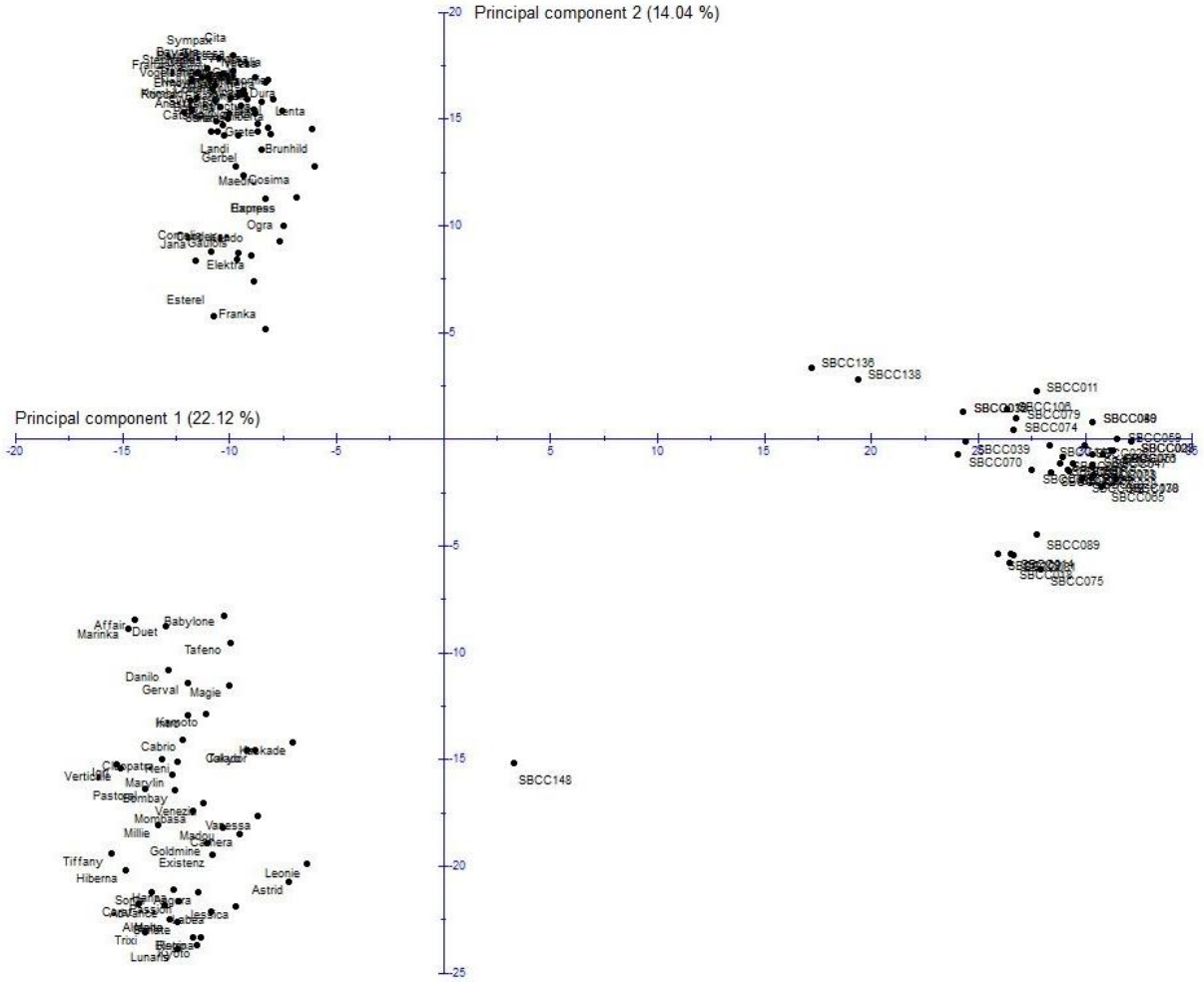

FIGURE 2: Principal component analysis (PCA) of the set of analysed genotypes utilising the dissimilarity matrix calculated out of the SNP marker set.

3.5 QUANTITATIVE TRAIT LOCI (QTL)

Genome wide association studies (GWAS) were conducted applying phenotypic data for drought stress response and leaf senescence, as well as relative expression data of genes differentially regulated by drought stress or leaf senescence. A lot of studies were carried out, e.g. in barley and wheat on the identification of quantitative trait loci (QTL) involved in the response to different abiotic stresses (Ashraf 2010; Cattivelli et al. 2008;

Nevo and Chen 2010). A huge amount of respective QTL were published but most of them have not been validated until now (Salvi and Tuberosa 2015). In the present study, both QTL (Chapter 2.2) and accompanying expression quantitative trait loci (eQTL, Chapter 2.3) involved in drought stress tolerance and drought stress induced leaf senescence were analysed. With the phenotypic data of the six physiological traits, 47 QTL, which were distributed over all barley chromosomes were observed under stress conditions for all physiological traits except content of free proline (CFP) (Table 4, Chapter 2.2 and Figure 3). In comparison, 15 eQTL were found for the relative expression of 10 out of the 14 differentially expressed genes analysed, which were located on all barley chromosomes except chromosome 4H (Table 4, Chapter 2.3 and Figure 3). Three of these 10 genes were drought stress related genes, four genes were involved in leaf senescence and three were genes coding for proteins detected in GWAS (Table 1, Chapter 2.3). Interestingly, most eQTL were located close to QTL, except GAD3 on barley chromosome 1H (Figure 3).

The eQTL for the relative expression of the genes coding for proteins identified was based on QTL analyses of physiological traits, which were mostly located close to the respective QTL. For instance, the eQTL for the relative expression of TRIUR 3, which is a gene identified out of a marker associated to SPAD was located close to the QTL for SPAD on chromosome 5H at 45 cM (Figure 3). TRIUR3 is described as a gene coding for abscisic acid- inducible protein kinase which is involved in dehydration stress response (Anderberg and Walker-Simmons 1992). However, abscisic acid as a phytohormone also promotes chlorophyll breakdown and leads to leaf senescence (Lim et al. 2007). Thus, a connection to the QTL for SPAD as a characteristic of leaf senescence is provided. The same holds true for AVP1, which was identified out of the marker associated to BY, and the eQTL for the relative expression of this gene was located near to the QTL for BY on 5H at 60 cM (Figure 3). AVP1 is described as a homolog to the HVP1 gene in barley on chromosome 7H (Shavrukov 2014), encoding for a vacuolar pyrophosphatase which regulates auxin- mediated developmental processes (Schilling et al. 2013). The phytohormone auxin regulates plant growth and thus, a link to the QTL for biomass production is provided. The eQTL for the relative expression of ETFQO was located near to the QTL for OA on chromosome 3H and to the QTL for OA and BY on chromosome 5H (Figure 3). Hence, the gene for an electron transfer flavoprotein- ubiquinone oxidoreductase (ETFQO) is described to be up-regulated during dark-induced leaf senescence and active in chlorophyll breakdown (Araújo et al. 2010), there is no direct link to the QTL for OA or BY.

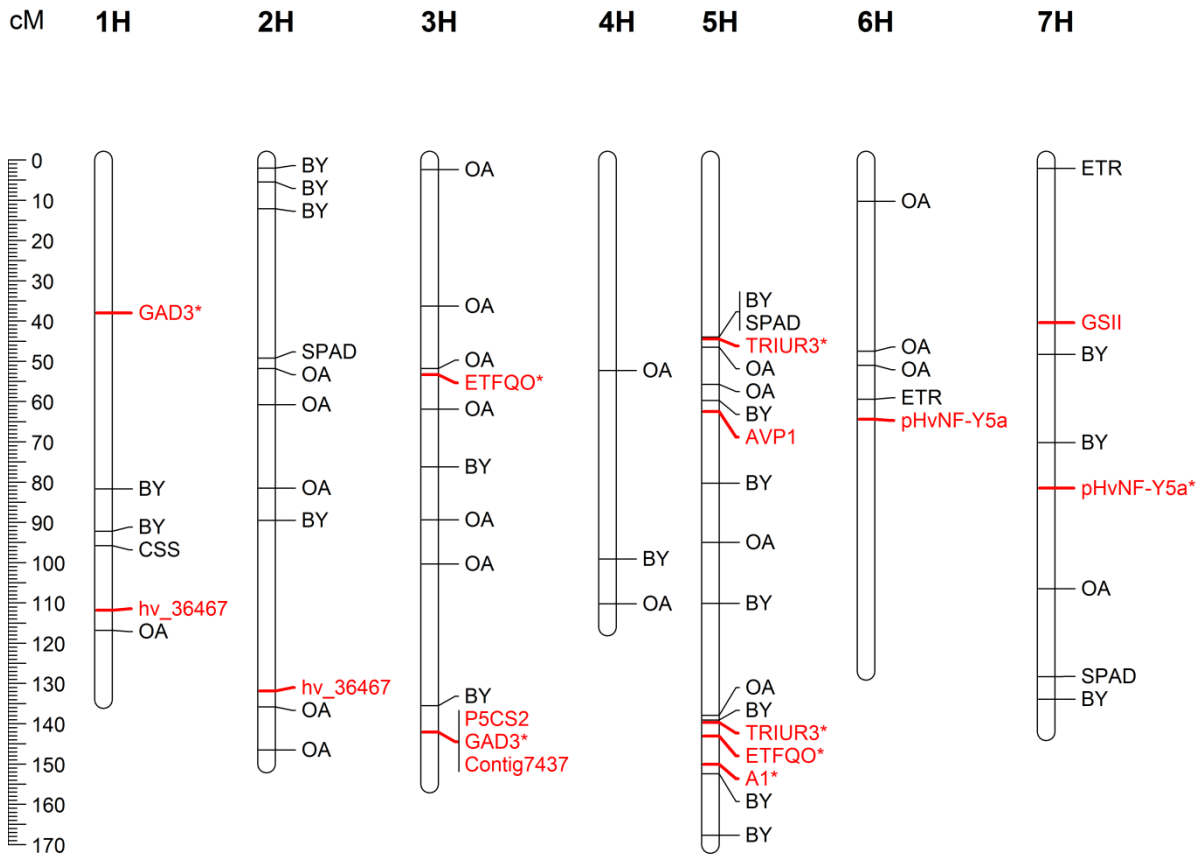

FIGURE 3: Significant (p< 0.001) associated QTL (black) for physiological traits under drought stress treatment (BY: biomass yield, SPAD: leaf colour, ETR: electron transport rate at PSII, OA: osmolality, CSS: total content of soluble sugars) and eQTL (red) for the relative expression of respective genes. Marked with a * are eQTL regulated in *cis*.

Moreover, eQTL for drought stress and leaf senescence associated genes were located next to QTL for physiological parameters. On chromosome 1H and 2H, the eQTL for the relative expression of hv_36467 was located near to a QTL for OA (Figure 3). The senescence associated gene hv_36467 is described as a gene similar to cystein protease. Cystein proteases are enzymes, e.g. hydrolases, which play an important role in the response on dehydration stress among others (Coupe et al. 2003). Thus, a link to OA seems to be present as both are involved in turgor maintenance. Besides this, the eQTL for the relative expression of pHvNF-Y5α was detected close to the QTL for ETR on chromosome 6H at 60 cM (Figure 3). As until now, little has been known on the functionality of this senescence associated gene, this link may be a hint to a function in leaf senescence. For the eQTL associated to the relative expression of the genes P5CS2, Contig7437, A1 and GSII no functional relationship to the closely located QTL for BY was found in literature.

Whereas more QTL were detected than eQTL, identified QTL explain less of the phenotypic variation (Additional file 2, Chapter 2.2 and Table 4, Chapter 2.3). With regard to multi gene controlled phenotypic changes, R^2 is much higher for eQTL as transcription variance concerning single genes is more specific than phenotypic variance (Holloway and Li 2010; Kliebenstein 2009). The SNP in QTL for physiological traits are associated to traits under drought stress or under well-watered conditions, while the markers in eQTL are associated to the relative expression representing an up- or down-regulation relative to the expression in the control variant. Thus, on the one hand, markers are associated to a phenotype, whose associated markers represent genes of interest. On the other hand, the same set of markers is associated to the relative expression of these genes. Most eQTL, and especially those, which are associated to genes of the respective QTL, are located next to this QTL, or are regulated in *cis,* based on the analysis of their location in Morex contigs (Table 5, Chapter 2.3 and Figure 3). They present a re-confirmation of the identified genomic regions for drought stress and leaf senescence. This close connection of eQTL to QTL, with comparable functions on a physiological and genetical level may be concerned as a confirmation of identified genomic regions, rendering these markers of special interest for barley breeding.

3.6 FUTURE PROSPECTS

After validating the results, the markers associated to physiological traits and differentially expressed genes involved in drought stress tolerance and leaf senescence may be included in marker based selection procedures on drought stress tolerance in future barley breeding. Breeders may save time by phenotyping only genotypes with the favourable alleles at these loci.

The screening protocol of drought stress response as well as leaf senescence which was described in this thesis is highly suitable for phenotyping and can be further applied in studies regarding drought stress tolerance and leaf senescence in barley, especially in early developmental stages. In order to get information on the response of the barley genotypes tested under drought stress in the generative stage compared to the results of the juvenile stage, respective genotypes may be phenotyped in additional pot trials till harvest or in rainout shelter trials in the field. With this approach, information on the transferability of respective QTL can be obtained.

Identified genomic regions should be validated, using ranked drought tolerant and sensitive genotypes in addition to other sets of barley genotypes. Besides, identified markers can be tested for these genotypes to test the specificity of the markers regarding drought or leaf senescence. With ongoing sequencing and marker development, the resolution of GWAS may be enhanced. With a higher density of markers, e.g. through GBS, exome capture or RNAseq, QTL localization will become more precise, and more detailed information on drought stress response in barley can be gained.

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7 ABBREVIATIONS

ΔΔCt	Relative expression
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
Blast	Basic Local Alignment Search Tool
BY	Biomass yield
CFP	Content of free proline
Chl	Chlorophyll
cM	Centimorgan
CSS	Total content of soluble sugars
Ct	Cycle threshold
CV	Coefficient of variation
das	Days after sowing
DH	Doubled haploid
DNA	Deoxyribonucleic acid
DSI	Drought susceptibility index; value across treatments
e.g.	for example
eQTL	Expression quantitative trait locus/ loci
ETR	Electron transport rate
Gbp	Giga base pairs
GBS	Genotyping by sequencing
GWAS	Genome wide association study
h^2	Heritability
i.e.	id est
k	number of subpopulations
LD	Linkage disequilibrium
LEA	Late embryogenesis abundant protein
LOD	Likelihood of odds
LSMeans	Last Square Means
MAF	Minor allele frequency
MAS	Marker assisted selection
MLM	Mixed linear model
mQTL	Metabolomic quantitative trait locus/ loci
OA	Osmolality
PAR	Photosynthetic active radiation
PCA	Principal component analysis
PCD	Programmed cell deth
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pQTL	Proteomic quantitative trait locus/ loci
PSII	Photosystem two
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative trait locus/ loci
RFLP	Restriction fragment length polymorphism
RILs	Recombinant inbreed lines
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAG	Senescence associated gene
SBCC	Spanish Barley Core Collection
SNP	Single nucleotide polymorphism
SPAD	Soil Plant Analysis Development; leaf colour
ssp	Subspecies
SSR	Simple sequence repeat

8 APPENDIX

ADDITIONAL FILE 1 (CHAPTER 2.2): Overview of the 156 analysed genotypes.

Nr	Genotype[a]	Row type
1	Alissa	six rowed
2	Alpaca	six rowed
3	Anastasia	six rowed
4	Andrea	six rowed
5	Angela	six rowed
6	Banteng	six rowed
7	Bayava	six rowed
8	Birgit	six rowed
9	Borwina	six rowed
10	Brunhild	six rowed
11	Candesse	six rowed
12	Carola	six rowed
13	Catania	six rowed
14	Catinka	six rowed
15	Cita	six rowed
16	Cornelia	six rowed
17	Corona	six rowed
18	Cosima	six rowed
19	Dura	six rowed
20	Elbany	six rowed
21	Elektra	six rowed
22	Ermo	six rowed
23	Esterel	six rowed
24	Express	six rowed
25	Franka	six rowed
26	Franziska	six rowed
27	SW16199	six rowed
28	Gaulois	six rowed
29	Gerbel	six rowed
30	Gilberta	six rowed
31	Grete	six rowed
32	Hampus	six rowed
33	Hasso	six rowed
34	Jana	six rowed
35	Julia	six rowed
36	Kendo	six rowed
37	Krimhild	six rowed
38	Landi	six rowed
39	Lenta	six rowed
40	Lomerit	six rowed
41	Ludmilla	six rowed
42	Mädru	six rowed
43	Mammut	six rowed
44	Masto	six rowed
45	Merlot	six rowed
46	Naomie	six rowed
47	Nebelia	six rowed
48	Nelly	six rowed
49	Nikel	six rowed
50	Noveta	six rowed
51	Ogra	six rowed
52	Petra	six rowed
53	Plana	six rowed

Nr	Genotype[a]	Row type
54	Rocca	six rowed
55	Sarah	six rowed
56	Stephanie	six rowed
57	Structura	six rowed
58	Sympax	six rowed
59	Tapir	six rowed
60	Theresa	six rowed
61	Traminer	six rowed
62	Venus	six rowed
63	Viresa	six rowed
64	Vogelsanger Gold	six rowed
65	Advance	two rowed
66	Affair	two rowed
67	Alraune	two rowed
68	Angora	two rowed
69	Astrid	two rowed
70	Babylone	two rowed
71	Bistro	two rowed
72	Bombay	two rowed
73	Cabrio	two rowed
74	Calador	two rowed
75	Camera	two rowed
76	Carat	two rowed
77	Cleopatra	two rowed
78	Danilo	two rowed
79	Duet	two rowed
80	Existenz	two rowed
81	Gerval	two rowed
82	Goldmine	two rowed
83	Hanna	two rowed
84	Hiberna	two rowed
85	Igri	two rowed
86	Intro	two rowed
87	Jessica	two rowed
88	Kamoto	two rowed
89	Kaskade	two rowed
90	Kyoto	two rowed
91	Labea	two rowed
92	Leonie	two rowed
93	Lunaris	two rowed
94	Madou	two rowed
95	Magie	two rowed
96	Malta	two rowed
97	Marinka	two rowed
98	Marylin	two rowed
99	Millie	two rowed
100	Mombasa	two rowed
101	Passion	two rowed
102	Pastoral	two rowed
103	Regina	two rowed
104	Reni	two rowed
105	Sonate	two rowed
106	Sonja	two rowed
107	Tafeno	two rowed
108	Tiffany	two rowed
109	Tokyo	two rowed
110	Trixi	two rowed
111	Vanessa	two rowed
112	Venezia	two rowed
113	Verticale	two rowed

Nr	Genotype[a]	Row type
114	SBCC3	six rowed
115	SBCC11	six rowed
116	SBCC12	six rowed
117	SBCC14	six rowed
118	SBCC16	six rowed
119	SBCC18	six rowed
120	SBCC23	six rowed
121	SBCC25	six rowed
122	SBCC27	six rowed
123	SBCC28	six rowed
124	SBCC32	six rowed
125	SBCC38	six rowed
126	SBCC39	six rowed
127	SBCC42	six rowed
128	SBCC47	six rowed
129	SBCC49	six rowed
130	SBCC51	six rowed
131	SBCC54	six rowed
132	SBCC59	six rowed
133	SBCC63	six rowed
134	SBCC65	six rowed
135	SBCC67	six rowed
136	SBCC70	six rowed
137	SBCC73	six rowed
138	SBCC74	six rowed
139	SBCC75	six rowed
140	SBCC76	six rowed
141	SBCC78	six rowed
142	SBCC79	six rowed
143	SBCC80	six rowed
144	SBCC81	six rowed
145	SBCC89	six rowed
146	SBCC91	six rowed
147	SBCC92	six rowed
148	SBCC97	six rowed
149	SBCC106	six rowed
150	SBCC109	six rowed
151	SBCC119	six rowed
152	SBCC130	six rowed
153	SBCC136	six rowed
154	SBCC138	six rowed
155	SBCC140	six rowed
156	SBCC148	two rowed

[a] SBCC: Spanish Barley Core Collection

Additional files of the publications (Additional file 2 of Chapter 2.2 and Additional file 1 of Chapter 2.3) are not displayed here. These files can be downloaded from BMC Plant Biology:

Additional file 2, Chapter 2.2: http://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-015-0524-3

Additional file 1, Chapter 2.3: http://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-015-0701-4

ADDITIONAL FILE 2: Differences in favourable alleles of the Spanish Barley Core Collection (SBCC) in comparison to the German cultivars for the most significantly associated markers for leaf colour (SPAD) and the electron transport rate at PSII (ETR).

Associated markers	SCRI_RS_175370	SCRI_RS_11645	SCRI_RS_160297	SCRI_RS_68142	BOPA1_ABC08327-1-1-353	BOPA2_12_30761
Trait	SPAD	SPAD	ETR	SPAD	SPAD	SPAD
Treatment	Control	DSI	Drought	Drought	Drought	Drought
LOD	4.8	4.37	3.6	3.36	3.14	3.08
R^2	3.4	5.4	5.5	3.3	3.06	3.1
SNP	[G/C]	[T/C]	[T/C]	[A/G]	[A/G]	[A/G]
Fav. allele	C	C	C	A	A	A
SBCC	G	T	T	G	G	G
	G	T	C	A	A	G
	G	T	C	G	G	A
	G	T	C	G	A	G
	G	T	T	A	A	G
	G	T	C	G	A	A
	G	T	C	A	A	G
	G	T	C	A	A	G
	G	T	C	A	A	G
	G	T	C	R	R	N
	G	T	C	G	G	A
	C	T	T	A	A	G
	G	T	C	A	A	G
	G	T	C	G	G	G
	G	T	C	G	A	G
	G	T	C	G	G	G
	G	T	C	G	A	G
	G	T	C	G	G	G
	C	T	T	A	A	G
	G	T	C	A	A	G
	C	T	T	A	A	G
	G	T	C	G	A	G
	G	T	C	G	A	G
	G	C	C	G	A	A
	G	T	C	G	G	A
	G	T	T	A	A	G
	G	T	C	G	G	G
	G	T	Y	R	A	N
	G	T	C	A	A	G
	G	T	C	G	N	G
	G	T	C	A	A	G
	G	T	C	A	A	G
	G	T	T	A	A	G
	G	T	C	G	G	A
	G	T	C	G	A	A
	G	T	C	G	G	G
	G	C	C	G	A	A
	G	T	T	A	A	G
	G	T	C	A	A	G
	G	T	Y	R	A	R
	S	T	Y	R	A	G
German Cultivars	G	C	C	A	A	A
	G	C	C	R	R	A
	C	C	C	A	A	A
	G	C	C	A	G	A
	G	C	C	A	A	A
	C	C	C	A	A	A
	G	C	C	A	G	A
	C	C	C	A	A	G
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	G	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	G	C	C	A	A	G
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
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	G	C	C	A	A	G
	C	C	C	A	A	A
	G	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	G	C	C	A	A	A
	G	C	C	A	A	A

Associated markers	SCRI_RS_175370	SCRI_RS_11645	SCRI_RS_160297	SCRI_RS_68142	BOPA1_ABC08327-1-1-353	BOPA2_12_30761
Trait	SPAD	SPAD	ETR	SPAD	SPAD	SPAD
Treatment	Control	DSI	Drought	Drought	Drought	Drought
LOD	4.8	4.37	3.6	3.36	3.14	3.08
R^2	3.4	5.4	5.5	3.3	3.06	3.1
SNP	[G/C]	[T/C]	[T/C]	[A/G]	[A/G]	[A/G]
Fav. allele	C	C	C	A	A	A
	C	C	C	A	A	A
	G	C	C	G	G	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
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	G	C	C	A	A	A
	C	T	C	A	A	G
	C	C	C	A	A	A
	G	C	C	A	A	A
	C	C	C	A	A	G
	C	C	C	A	A	A
	G	C	C	A	A	A
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	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	G
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	G
	C	C	C	A	A	A
	G	C	C	A	A	A
	N	C	C	A	A	A
	C	C	C	A	A	G
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	C	C	C	A	A	A
	G	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
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	G	C	C	A	A	A
	G	C	C	A	A	A
	C	C	C	A	A	A
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	C	C	C	A	A	G
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	G	C	C	A	A	G
	C	C	C	G	G	A
	G	C	C	A	A	A
	C	C	C	A	A	A
	C	C	C	A	A	A
	S	C	C	A	A	R
	G	C	C	A	A	G
	G	C	C	A	A	G
	C	C	C	A	A	A

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PUBLICATIONS AND PROCEEDINGS

Publications (out of Chapter 2)

Wehner, G.; Balko, C.; Ordon, F., (2016) Experimental Design to Determine Drought Stress Response and Early Leaf Senescence in Barley (*Hordeum vulgare* L.), *Bio-protocol* 6(5): e1749, http://www.bio-protocol.org/e1749

Wehner, G.; Balko, C.; Humbeck, K.; Zyprian, E.; Ordon, F., (2016) Expression profile of genes involved in drought stress and leaf senescence in juvenile barley, *BMC plant biology*, 16(3)

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 biology,* 15(125)

Presentations

Wehner, G., Identifikation von Genomregionen mit Beteiligung an der Trockenstresstoleranz und Blattseneszenz in frühen Entwicklungsstadien der Gerste, Annnual Meeting of the Gemeinschaft zur Förderung von Pflanzeninnovation (GFPi), 2015-11-04, Bonn, Germany

Wehner, G., Identification of genomic regions involved in tolerance to drought stress and drought stress induced leaf senescence in juvenile barley, 8th Young Scientists Meeting, 2015-10-21, Quedlinburg, Germany

Wehner, G., Identification of genomic regions involved in tolerance to drought stress and drought stress induced leaf senescence in juvenile barley, Gesellschaft für Pflanzenzüchtung e. V. (GPZ) Conference “Genome Research”, 2015-09-23, Düsseldorf, Germany

Wehner, G., Identifizierung von Genomregionen mit Beteiligung an trockenstressinduzierter Blattseneszenz in juveniler Gerste, GPZ Conference “Ertrags- und Stressphysiologie“, 2015-06-12, Nordsaat Gudow, Germany

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Poster

Wehner, G.; Balko, C.; Humbeck, H.; Zyprian, E.; Ordon, F., QTL for drought stress tolerance and leaf senescence in juvenile barley, GPZ Conference “German Plant Breeding Conference“, 2016-03-09, Bonn, Germany

Wehner, G.; Balko, C.; Zyprian, E.; Ordon, F., Identification of genomic regions involved in drought stress induced leaf senescence and drought stress tolerance in juvenile barley, 7th Young Scientists Meeting, 2014-11-26, Quedlinburg, Germany

Wehner, G.; Balko, C.; Zyprian, E.; Ordon, F., Identification of genomic regions involved in drought stress induced leaf senescence and drought stress tolerance in juvenile barley, GPZ Conference “Genetic variation in plant breeding“, 2014-09-23, Kiel, Germany

Wehner, G.; Balko, C.; Ordon, F., Genome wide association study (GWAS) for drought stress induced leaf senescence and drought stress tolerance in juvenile barley (*Hordeum vulgare* L.) genotypes, EUCARPIA Cereal Section & ITMI Conference, 2014-06-30, Wernigerode, Germany

Wehner, G.; Fettköther, T.; Balko, C.; Ordon, F., Drought stress induced leaf senescence of barley genotypes in the juvenile and generative stage, 6th European Workshop on Leaf Senescence, 2013-10-16, Versailles, France

Wehner, G.; Balko, C.; Enders, M.; Ordon, F., Identification of QTLs for drought stress induced leaf senescence in barley using genome wide association studies, 5th Young Scientists Meeting, 2012-12-05, Quedlinburg, Germany

EIDESSTATTLICHE ERKLÄRUNG/ DECLARATION UNDER OATH

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

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

Rostock, 27.04.2016

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