Genetic studies on ABA responsiveness and abiotic stress tolerance in hexaploid wheat

Iehisa Ouchi Julio Cesar Masaru

(Degree)
博士（学術）

(Date of Degree)
2013-09-25

(Date of Publication)
2014-09-01

(Resource Type)
doctoral thesis

(Report Number)
甲第5927号

(URL)
https://hdl.handle.net/20.500.14094/D1005927

Doctoral Dissertation

Genetic studies on ABA responsiveness and abiotic stress tolerance in hexaploid wheat

6 倍性コムギにおける ABA 応答性と

非生物的ストレス耐性に関する遺伝学的研究

July 2013

Graduate School of Agricultural Science

Kobe University

Julio Cesar Masaru Iehisa Ouchi

Contents

Chapter I

General introduction

1-1. Study of intraspecific natural variation and its importance

The huge diversity of life on Earth was originated by speciation of organisms and adaptation to the environment. In addition, many of these species show intraspecific natural variation. The analysis of natural variation in wild species is an important tool for functional and evolutionary biology. It has been applied mainly to the model plant *Arabidopsis thaliana* (Tonsor et al. 2005; Alonso-Blanco et al. 2009) and has elucidated the molecular basis of several traits such as flowering time variation (Johanson et al. 2000; Michaels et al. 2003; Werner et al. 2005), photoperiod response (Maloof et al. 2001; Filiault et al. 2008), seed dormancy (Bentsink et al. 2006), root development (Mouchel et al. 2004), and very recently, salt tolerance and abscisic acid (ABA) sensitivity (Ren et al. 2010). This approach can also be used in crop plant species, including wheat, to improve agronomic important traits.

1-2. Evolution of common wheat

Common wheat (*Triticum aestivum* L., 2n=6x=42, genome constitution AABBDD) is an allohexaploid species counted among the big three cereal crops, with about 650 million tons produced annually worldwide (FAOstat 2010). Hexaploid wheat arose by natural hybridization between tetraploid wheat (*Triticum turgidum* L., 2n=4x=28, AABB), including emmer and durum wheat, and diploid *Aegilops tauschii* Coss (syn. *Ae. squarrosa* Eig, 2n=2x=14, DD) (Kihara 1944; McFadden and Sears 1944) (Figure I-1).

Wild emmer itself is supposed to be an allotetraploid derived through hybridization between *Triticum urartu* (AA) (Chapman et al. 1976; Dvorak et al. 1993; Takumi et al. 1993) and possibly *Aegilops speltoides* (SS) (Dvorak and Zhang 1990; Sasanuma et al. 1995). The origin of the B genome remains controversial. Recent studies show that the B genome could be related to several *Ae. speltoides* lines but not to other species of the Sitopsis section (Wang et al. 2007), or suggest that *Ae. speltoides* have diverged very early from the B genome progenitor (Salse et al. 2008).

Ae. tauschii, a wild relative of common wheat, is widely distributed in Eurasia and shows abundant genetic variation (Dudnikov and Goncharov 1993; Dvorak et al. 1998; Dudnikov and Kawahara 2006; Matsuoka et al. 2007, 2008, 2009). Traditionally, *Ae. tauschii* has been categorized into two subspecies, *Ae. tauschii* Coss. ssp. *tauschii* and *Ae. tauschii* Coss. ssp. *strangulata* (Eig) Tzvel. (Eig 1929; Hammer 1980). Accessions

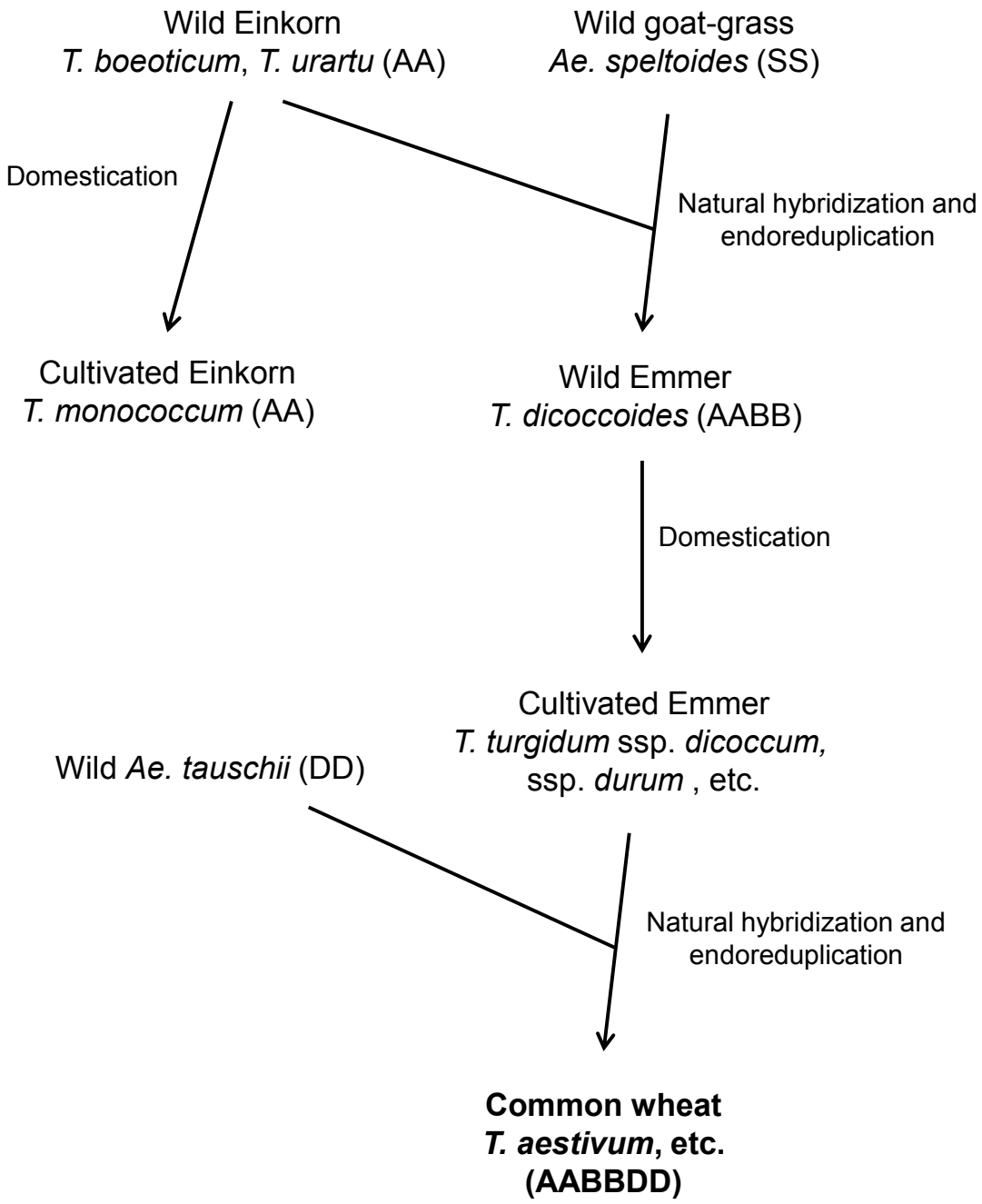

Figure I-1. Polyploid evolution and phylogenetic relationship of diploid, tetraploid and hexaploid wheat species.

of subspecies *tauschii* have elongated cylindrical spikelets, whereas subspecies *strangulata* is characterized by quadrate spikelets. These subspecies show distinct patterns of geographical distribution: subspecies *tauschii* is distributed widely throughout the species range, whereas subspecies *strangulata* is limited to the southeastern Caspian coastal region and the Caucasus (Eig 1929). However, in the monograph of Van Slageren (1994), these subspecies were not formally described, mainly because morphologically and genetically intermediate forms also exist. Among molecular studies, some support the subspecific division (Lubbers et al. 1991; Dvorak et al. 1998; Pestsova et al. 2000), while others do not (Lelley et al. 2000; Saeidi et al. 2006). Recent study based on variation in spikelet-related traits and chloroplast DNA suggested that subspecies *strangulata* diverged from an ancestor that carried a specific chloroplast DNA type, whereas, after divergence, this subspecies became polyphyletic, likely through hybridization (Matsuoka et al. 2009; Takumi et al. 2009a). Based on the population structure analyses, *Ae. tauschii* has been divided into two major genealogical lineages, lineage 1 (L1) and L2, and a minor lineage HGL17 (Mizuno et al. 2010a; Sohail et al. 2012; Wang et al. 2013). The L1 had a wide geographic distribution, from western to eastern habitats, whereas L2 was specific to western habitats. The diversification of L1 and L2 did not correspond to the differentiation of the two subspecies, *tauschii* and *strangulata*. Genetic differentiation between L1 and L2 was much more remarkable than that between two subspecies. The *strangulata* accessions belonged to L2, and most were found to a limited extent in sublineage 2-3 (Mizuno et al. 2010a). The birthplace of common wheat is considered to lie within the area comprising Transcaucasia and the southern coastal region of the Caspian Sea (Tsunewaki 1966; Dvorak et al. 1998). However, *Aegilops tauschii* do not share a common geographical distribution with tetraploid wheat. It is supposed that the *Ae. tauschii* populations involved in the origin of common wheat are limited to a narrow distribution range, probably belong to sublineage 3 of L2, this has given rise to a founder effect in hexaploid wheat (Feldman 2001; Mizuno et al. 2010a). Tetraploid wheat and *Ae. tauschii* can be crossed artificially to produce synthetic hexaploid wheat (Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004). These synthetics are agronomically poor, difficult to thresh, generally tall, low yielding, and frequently have poor quality. However, they have the potential to provide new genetic variation for disease resistance, tolerance to drought, salinity, frost, high temperatures, and soil nutrient stress (Trethowan and Mujeeb-Kazi 2008; Jones et al. 2013). These economically important traits can be transferred to cultivated wheat by crossing with synthetics. Therefore, *Ae. tauschii* is considered as one of the potential sources of new genetic variation for abiotic

stress tolerance in hexaploid wheat improvement.

1-3. Plant responses to abiotic stress

Higher plants, as sessile organisms, are exposed to several environmental stresses. To survive under both biotic and abiotic stresses, they have evolved complex mechanisms to rapidly sense and adapt to changing environmental conditions. Abiotic stress includes drought, high salinity, extreme temperatures, hypoxia, chemical toxicity and oxidative stress. Plants respond to these stresses through ABA-dependent and ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 2005) (Figure I-2).

Promoter analyses of drought- and/or cold-inducible genes have identified at least four independent regulatory systems for gene expression. Two are ABA-dependent and the other two are ABA-independent. Many drought-induced genes are also induced by high salinity and cold stress. This suggests that there is an interaction of different *cis*-acting elements in the cross-talk of stress signaling pathways (Yamaguchi-Shinozaki and Shinozaki 2005).

ABRE (ABA-responsive element) is a major *cis*-acting element in ABA-responsive gene expression. It was first identified in the wheat *Em* gene, with a conserved PyACGTGGC sequence (Marcotte et al. 1989). However, a single copy of ABRE is not sufficient for ABA-responsive transcription. The promoter of the barley ABA-responsive *HVA1* and *HVA22* genes contains ABRE and coupling elements such as CE1 and CE3, which constitute an ABA-responsive complex in transcriptional regulation (Shen and Ho 1995; Shen et al. 1996). In *Arabidopsis*, *RD29B* gene requires two ABRE sequences for its expression (Uno et al. 2000). ACGT-containing ABREs and CEs are functionally equivalent *cis*-acting elements (Hobo et al. 1999). Transcription factors that interact with ABREs are basic-domain leucine zipper (bZIP) proteins named ABRE-binding factors (ABFs) or ABRE-binding proteins (AREBs). Activation of AREB/ABFs has been shown to require an ABA-dependent phosphorylation (Hobo et al. 1999; Uno et al. 2000). On the other hand, an ERF/AP2-type transcription factor ABI4 was shown to bind to the CE1 element (Niu et al. 2002).

Other *cis*-acting elements in ABA-dependent gene expression are MYC and MYB recognition sites. These sites are bound by MYC-like basic helix-loop-helix (bHLH) transcription factor, and MYB transcription factor, respectively. MYC and MYB transcription factors are synthesized after the accumulation of endogenous ABA, indicating that they play roles at late stage of stress response. NAC recognition site also function as *cis*-acting element in this pathway (Yamaguchi-Shinozaki and Shinozaki

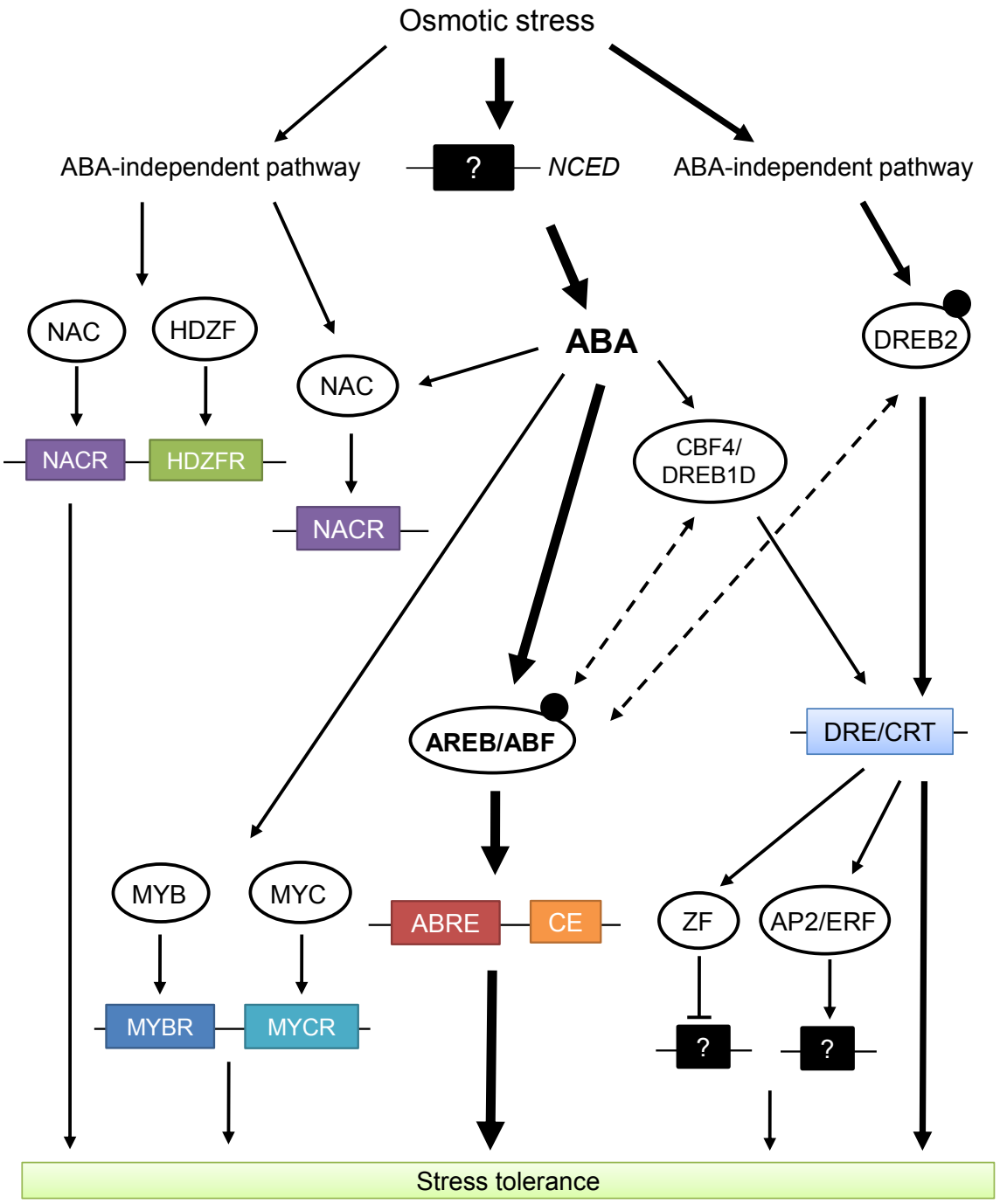

Figure I-2. Transcriptional regulatory networks of *cis*-acting elements and transcription factors (TFs) involved in osmotic stress-responsive gene expression in *Arabidopsis*. TFs are shown in ellipses and *cis*-acting elements in boxes. Small filled circles indicate the post-translational modifications in response to stress signals. Thick arrows indicate the major signaling pathways, and broken arrows, protein-protein interactions.

2005).

In ABA-independent gene expression, DRE (dehydration responsive element)/CRT (C-repeat)/LTRE (low-temperature-responsive element) acts as a major *cis*-acting element. Deletion and base-substitution analysis of the *rd29A* promoter region revealed that the 9-bp conserved core sequence (TACCGACAT) is essential for the regulation of the expression of *rd29A* under dehydration, low-temperature and high-salinity conditions. This *cis*-acting element named DRE, unlike ABRE, does not require other elements for its function (Yamaguchi-Shinozaki and Shinozaki 1994). CRT and LTRE are similar *cis*-acting elements that regulate cold-inducible promoters, and contain A/GCCGAC motif that forms the core of the DRE sequences (Baker et al. 1994; Jiang et al. 1996). Transcription factors that interact with this *cis*-element contain the conserved DNA-binding domain found in the ERF (ethylene-responsive element-binding factor) and AP2 proteins. These transcription factors are named CBF (CRT binding factor)/DREB (DRE binding protein), there are three major DREB1/CBF (DREB1B/CBF1, DREB1A/CBF3 and DREB1C/CBF2) and two major DREB2 proteins (DREB2A and DREB2B) (Yamaguchi-Shinozaki and Shinozaki 2006).

Other *cis*-acting element regulating ABA-independent gene expression was found in *ERD1* (*early response to dehydration 1*) gene promoter. Its promoter region contains two different *cis*-elements, a MYC-like sequence (CATGTG) and a 14-bp *rps1* site 1-like sequence. Three MYC-like sequence-binding genes (*ANAC019*, *ANAC055*, and *ANAC072*) and a zinc-finger homeodomain (ZFHD) protein, ZFHD1, were found to bind to *ERD1* promoter through yeast one-hybrid screening. Both transcription factors are necessary for expression of *ERD1* (Yamaguchi-Shinozaki and Shinozaki 2006).

Transcriptome analysis using microarray technology has been used in the discovery of many stress-inducible genes involved in stress response and tolerance. The functions of these gene products are classified into two major groups, one involved in stress tolerance, such as chaperones, late embryogenesis abundant (LEA) proteins, enzymes for osmolyte biosynthesis and detoxification enzymes; and the other in gene expression and signal transduction in abiotic stress response, such as protein kinases, transcription factors and enzymes in phospholipids metabolism (Yamaguchi-Shinozaki and Shinozaki 2005).

COR (cold-responsive)/LEA proteins promote stress tolerance by protecting cellular components from stress (Thomashow 1999). A number of *Cor/Lea* genes contain both CRT/DRE and ABRE motifs in their promoters. Expression of these *Cor/Lea* genes is regulated by major transcription factors in the CBF/DREB and AREB/ABF (Yamaguchi-Shinozaki and Shinozaki 2006).

1-4. ABA signaling pathway in *Arabidopsis thaliana*

Identification of the ABA receptor has been one of the most active areas in the plant hormone research. Several proteins have been reported as a putative ABA receptor: the RNA-binding protein FCA (Razem et al. 2006), Mg-chelatase H subunit (Shen et al. 2006), G protein-coupled receptor (GPCR) GCR2 (Liu et al. 2007), and GPCR-type G protein 1 and 2 (GTG1 and GTG2) (Pandey et al. 2009). However their exact roles in ABA signaling remained controversial (McCourt and Creelman 2008).

In May 2009, two independent groups reported a new family of proteins as a candidate ABA receptor using chemical genetics (Park et al. 2009) and proteomics (Ma et al. 2009) approaches. Members of this family, known as PYR (PYrabactin Resistance)/PYL (PYR-Like)/RCAR (Regulatory Component of ABA Response) proteins, were found to bind ABA and inhibit the activity of protein phosphatase type 2C (PP2C). Seven month later, many research groups defined the structural and functional mechanisms by which ABA is sensed by this receptor (Melcher et al. 2009; Miyazono et al. 2009; Fujii et al. 2009; Santiago et al. 2009; Nishimura et al. 2009; Yin et al. 2009).

The ABA signaling pathway revealed is as follows. In the absence of ABA, the phosphatase PP2C is free to inhibit phosphorylation of SnRK2 (sucrose-nonfermenting kinase1 (SNF1)-related kinases 2) (Figure I-3A). The activation loop of SnRK2 is packed against the active site of PP2C (such as ABI1, ABI2, HAB1). When ABA is present, it enters into PYR/PYL/RCAR protein's cavity and initiates an allosteric change and allows the receptor to dock into the PP2C active site, thus inhibiting PP2C activity by blocking substrate access. This relieves inhibition on the SnRK2 kinase, which becomes auto-activated and can subsequently phosphorylate and activate downstream transcription factors called ABFs/AREBs. The ABFs bind to ABA-responsive promoter elements (ABRE) and induce the expression of ABA-responsive genes (Figure I-3B).

Three members of SnRK2s, OST1 (Open Stomata1)/SnRK2.6/SnRK2E, SnRK2.2/SnRK2D and SnRK2.3/SnRK2I, play central roles in *Arabidopsis* as positive regulators in ABA signal transduction (Klingler et al. 2010; Raghavendra et al. 2010). Group A PP2Cs interact physically with SnRK2s and directly inactivate them via dephosphorylation of multiple Ser/Thr residues in the activation loop (Umezawa et al. 2009). SnRK2s act as positive regulators in stomatal closure by activating the anion channel SLAC1 and inhibiting the cation channel KAT1 (Raghavendra et al. 2010).

Two of the ABF/AREB transcription factors, ABF2/AREB1 and ABI5 (ABA Insensitive 5), are directly phosphorylated and activated by the three SnRK2s

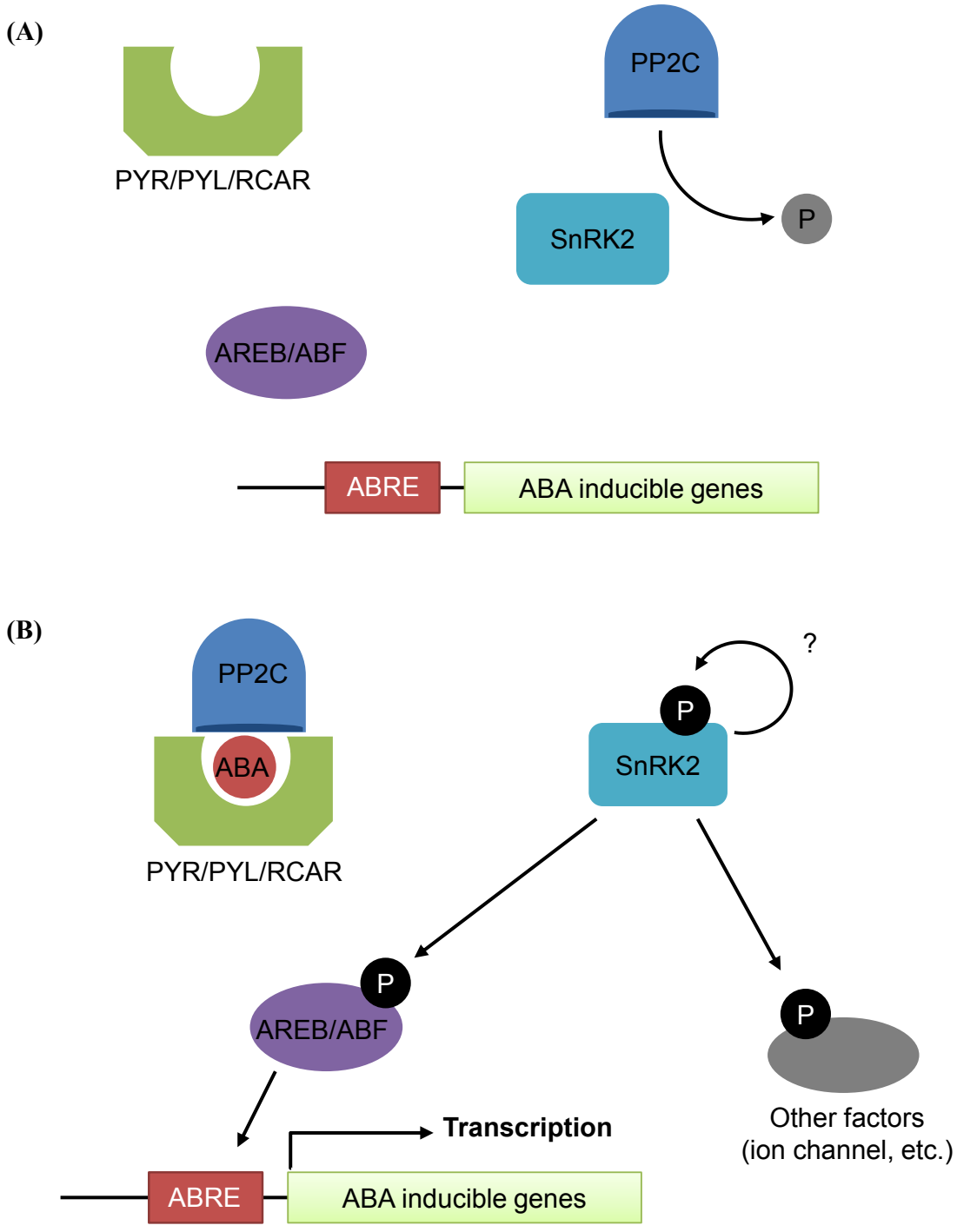

Figure I-3. Proposed model of the core ABA-signaling pathway. (A) In the absence of ABA, PP2C dephosphorylates SnRK2 preventing its activation. (B) In the presence of ABA, PYR/PYL/RCAR binds to ABA and inhibits PP2C by covering the phosphatase active site. This permits the activation of SnRK2 which activates the downstream factors through phosphorylation. The active site of PP2C is indicated in dark blue color.

(OST1, SnRK2.2/SnRK2D and SnRK2.3/SnRK2I) (Lopez-Molina et al. 2001; Furihata et al. 2006). Other transcriptional regulators also contribute to ABA-specific transcription. ABI3 (ABA Insensitive 3), belonging to the B3 transcription factor, binds to ABI5 and enhances its action. In addition, ABI4 and a number of additional transcription factors including MYC/MYB-type regulators act as positive ABA response regulators (Raghavendra et al. 2010).

Another putative ABA receptor, Mg-chelatase H subunit (CHLH), also known as ABA receptor (ABAR), spans the chloroplast envelope and both the N- and C-terminal portions of the protein are exposed to the cytoplasm. The C-terminal part of ABAR binds ABA (Wu et al. 2009) and also interacts with AtWRKY40 transcription factor and with a lower affinity with AtWRKY18 and AtWRKY60 (Shang et al. 2010). Knockout mutants of *AtWRKY18*, *AtWRKY40* and *AtWRKY60* all show ABA-hypersensitive phenotypes in ABA-induced postgermination growth arrest and inhibition of seed germination, suggesting that the three WRKY TFs cooperate to negatively regulate ABA signaling. The ABA-induced movement of AtWRKY40 from the nucleus to the cytoplasm therefore represents a de-repression of ABA signaling and reveals the first stages in a novel mechanism where ABA induces gene expression (Rushton et al. 2012). An additional report also demonstrated that these three WRKY transcription factors are involved in abiotic stress (Chen et al. 2010a).

1-5. Regulation of *Cor/Lea* genes expression through ABA in wheat

In wheat, CRT/DRE, ABRE and other cold-responsive motifs have been identified in the promoter region of many *Cor/Lea* genes such as *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al. 2008a). These *Cor/Lea* genes are responsive to low temperature, drought and ABA (Egawa et al. 2006; Kobayashi et al. 2004, 2006; Ohno et al. 2003). Some of the transcription factors acting in ABA signaling are: WLIP19, TaOBF1 (Kobayashi et al. 2008b), and WABI5 (Kobayashi et al. 2008c), corresponding to bZIP-type transcription factor; and ERF/AP2 domain containing WDREB2 (Egawa et al. 2006) and TaDREB1 (Shen et al. 2003). *Trans*-activation analysis of these *Cor/Lea* genes promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2; however, *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al. 2008a, 2008b, 2008c) (Figure I-4).

Although *Arabidopsis thaliana* DREB2 homolog in barley (HvDRF1) also bind to CRT/DRE motif, they preferably bind to a CT-rich element (T(T/A)ACCGCCTT) where the core sequence is called DRF1E motif (ACCGCC) (Xue and Loveridge 2004). WDREB2 presents high homology to barley HvDRF1. Unlike AtDREB2s, WDREB2

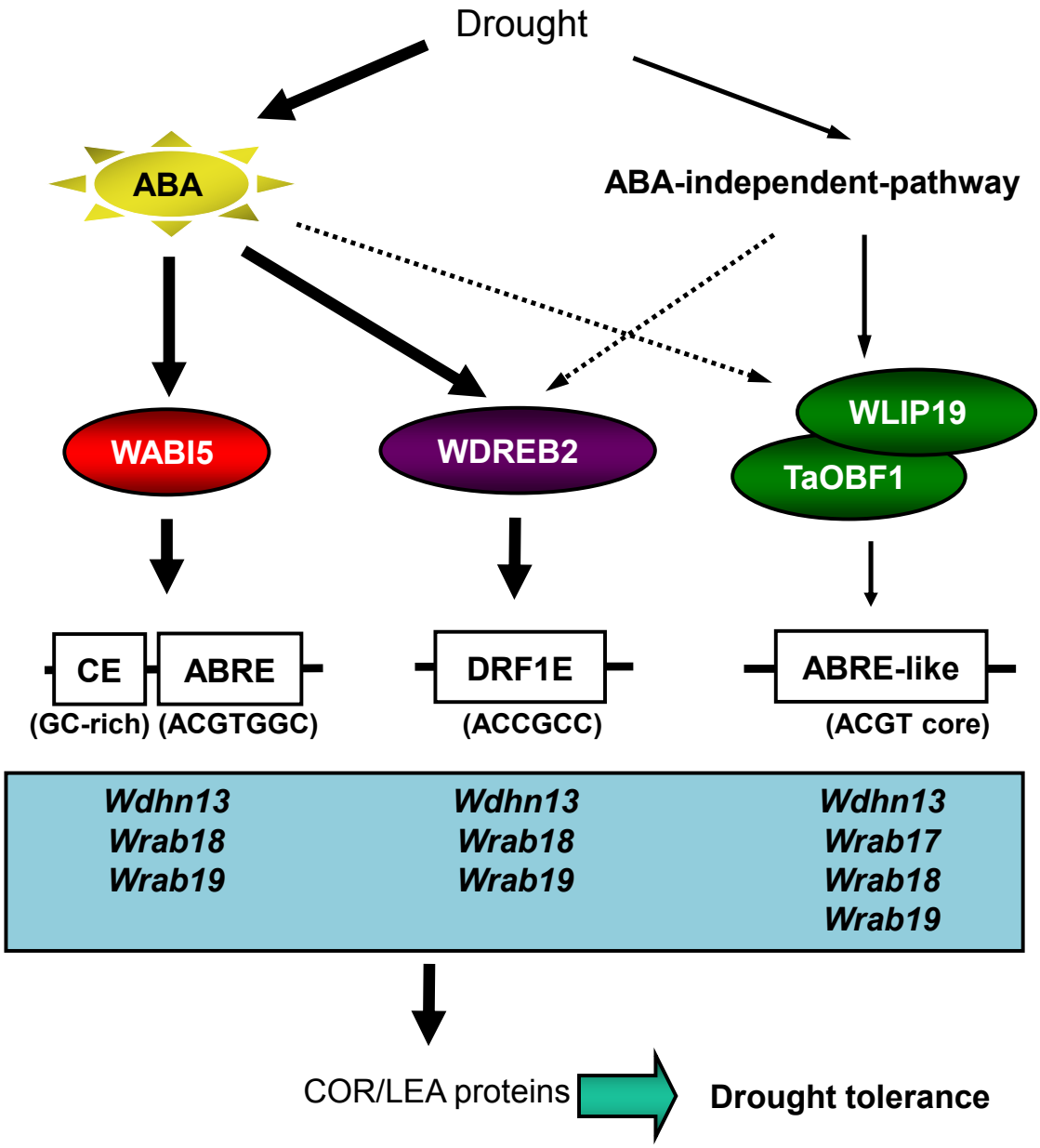

Figure I-4. Transcriptional regulatory networks of *cis*-acting elements and transcription factors (TFs) involved in drought stress tolerance in common wheat. TFs are shown in ellipses and *cis*-acting elements in boxes. Expression of *Cor/Lea* genes controlled by each TF is listed in the light blue box. Thick arrows indicate the major signaling pathways.

and HvDRF1 are also responsive to ABA and cold stress, and their transcripts where shown to be regulated by alternative splicing (Xue and Loveridge 2004; Egawa et al. 2006). *WDREB2* presents three splicing forms: *WDREB2α*, *WDREB2β*, and *WDREB2γ*, of which *WDREB2β* is the most abundant transcript form and putatively encodes a truncated polypeptide of 66 amino acids lacking ERF/AP2 domain (Egawa et al. 2006). It was also showed that the increase in ploidy level during wheat polyploid evolution reduced the alternative splicing efficiency of *WDREB2* transcripts, possibly due to defective heteromeric protein complexes formation caused by allopolyploidization (Terashima and Takumi 2009).

Quantitative trait locus (QTL) analysis for ABA responsiveness at the seedling stage were performed using a mapping population of recombinant inbred lines (RILs) derived from the cross between two common wheat cultivars 'Chinese Spring' (CS) (low ABA responsiveness) and 'Mironovskaya 808' (M808) (high ABA responsiveness). Five QTLs were found, of which 2A, 3A, 6D and 7B QTLs regulated both root-growth arrest and *Cor/Lea* gene expression in ABA-treated seedlings. The QTL located on chromosome 6D had the greatest effect and explained 11.12% of the variance in the ABA responsiveness. Allelic differences in QTLs on chromosome 2A, 6D and 7B influenced the expression of *Wdhn13*, *Wrab15* and *Wrab17*. In contrast, the 3A QTL is likely to involve in the regulation of *Wdhn13* and *Wrab15*, but not the *Wrab17* expression (Kobayashi et al. 2010).

Some other ABA-signaling components cloned in wheat are: two SnRK2, PKABA1 (Johnson et al. 2002) and TaSnRK2.4 (Mao et al. 2010); *TaABI1*, encoding a PP2C (Nakamura et al. 2007); *TaVP1*, encoding a B3-type transcription factor (homolog of *AtABI3*) (Nakamura and Toyama 2001; McKibbin et al. 2002); TaABF, a homolog of *AtABI5* (Johnson et al. 2002); and many homologues identified in diploid wheat *Triticum monococcum* such as TmABI8, TmERA1 (enhanced response to ABA1), and TmERA3 (Nakamura et al. 2007). Among them, only PKABA1 and TaSnRK2.4 are involved in stress response (Johnson et al. 2002; Mao et al. 2010). TaABI1, acting as negative regulator of ABA signaling pathway, is up-regulated by ABA (Kobayashi et al. 2006, appears as an EST clone whyd2h10). The rest of the components mentioned here are involved in ABA-signaling in seeds of wheat. Although several ABA-signaling components were identified in wheat, many of such component involved in the ABA-responsiveness at vegetative stage, seed dormancy and pre-harvest sprouting, still remain uncharacterized.

1-6. Plant responses to low temperature

Low temperature may cause serious damage and losses in agricultural productivity of crops. Common wheat and its related species are able to grow under wide range of environmental conditions. Wheat and its relatives show broad genetic diversity in the ability to tolerate various abiotic stresses such as low temperature (Fowler and Gusta 1979). Cold acclimation, or hardening, is an adaptive process in which the short-term exposure of plants to low, non-freezing temperatures leads to an increase in freezing stress tolerance. This complex process involves drastic physiological, biochemical and metabolic changes. Most of these alterations are regulated through changes in gene expression. One of the mechanisms behind development of freezing tolerance is induction of the *Cor/Lea* gene family (Thomashow 1999). Freezing-tolerant cultivars show greater ability for cold acclimation than susceptible varieties (Sakai and Larcher 1987). Another response to low temperature is vernalization, where long-term exposure leads to acceleration of competence to flowering (Sung and Amasino 2005). Winter wheat cultivars are freezing-tolerant and tend to exhibit requirement for vernalization, whereas spring cultivars are generally freezing-sensitive and do not require vernalization for flowering.

In common wheat, major loci controlling freezing tolerance (*Fr-1*) and vernalization requirement (*Vrn-1*) have been assigned to the long arm of chromosomes 5A (*Fr-A1*/Vrn-A1), 5B (*Fr-B1*/*Vrn-B1*) and 5D (*Fr-D1*/*Vrn-D1*) (Galiba et al. 1995, Snape et al. 1997, Tóth et al. 2003). Previous studies showed that the *Fr-A1* locus was located 2 cM proximal to the *Vrn-A1* (Galiba et al. 1995), *Fr-B1* and *Vrn-B1* interval was 40 cM (Tóth et al. 2003) and that of *Fr-D1* and *Vrn-D1* was 10 cM (Snape et al. 1997). Physical mapping study using CS deletion lines indicated that *Fr-A1* and *Vrn-A1* loci are closely linked, but physically separated (Sutka et al. 1999). However, a recent study suggests that freezing tolerance is a pleiotropic effect of *Vrn-1* rather than the effect of *Fr-1* (Dhillon et al. 2010). *Fr-1* is either tightly linked to *Vrn-1* or is tightly associated with the pleiotropic effects of *Vrn-1*; chromosomal relationship between *Fr-1* and *Vrn-1* is unknown in wheat and barley (Galiba et al. 1995, 2009, Snape et al. 1997, Casao et al. 2011). On the other hand, a second locus for freezing tolerance, *Fr-A2*, has been identified on a distal region of the long arm of chromosome 5A in einkorn and common wheat (Vágújfalvi et al. 2003, 2005, Båga et al. 2007). The recent advances clarified that the *Fr-B1* locus was orthologous to *Fr-2* rather than *Fr-1* according to the marker position and the location of *Fr-B1* QTL (Catalogue of gene symbols, http://www.shigen.nig.ac.jp/wheat/komugi/). *Fr-2* is coincident with a cluster of genes encoding CBFs (Miller et al. 2006), which are transcription factors that induce

downstream genes during cold acclimation (Thomashow et al. 2001). However, relationship between the *Fr* loci and cold-responsive gene expression patterns still remains unclear in common wheat.

1-7. Cold stress signaling pathway

Arabidopsis CBF/DREB1 genes, *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* are rapidly induced by low-temperature treatment (Mizoi et al. 2012). These three genes are organized in tandem on chromosome IV of *Arabidopsis* (Gilmour et al. 1998, Medina et al. 1999). Natural variation in freezing tolerance between ecotypes is correlated with the expression levels of *CBF* genes, indicating that regulation of their expression levels is a major regulatory mechanism for the CBF activity (Mizoi et al. 2012). ICE1 (Inducer of *CBF* Expression 1), a MYC-like basic helix-loop-helix (bHLH) transcription factor, binds to the MYC recognition site in the *CBF3* promoter and activates its expression (Chinnusamy et al. 2003). On the other hand, ICE2, an ICE1-like protein, has been shown to activate *CBF1* expression (Fursova et al. 2009). *CBF2* promoter contains two *cis*-acting elements ICEr1 and ICEr2 (Induction of *CBF* Expression region 1 and 2), that is sufficient to impart cold-responsive gene expression (Zarka et al. 2003). It was found that the binding site of a calmodulin binding transcription activator (CAMTA) protein, CAMTA3, overlaps with ICEr2. The *camta3* mutation reduces cold-induced transcript accumulation of *CBF1* but not *CBF3* (Doherty et al. 2009). A negative regulator of *CBFs* was also identified. MYB15, an R2R3-MYB transcription factor, binds to the MYB recognition sites in the promoters of *CBF1*, *CBF2* and *CBF3*. MYB15 is up-regulated by cold and physically interacts with ICE1. The expression of *MYB15* is down-regulated by ICE1 (Agarwal et al. 2006).

As in *Arabidopsis*, a cluster of *CBF* genes has been mapped on chromosome 5A, at the *Fr-A*^{m}*2* and *Fr-A2* loci in einkorn and common wheat, respectively (Miller et al. 2006, Badawi et al. 2007). There are up to 25 CBF genes in the wheat genome, and at least 11 *CBF* copies are located as a gene cluster on the *Fr-A*^{m}*2* region (Miller et al. 2006, Badawi et al. 2007). Phylogenetic analysis revealed that the *Poaceae CBFs* can be classified into 10 groups, of which six groups are found only in the Pooideae (a subfamily containing the temperate cereals as wheat, barley and rye) suggesting that they represent the CBF machinery that evolved recently during colonization of temperate habitats (Badawi et al. 2007). Expression analysis revealed that five of the Pooideae-specific groups display higher constitutive and low temperature inducible expression in the winter cultivars, and may contribute to the superior low temperature tolerance of these cultivars (Badawi et al. 2007). In common wheat, the cultivar

difference in freezing tolerance has also been associated with the difference in *WCBF2* expression pattern (Kume et al. 2005). WCBF2 directly activates downstream *Cor*/*Lea* genes through binding the *Cor*/*Lea* gene promoters (Figure I-5, Takumi et al. 2008). Two inducers of CBF expression (*ICE1*-like genes), *TaICE87* and *TaICE41*, were also isolated in wheat, and belonged respectively to ICE1 and ICE2 clades (Badawi et al. 2008). Both ICE transcription factors bind to different MYC elements in the *TaCBFIVd-B9* promoterand activate its transcription (Figure I-5, Badawi et al. 2008). Therefore, the *ICE*-*CBF*-*Cor*/*Lea* signal pathway is evolutionally and functionally conserved between *Arabidopsis* and wheat.

Cold can also cause osmotic stress in addition to their direct effect on metabolism. Therefore, osmotic stress appears to be common consequences of exposure to drought, salinity and cold (Huang et al. 2012). The osmotic stress-responsive gene expression is regulated through ABA dependent and independent pathways.

1-8. Genetic dissection of complex traits at transcript levels

Natural genetic variations are useful resource for crop improvement. Most of the natural variation is a result of polygenic variation, which may complicate the dissection of molecular causes (Alonso-Blanco et al. 2009). Diversity at the molecular level usually far exceeds phenotypic variation, described as phenotypic buffering or robustness (Fu et al. 2009, Delker and Quint 2011). Through QTL analysis, many of the large effect phenotypes have been shown to be the result of alterations in amino acid sequences as well as alterations in non-coding genomics regions such as promoters and introns that affect transcript accumulation level (Alonso-Blanco et al. 2009). Expression-level polymorphisms, which are natural variations in gene expression between accessions, are considered more sensitive to molecular diversity than phenotypic differences. Most expression level polymorphisms are regulated in *trans* or in *cis* by multiple eQTLs (Delker and Quint 2011). Mapping of eQTLs is an efficient approach to identify genetic loci controlling complex traits such as seed development and disease resistance in higher plants (Jordan et al. 2007, Chen et al. 2010b, 2010c).

As described in above sections, a correlation between both *CBF* and *Cor/Lea* gene expression patterns and freezing tolerance has been reported in various studies. In cultivated einkorn wheat, a diploid wheat species *Triticum monococcum*, eQTL for *Cor/Lea* gene *Cor14b* have been performed in a RIL population derived from winter and spring habit varieties (Vágújfalvi et al. 2003). The parental winter-type accession G3116 was significantly more freezing tolerant and accumulated more *Cor14b* transcripts at 15°C than the parental spring-type accession DV92. The eQTL for *Cor14b*

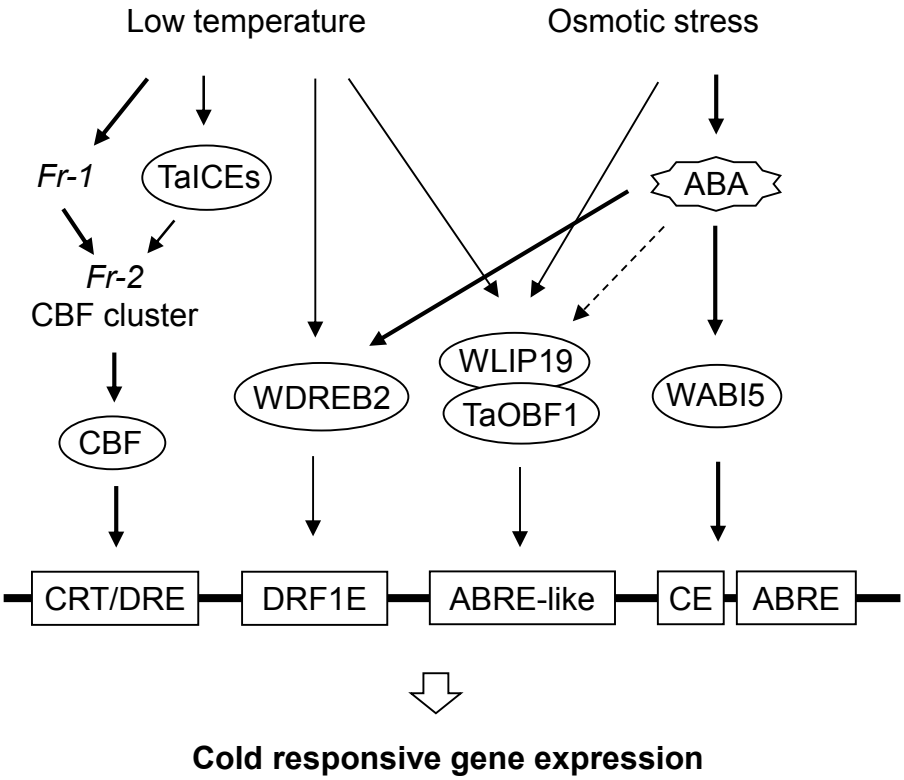

Figure I-5. Cold stress signaling pathways in common wheat. Low temperature leads to accumulation of transcription factors (indicated by circles) through ABA-dependent and -independent pathways. Specific binding of each transcription factor to the *cis*-acting elements (indicated by boxes) activates the cold responsive gene expression. Dashed lines indicate putative interaction.

overlapped with the QTL for freezing tolerance on the long arm of chromosome $5A^m$, coinciding with freezing tolerance locus *Fr-A^{m}2*. The einkorn wheat ortholog of barley *CBF3* was also mapped at the chromosomal region of the *Cor14b* eQTL and *Fr-A^{m}2* QTL, suggesting that allelic variance at the *CBF* locus might result in differential regulation of *Cor14b* expression and freezing tolerance in einkorn wheat (Vágújfalvi et al. 2003).

In common wheat, two loci on the long arm of chromosome 5A have been suggested to control the threshold induction temperature of *Cor14b* using nine single-chromosome recombinant lines derived from a cross between common wheat accessions CS/*Triticum spelta* 5A and CS/Cheyenne (Cnn) 5A (Vágújfalvi et al. 2000). These two loci are corresponding to *Fr-A^{m}1* and *Fr-A^{m}2* of einkorn wheat (Vágújfalvi et al. 2000, 2005). In another study, it was also found that three of eight *CBF* copies (*CBF1A*, *CBF1C* and *CBF7*), mapped at the putative *Fr-A2* region (originally named as *Rcg1*), showed significantly higher levels of the *Cor14b* transcript accumulation in lines with putatively distinct alleles of *Fr-A2* (Vágújfalvi et al. 2005).

M808 is well known as one of the most tolerant wheat cultivars to freezing stress (Veisz and Sutka 1990). eQTL analysis performed in a RIL population derived from the cross between freezing-sensitive cultivar CS and freezing-tolerant M808 found eQTLs for *WCBF2* and three *Cor/Lea* genes (*Wlt10*, *Wdhn13* and *Wcor14*) on long arm of chromosome 5A and coincided with the *Fr-A2* locus (Figure I-6, Motomura et al. 2013). An eQTL for *TaCFB12* was located on chromosome 2A, and additional eQTLs for *Wlt10* and *Wcor14* were detected on chromosomes 1D and 4B, respectively. The bioassay results for freezing tolerance validated that the 1D and 5A eQTLs play important roles in development of freezing tolerance, and that the M808 allele of the 5A eQTL contributes to the high freezing tolerance of M808. The chromosomal location of the 1D eQTL seems to coincide with a freezing tolerance QTL as reported by Båga et al. (2007), and this locus might act as a positive regulator of the *Cor/Lea* gene expression during cold acclimation in common wheat. The 5A eQTL regulated not only the *Cor/Lea* genes in *trans* but also the *CBF* copies in *cis*, suggesting that one or some of the *CBF* copies at the *Fr-2* region positively regulate other copies, which might amplify the positive effects of the *CBF* cluster on the downstream *Cor/Lea* gene activation. Allelic difference at *Fr-A2* might be a major cause for cultivar differences of freezing tolerance level in common wheat.

Previous studies using ABA-sensitive and less-sensitive mutants of common wheat suggested that the regulation of ABA response is involved in the basic mechanisms for development of freezing tolerance (Kobayashi et al. 2006, 2008d). Cultivar difference in

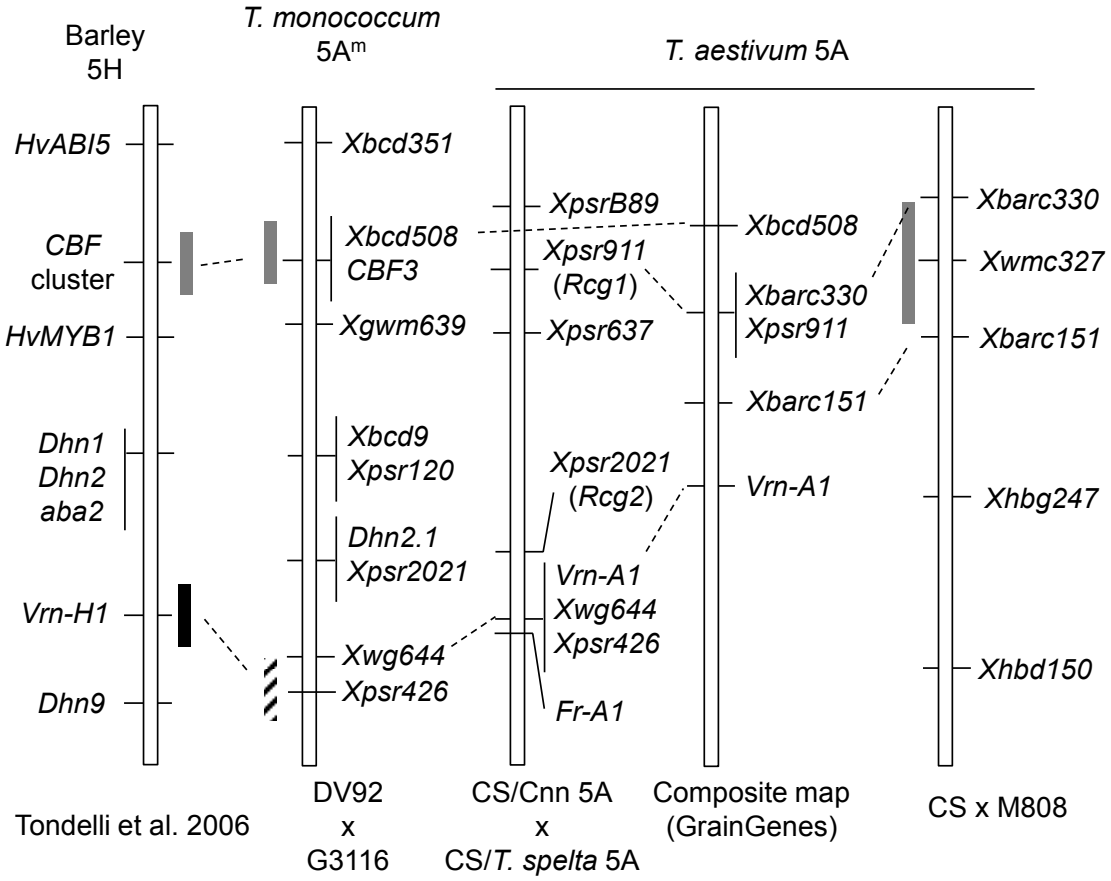

Figure I-6. Chromosomal synteny of homoeologous group 5 chromosomes with *Vrn-1* and frost resistance loci among barley, *T. monococcum* and common wheat. Chromosomal positions of QTLs for *Fr-1* (black bar), *Fr-2* (gray bars) and *Vrn-1* (shaded bar) are indicated. Gray bar indicates the position of eQTL for four cold responsive genes in the genetic map of CS x M808 RILs. *Rcg1* and *Rcg2*, involved in the *Cor14b* regulation, were originally identified in Vágújfalvi et al. (2000).

ABA responsiveness has also been associated with differential expression levels of ABA-inducible genes (Egawa et al. 2006, Kobayashi et al. 2004, 2006, 2008b, 2008c, 2008d). Therefore, the induction levels of ABA-responsive genes are tightly associated with abiotic stress tolerance levels. On the other hand, QTL analysis for ABA responsiveness in a RIL population obtained from CS x M808 found major QTL on chromosome 6D and minor QTLs on chromosomes 1B, 2A, 3A and 7B, and they appeared to be involved in the regulatory system of *Cor/Lea* expression (Kobayashi et al. 2010). These results suggest that eQTL analysis of ABA-inducible genes might be useful in dissecting genetically not only ABA responsiveness but also abiotic stress tolerance in common wheat.

1-9. Molecular markers development in wheat

High-resolution genetic map construction is important for genetic and genomic research as well as for molecular breeding (Yano 2001). In wheat, genetic maps are commonly constructed using SSR markers (Röder et al. 1998; Somers et al. 2004), but the number of SSR markers reported in public databases such as the National BioResource Project (NBRP) KOMUGI web site (http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp) and GrainGene web site (http://wheat.pw.usda.gov/GG2/maps.shtml) are not sufficient for high-resolution maps. Although simple sequence repeats (SSR) are the most popular marker system, the constructed map resolution remains low in plant species without a known genome sequence. An alternative marker system, single nucleotide polymorphism (SNP) has received considerable attention because it is the predominant class of genetic variation (Deschamps and Campbell 2010).

Next generation whole genome resequencing has identified large number of SNPs in plants with reference genome such as *Arabidopsis* (Ossowski et al. 2008), rice (Deschamps et al. 2010; Yamamoto et al. 2010; Subbaiyan et al. 2012), soybean (Deschamps et al. 2010; Lam et al. 2010) and maize (Lai et al. 2010). High-throughput SNP-typing systems can be developed for organisms with a reference genome or comprehensive expressed sequence tag (EST) database, i.e., barley (Close et al. 2009). But in many plants having large and complex genomes and insufficient reference information, genome-wide SNP discovery is insufficient because of the presence of highly repetitive regions.

SNP discovery in wheat progressed more slowly due to highly repetitive and polyploid nature of its genome, which often difficult the discrimination between homoeologous (intravarietal) and intervarietal SNPs (Barker and Edwards 2009; Kaur et

al. 2012). In tetraploid durum wheat, complexity reduction of polymorphic sequences (CRoPS®) technology were applied and identified 2659 SNPs on 1206 consensus sequences from four cultivars (Trebbi et al. 2011). CRoPS combines genomic complexity reduction based on AFLP and next-generation sequencing. Application of the *Pst*I/*Taq*I enzyme combination reduces genome complexity mainly based on the discriminating selection between homoeologous regions, thus significantly reducing repetitive and duplicated sequences during marker discovery. Only 32.2% of the SNP-harboring sequences were classified as repetitive, while the percentage of repetitiveness in the wheat genome is around 77 to 86% (Trebbi et al. 2011).

In *Ae. tauschii*, a diploid organism with large and complex genome and insufficient reference information, annotation-based genome-wide SNP discovery has developed to overcome these problems. In this strategy, Roche 454 shotgun reads with low genome coverage of one genotype were annotated and then genomic and cDNA shotgun reads of another genotype were generated on SOLiD or Solexa platforms to identify putative SNPs. However, around 56% of the sequence length, characterized as repetitive region, was excluded from analysis (You et al. 2011). Another method for SNP discovery is RNA-seq, a next generation sequencing technology for transcripts, which is cost-effective in organisms with complex genomes (Hansey et al. 2012) and simpler means than annotation. This transcriptome sequencing was applied in hexaploid common wheat (Allen et al. 2011) and durum wheat (Trick et al. 2012).

Based on SNPs information generated by next generation sequencing, whole genome genotyping can be performed developing high-throughput genotyping systems. However, in wheat, the genetic map is commonly constructed using SSR markers and there is a need for saturating only the chromosomal region of interest with more markers. Recently, a draft genome sequence of *Ae. tauschii* (Jia et al. 2013) and barley (International Barley Genome Sequencing Consortium, IBGSC 2012) has been published. A survey genome sequences of wheat chromosome arms 3B (Paux et al. 2008; Rustenholz et al. 2011), 4A (Hernandez et al. 2012), 5A (Vitulo et al. 2011), 7B (Berkman et al. 2012) and 7D (Berkman et al. 2011) have also been publicly available for development of markers. These informations would facilitate the development of molecular markers in wheat.

1-10. Objectives of this study

Despite the roll of ABA in abiotic stress tolerance, the genes determining the difference in ABA-responsiveness among hexaploid wheat accessions have not been identified. The genes controlling ABA-responsiveness might also regulate the expression of

ABA-inducible genes, abiotic stress tolerance and/or seed dormancy. The aim of this study is the investigation of genetic factors determining such difference using seedlings of hexaploid wheat lines. This is important for the improvement of abiotic stress tolerance in common wheat (e.g. by marker assisted selection). Two different plant material sets were used: common wheat cultivars and synthetic hexaploid wheat lines.

Chapter II

Identification of chromosomes controlling abscisic acid responsiveness and transcript accumulation of *Cor-Lea* genes in common wheat seedlings

1. Abstract

Abiotic stresses, such as cold, drought, or high salinity, seriously affect plant growth and reduce yield in crop species including common wheat (*Triticum aestivum* L.). The phytohormone ABA plays important roles in plant adaptation to abiotic stress. We compared responsiveness to exogenous ABA, based on root growth inhibition by ABA, among three common wheat cultivars. Seedlings of the cultivars Cheyenne (Cnn) and Hope showed higher ABA responsiveness and higher levels of *Cor* (cold-responsive)-*Lea* (late embryogenesis abundant) gene expression than seedlings of Chinese Spring (CS). The chromosomes involved in the regulation of ABA responsiveness and *Cor-Lea* expression were identified using chromosome substitution lines, in which a chromosome pair of CS was substituted for the corresponding homologous pair of Cnn or Hope. In the CS-Cnn substitution lines, chromosomes 3A, 5A, 5D, and 7A increased the ABA responsiveness of CS. Chromosomes 3A and 5A were also involved in the regulation of *Cor-Lea* gene expression and stomatal response during leaf dehydration. Substitution of CS chromosomes 3A or 5A with the respective homologous pair from Hope also enhanced ABA responsiveness and *Cor-Lea* expression. In addition, the factors present on chromosomes 4D and 7B of highly responsive cultivars increased *Wrab17* expression but had little or no effect on ABA responsiveness. Cultivar differences in ABA responsiveness appear to be determined by genes present on these specific chromosomes in common wheat.

2. Introduction

Abiotic stresses, such as cold, drought, or high salinity, seriously affects plant growth and reduces yield in crop species. ABA plays important roles in plant adaptation to abiotic stress as well as in plant growth and development including seed maturation and dormancy (Finkelstein et al. 2002). ABA induces expression of a variety of genes that function in the regulation of gene expression, signal transduction and stress tolerance (Yamaguchi-Shinozaki and Shinozaki 2006). One family of genes encodes the COR-LEA proteins, which promote stress tolerance by protecting cellular components from environmental stress (Thomashow 1999). Several *Cor-Lea* genes contain C-repeat (CRT), dehydration-responsive element (DRE) and ABA-responsive element (ABRE)

motifs in their promoters, and these *cis*-acting elements are considered to function independently. Expression of these *Cor-Lea* genes is regulated by major transcription factors in the CBF-DREB and AREB-ABF families (Yamaguchi-Shinozaki and Shinozaki 2006). In *Arabidopsis thaliana* (L.) Heynh., the recently defined core ABA signaling pathway is composed of the ABA receptor, PP2C, and SnRK2s (reviewed in Raghavendra et al. 2010). SnRK2s activate bZIP transcription factors such as AREB-ABFs and ABI5 through phosphorylation (Fujii et al. 2009; Nakashima et al. 2009). However, there is little information about causal genes inducing intraspecific variations in abiotic stress tolerance levels through ABA signaling pathways.

In common wheat (*Triticum aestivum* L.), CRT-DRE, ABRE and other cold-responsive motifs have been identified in the promoter regions of four *Cor-Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al. 2008a). These *Cor-Lea* genes are responsive to low temperature, drought and ABA (Ohno et al. 2003; Kobayashi et al. 2004, 2006; Egawa et al. 2006). Some of the transcription factors acting in ABA signaling are bZIP-type transcription factors WLIP19 (Kobayashi et al. 2008b) and WABI5 (Kobayashi et al. 2008c), and ethylene-responsive factor (ERF)-APETALA2 (AP2) domain-containing transcription factors WDREB2 (Egawa et al. 2006) and TaDREB1 (Shen et al. 2003). *Trans*-activation analysis of the *Cor-Lea* gene promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2, whereas *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al. 2008a, 2008b, 2008c).

Other ABA-signaling components have been cloned in wheat, such as *PKABA1* (Johnson et al. 2002) and *TaSnRK2.4* (Mao et al. 2010), which are two *SnRK2*, while *TaABI1* encodes a PP2C (Nakamura et al. 2007), *TaVP1* encodes a B3-type transcription factor (a homolog of *AtABI3*) (Nakamura and Toyama 2001; McKibbin et al. 2002), and *TaABF* is an *AtABI5* homolog (Johnson et al. 2002). Of these, *PKABA1* and *TaSnRK2.4* are involved in stress responses (Johnson et al. 2002; Mao et al. 2010). Although several ABA-signaling components have been identified in wheat, many components involved in ABA responsiveness at the vegetative, seed dormancy and pre-harvest sprouting stages remain uncharacterized.

In a previous study, quantitative trait locus (QTL) analysis for ABA responsiveness at the seedling stage was performed using a mapping population of recombinant inbred lines (RILs) derived from a cross between two common wheat cultivars, Chinese Spring (CS) (a low ABA-responsive variety) and Mironovskaya 808 (M808) (a highly ABA-responsive variety). Five significant QTLs were found, with 2A, 3A, 6D and 7B QTLs regulating both root growth arrest and *Cor-Lea* gene expression in ABA-treated

seedlings (Kobayashi et al. 2010). In the present study, we aimed to identify chromosomes controlling the ABA responsiveness of common wheat using two sets of chromosome substitution lines. Significant differences of ABA responsiveness at the seedling stage could be detected among three cultivars, the parental cultivars of the chromosome substitution lines. Therefore, we attempted to identify different chromosomes controlling ABA responsiveness from the previously reported ones, and then studied the effects of the substituted chromosomes on ABA responsiveness, *Cor-Lea* expression and abiotic stress tolerance.

3. Materials and methods

3-1. Plant materials

Three common wheat cultivars, *Triticum aestivum* L. cv. Chinese Spring (CS), *T. aestivum* cv. Cheyenne (Cnn) and *T. aestivum* cv. Hope, and two sets of their chromosome substitution lines (CS-Cnn and CS-Hope) were used in this study. The CS-Cnn and CS-Hope substitution lines consisted of CS background (recipient), in which a chromosome pair was substituted with the corresponding homologous pair of the Cnn and Hope donor, respectively. The CS-Cnn substitution lines were developed by R. Morris at the University of Nebraska, and that of CS-Hope by E. R. Sears at the University of Missouri, as described in some previous studies (Sutka 1981; Szalai et al. 2001). Both substitution lines were supplied by the National BioResource Project-Wheat, Japan (http://www.nbrp.jp/report/reportProject.jsp?project=wheat).

3-2. Bioassay for ABA responsiveness

After imbibition for 5 hours, seeds were placed in a glass Petri dish containing filter paper wetted with distilled water, then germinated for 24 h at 23ºC in the dark. Five to six synchronously germinated seeds were placed in a plastic Petri dish containing two overlapping pieces of filter paper wetted with 6 ml of distilled water (control group) or 20 μM ABA solution, then incubated at 23ºC in the dark. Two days after treatment, the length of shoots and roots was recorded in three independent experiments. Relative growth inhibition was calculated as the growth difference between the control and ABA-treated groups, relative to control.

3-3. Expression analysis of ABA-responsive genes

Seven-day-old seedlings, grown at 23ºC under long day conditions (16 h:8 h), were treated with 20 μM ABA solution containing 0.1% (w/v) Tween 20 (Nakalai Tesque, Kyoto, Japan) by foliar spray. Total RNA was extracted from leaves at various times

after ABA treatment using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and an oligo$(dT)_{20}$ primer. The transcript accumulation of each gene was detected by quantitative reverse transcription-PCR (RT-PCR) using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with Thunderbird SYBR qPCR Mix (Toyobo) and the following gene-specific primer sets: 5'-GGCGAGAAGAAGGGCGTCAT-3' and 5'-GTGGTGGCTGTGGTGGCAT-3' for *Wdhn13*, 5'-AGACGGGGAAGACCATTCAAGA-3' and 5'-CTCCTTGGTGGCATCGGCA-3' for *Wrab17*, 5'-CACCTCAGCGCCAAGAC-3' and 5'-CTCCCATACCAACTGCCCTC-3' for *WABI5*, 5'-CAACATCGACGGCGGCAG-3' and 5'-GGCTCAGAACTGGAACGCGTC-3' for *WLIP19*, and 5'- GCCGTGCTTTCCCTCTATG-3' and 5'-GCTTCTCCTTGATGTCCCTTA-3' for *Actin*. The *Actin* gene was used as an internal control. The relative expression level was calculated as $2^{-\Delta\Delta Ct}$, representing value relative to the transcript levels in CS samples obtained at 0 h.

3-4. Determination of water loss

Plants were grown under standard conditions as for expression analysis, and the first leaves of 7-day-old seedlings (n = 10-15) were detached. The leaves were kept in a growth chamber (23ºC, 40-60% relative humidity) and their fresh weight was measured. Water loss was calculated as the weight difference between initial fresh weight and weight at the indicated time, divided by initial fresh weight. Two independent experiments were performed.

3-5. Bioassay for salt stress tolerance

Seven-day-old seedlings grown under standard conditions were removed from the soil and the roots were washed with tap water. They were then transferred to 0.2% Hyponex (Hyponex Japan, Osaka, Japan) solution with or without (control group) 0.1 M NaCl and grown under standard conditions for 1 week. Fresh weight of plants was measured every day, and weight gain was calculated as the percentage of weight gain (weight at the indicated time - initial fresh weight) of the salt-treated group versus weight gain of the control group. Three independent experiments were performed using 10-12 individuals in each group.

4. Results

4-1. Cnn and Hope are highly ABA-responsive common wheat cultivars

ABA responsiveness of CS, Cnn and Hope was evaluated based on inhibition of root and shoot growth by exogenous ABA. In the presence of 20 μM ABA, the growth rates of Cnn and Hope were greatly reduced compared to CS (Figure II-1). The root growth inhibition relative to the control condition (absence of ABA) was significantly greater in Cnn (76.7 ± 4.0%, $P < 0.001$) and Hope (63.6 ± 1.0%, $P < 0.01$) than in CS (55.6 ± 4.5%). The relative shoot growth inhibition was also significantly greater in Cnn (85.9 ± 0.4%, $P < 0.01$) and Hope (86.7 ± 1.9%, $P < 0.01$) compared to CS (81.3 ± 1.9%). These results indicated that Cnn and Hope are more sensitive to ABA than CS.

We compared the expression patterns of ABA-inducible genes between wheat cultivars CS and Cnn. Expression profiles of two transcription factor genes, *WLIP19* and *WABI5*, and two *Cor-Lea* genes, *Wrab17* and *Wdhn13*, were analyzed. Transcript accumulation levels of the two transcription factor genes were similar in both cultivars. *WLIP19* was rapidly induced by ABA and reached a maximum 0.25 h after ABA treatment and then decreased slowly (Figure II-2A). The highest transcript accumulation of *WABI5* was observed 1 hour after treatment (Figure II-2B). Although there was no difference in the expression level of the transcription factors, the transcript accumulation level of the two *Cor-Lea* genes was significantly higher in Cnn (Figure II-2C, 2D). The highest expression level was reached 5 h after ABA treatment for *Wrab17* (15.9 ± 0.5) and 2 h after treatment for *Wdhn13* (114.9 ± 2.8) in Cnn. In CS, both genes reached a maximum 2 h after ABA treatment (9.1 ± 0.5 for *Wrab17* and 43.3 ± 1.2 for *Wdhn13*), but exhibited lower expression levels than Cnn.

The transcript accumulation of the two *Cor-Lea* genes was also higher in Hope than in CS. As in Cnn, *Wrab17* reached its highest expression level 5 h after ABA treatment in Hope (13.5 ± 0.7) (Figure II-2C), and the expression level of *Wdhn13* reached a maximum 2 h after treatment (76.7 ± 3.5) (Figure II-2D). The expression levels of *Wrab17* and *Wdhn13* differed greatly for CS and Hope 5 h after ABA treatment.

4-2. Identification of wheat chromosomes regulating ABA responsiveness using chromosome substitution lines

To identify chromosomes involved in the regulation of seedling growth inhibition by ABA, two chromosome substitution lines were used. In the CS-Cnn substitution lines, a CS chromosome pair was substituted for the corresponding homologous pair of Cnn. Because the cultivar difference in root growth inhibition was clearer than shoot growth inhibition, relative root growth inhibition is equated to ABA responsiveness hereafter.

Among the CS-Cnn substitution lines, root growth inhibition was significantly different from CS in five lines (Figure II-3A). The substitution of any of the

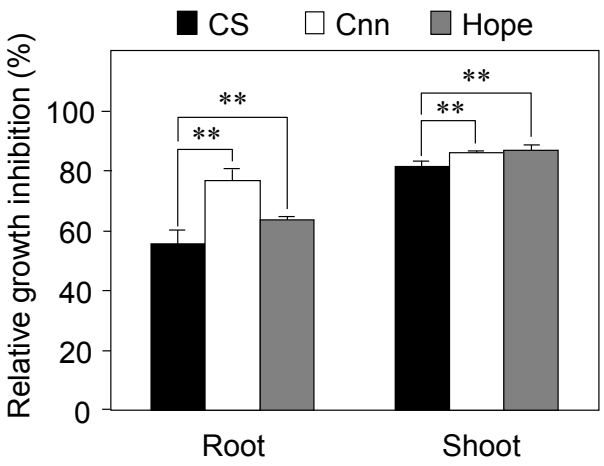

Figure II-1. Comparison of ABA responsiveness in common wheat cultivars. Shoot and root growth inhibition by 20 μM ABA in CS (black bars), Cnn (white bars) and Hope (gray bars). One-day-old seedlings were grown for 2 days in the presence or absence of ABA. Student's *t*-test was used for statistical significance (**$P < 0.01$; ***$P < 0.001$).

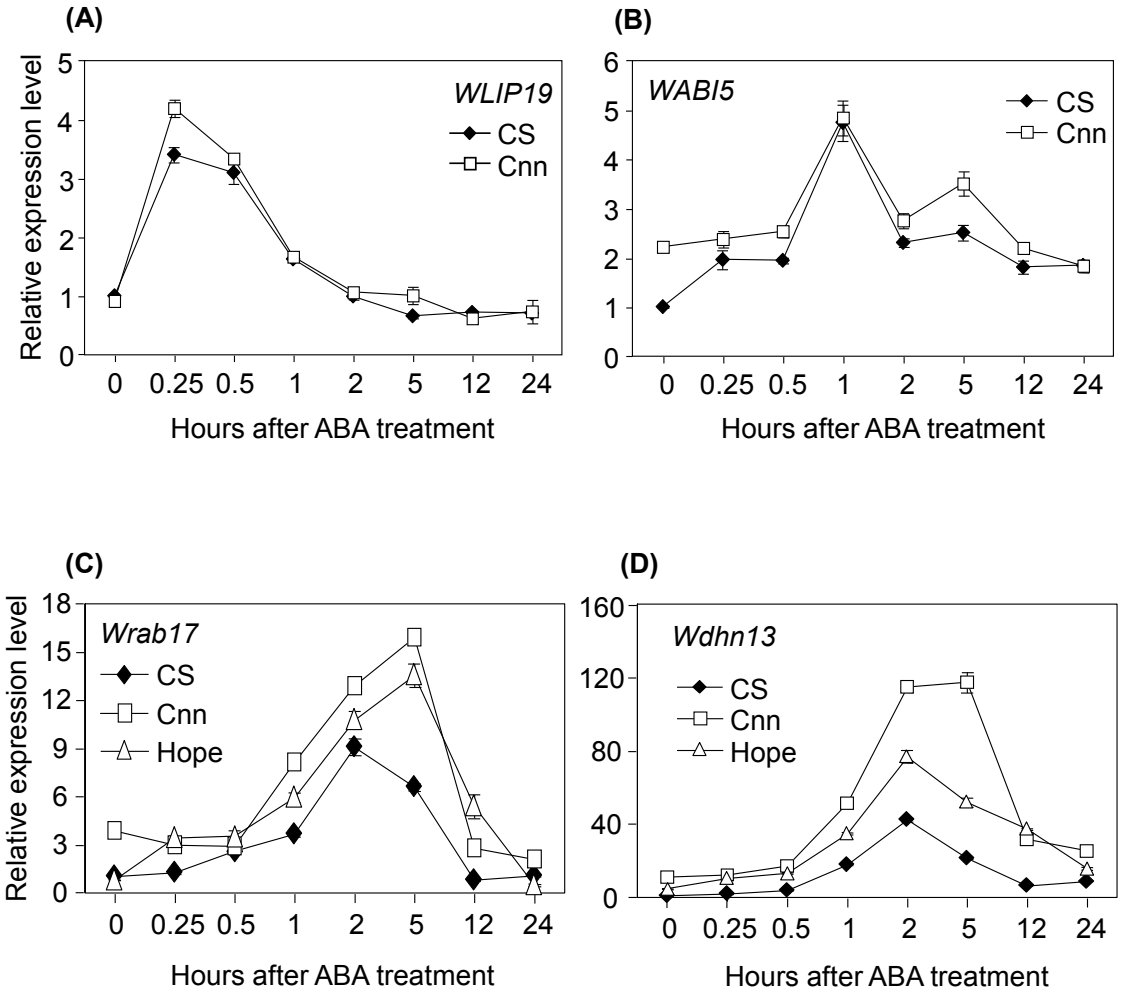

Figure II-2. Time-course analysis of transcript levels of ABA-inducible genes. Transcript accumulation levels of the transcription factors *WLIP19* (A) and *WABI5* (B), and *Cor-Lea* genes *Wrab17* (C) and *Wdhn13* (D) were estimated by quantitative RT-PCR analysis. Seven-day-old seedlings were treated with 20 μM ABA by foliar spray, and RNA samples were extracted at the indicated number of hours after ABA treatment. *Actin* was used as internal control. Each transcript level was represented as the value relative to that of the CS level at 0 h.

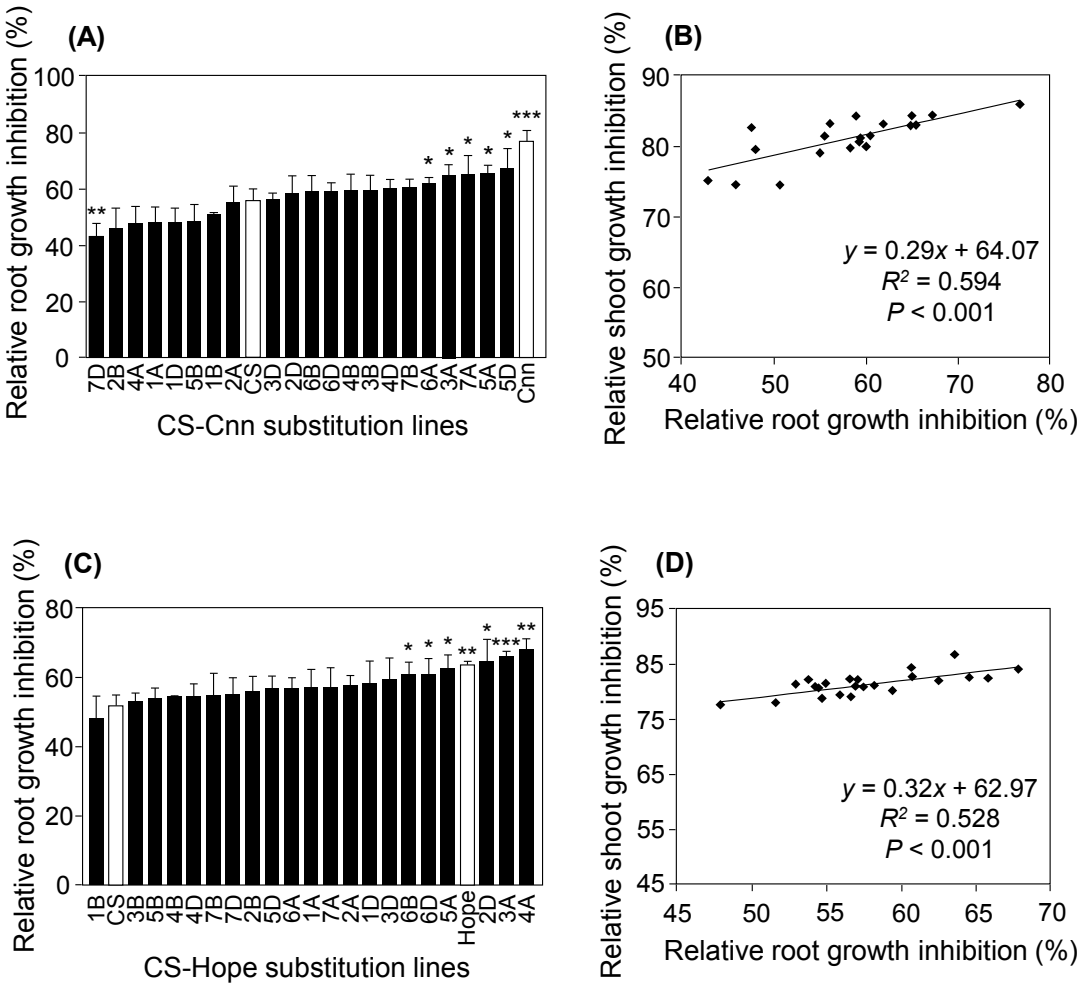

Figure II-3. Effect of substituted chromosomes on relative root growth inhibition in two chromosome substitution lines of CS. (A) Relative root growth inhibition in the CS-Cnn substitution lines. Relative growth inhibition is the difference of growth rate in the absence (control) and presence of ABA, relative to the control group. A higher percentage indicates higher ABA responsiveness. Data are represented as means ± s.d. Student's *t*-test was used to test for statistical significance (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). (B) Correlation between root and shoot relative growth inhibition in the CS-Cnn substitution lines. (C) Relative root growth inhibition in the CS-Hope substitution lines. (D) Correlation between root and shoot relative growth inhibition in the CS-Hope substitution lines.

homologous chromosome pairs 5D (67.3 ± 7.0%), 5A (65.5 ± 2.9%), 7A (65.0 ± 6.9%), and 3A (64.9 ± 3.7%) of CS by their corresponding chromosomes of Cnn increased the ABA responsiveness of CS. In contrast, the substitution of chromosome 7D (42.9 ± 4.6%) decreased ABA responsiveness. The relative shoot growth inhibition was highly correlated with that of the root (Figure II-3B, $r = 0.77$; $P < 0.001$).

Similarly, growth inhibition was significantly higher than that of CS in six of the CS-Hope substitution lines (Figure II-3C). All of these substitutions increased the ABA responsiveness of CS. The relative root growth inhibition was 67.9 ± 3.2% in the CS-Hope 4A line, 65.9 ± 1.7% in 3A, 64.5 ± 6.4% in 2D, 62.5 ± 3.9% in 5A, 60.7 ± 4.6% in 6D and 60.7 ± 3.6% in 6B. The correlation between root and shoot relative growth inhibition was also high (Figure II-3D, $r = 0.73$; $P < 0.001$), as in the CS-Cnn substitution lines. In the CS-Cnn and CS-Hope substitution lines, two common chromosome substitutions (3A and 5A) had a similar effect in both lines, an increase in the ABA responsiveness compared with CS.

4-3. Identification of wheat chromosomes controlling *Cor-Lea* transcript accumulation

Because the expression level of *Wrab17* and *Wdhn13* differed greatly between CS and Cnn 5 h after ABA treatment (Figure II-1B, 1C), total RNA was extracted from leaves of the CS-Cnn substitution lines at this time after ABA treatment. The chromosomes involved in the regulation of *Wrab17* and *Wdhn13* expression were identified by real-time RT-PCR analysis. For *Wrab17*, chromosome substitutions that increased the accumulation of CS transcript more than 2-fold (6.9 ± 0.3) were 4D, 7B, 6D, 2B and 1A (Figure II-4A). The highest increase was observed in the CS-Cnn 4D line, which had an expression level of 46.4 ± 0.2 relative to that of CS at 0 h. In contrast, the substitution of CS chromosome 2D into Cnn decreased the expression level more than 2-fold (3.3 ± 0.2). None of these chromosomes had a large effect on ABA responsiveness. A weakly positive, though non-significant ($r = 0.12$, $P = 0.60$), correlation between the transcript accumulation of *Wrab17* and root growth inhibition was observed (Figure II-5A).

For *Wdhn13*, all chromosome substitutions increased the expression level of CS (21.4 ± 0.2) to a greater or lesser extent (Figure II-4B). Upregulation of more than 2-fold was observed in the CS-Cnn 6D, 3A, 6A, 4D, 2A, 3B, 3D, 7A and 5A lines, of which 6D showed the largest effect (64.5 ± 3.3). CS-Cnn 3A, 5A and 7A lines also showed high ABA responsiveness. In contrast, chromosomes 4D and 6D were involved in the regulation of *Wrab17* and *Wdhn13*, but not in the control of ABA responsiveness. The expression level of *Wdhn13* positively correlated with relative root growth

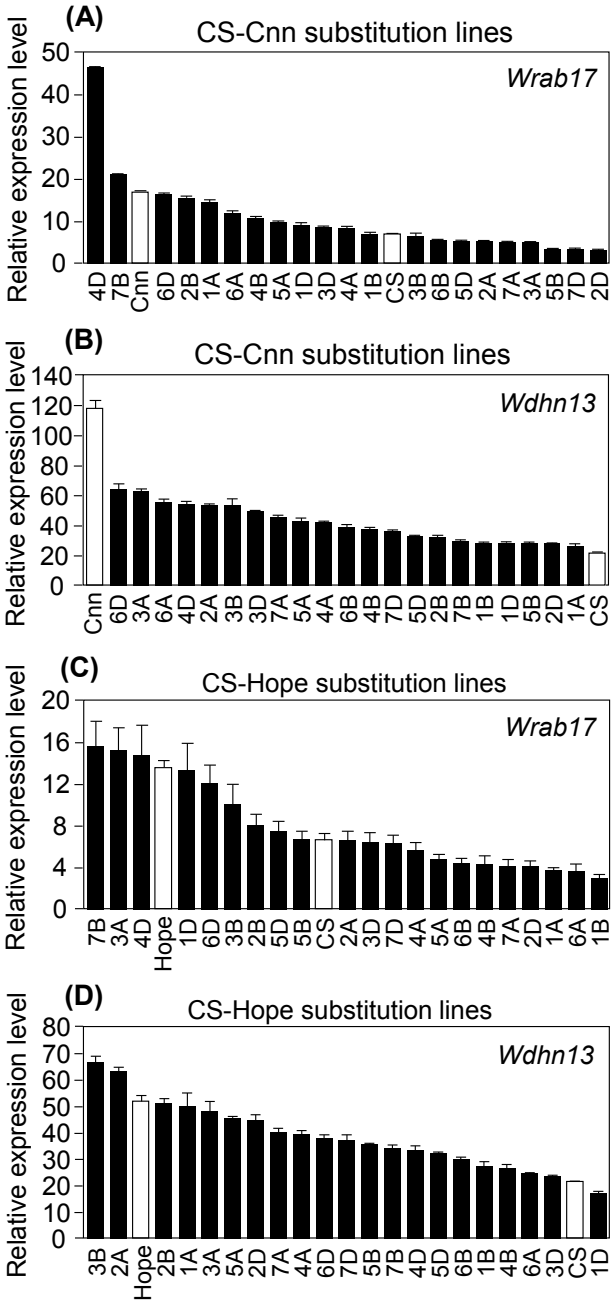

Figure II-4. Effect of substituted chromosomes on transcript accumulation of *Cor-Lea* genes in two chromosome substitution lines of CS. Quantitative RT-PCR was conducted using total RNA from 7-day-old seedlings obtained 5 h after 20 μM ABA treatment by foliar spray. Expression levels of the substitution lines are represented by black bars and those of CS, Cnn and Hope by open bars. The *Actin* gene was used as an internal control. Each transcript level was represented as the value relative to that of the CS level at 0 h. (A) Transcript accumulation levels of *Wrab17* in the CS-Cnn substitution lines. (B) Transcript accumulation levels of *Wdhn13* in the CS-Cnn substitution lines. (C) Transcript accumulation levels of *Wrab17* in the CS-Hope substitution lines. (D) Transcript accumulation levels of *Wdhn13* in the CS-Hope substitution lines.

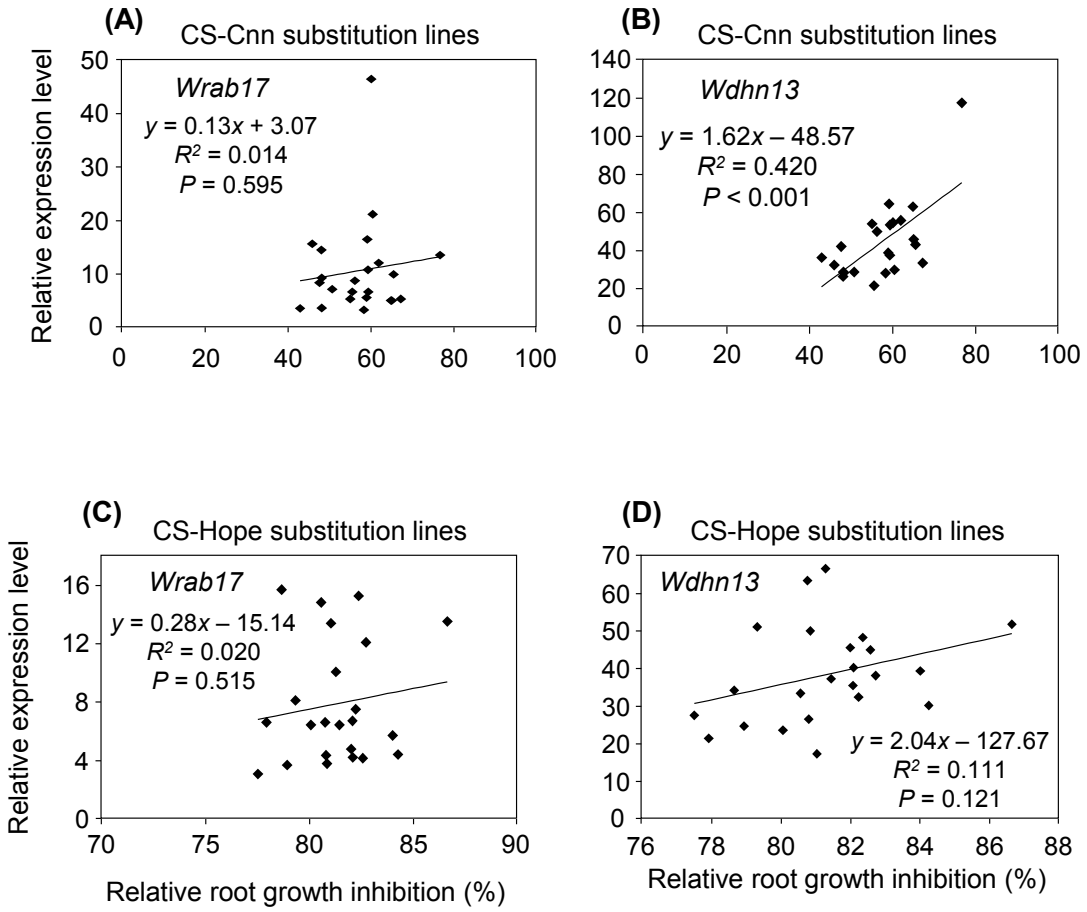

Figure II-5. Correlation between relative root growth inhibition and *Cor-Lea* transcript accumulation in chromosome substitution lines of CS. Scatter plot of relative root growth inhibition and transcript accumulation of *Wrab17* (A and C) and *Wdhn13* (B and D) in the CS-Cnn lines (A and B) and CS-Hope lines (C and D). (B) Scatter plot of relative root growth inhibition and transcript accumulation of *Wrab17* in the CS-Hope lines. (C) Scatter plot of relative root growth inhibition and transcript accumulation of *Wdhn13* in the CS-Hope lines.

inhibition (r = 0.66, P < 0.001) in CS-Cnn substitution lines (Figure II-5B). These results suggest that the regulation of *Wdhn13* expression and the growth arrest mediated by ABA share common signaling components.

Similarly, real-time RT-PCR analysis of *Wrab17* and *Wdhn13* expression was conducted in CS-Hope substitution lines. Expression analysis of *Wrab17* revealed that five chromosome substitutions had large effects on its transcript accumulation (Figure II-4C). The CS-Hope substitution lines 7B (15.7 ± 2.3), 3A (15.3 ± 2.1), 4D (14.8 ± 2.8) and 1D (13.4 ± 2.6) were upregulated more than 2-fold, and 1B (3.1 ± 0.3) was downregulated more than 2-fold compared with CS (6.6 ± 0.6). A positive correlation was found between the expression level of *Wrab17* and relative root growth inhibition in CS-Hope lines, but this correlation was not significant (r = 0.14, P = 0.52) (Figure II-5C). The effect of Cnn and Hope chromosomes 4D and 7B on the expression of *Wrab17* was similar when their homologous pairs were substituted in the CS background.

The CS-Hope substitution lines that upregulated *Wdhn13* expression more than 2-fold were 3B, 2A, 2B, 1A, 3A, 5A and 2D (Figure II-4D). The CS-Hope 3B line showed the highest expression level (66.6 ± 2.3), and no line showed great downregulation of *Wdhn13* transcript accumulation. Chromosomes 5A and 2D regulated both the level of *Wdhn13* expression and ABA responsiveness; in addition, chromosome 3A regulated the accumulation of *Wrab17* transcript. The correlation between *Wdhn13* expression and relative root growth inhibition was positive, but unlike in CS-Cnn lines, it was not significant (r = 0.33, P = 0.12) (Figure II-5D). Chromosomes common to the CS-Cnn and CS-Hope lines that regulated the level of expression of *Wdhn13* in a similar manner were 3A, 2A, 3B and 5A.

4-4. Water loss and salt stress tolerance of CS-Cnn 3A and CS-Cnn 5A

Because chromosomes 3A and 5A were found to control ABA responsiveness and *Wdhn13* expression in both CS-Cnn and CS-Hope substitution lines, we compared the time course of expression of the ABA-inducible genes among CS, CS-Cnn 3A and CS-Cnn 5A. The expression levels of *WLIP19* and *WABI5* were similar for CS-Cnn 5A and CS. In CS-Cnn 3A, the level of *WLIP19* expression was 1.58-fold higher than in CS 0.5 h after ABA treatment, and the *WABI5* expression level 1.46-fold lower at the peak than in CS (Figure II-6A, II-6B). Although the level of *Wdhn13* expression did not increase more than 2-fold, as shown in Figure II-2C, it was 1.45- and 1.47-fold higher in the CS-Cnn 3A and 5A lines, respectively, than in CS at 5 h after ABA treatment (Figure II-6C). The transcript accumulation of *Wdhn13* increased between 0.25 and 2 h after

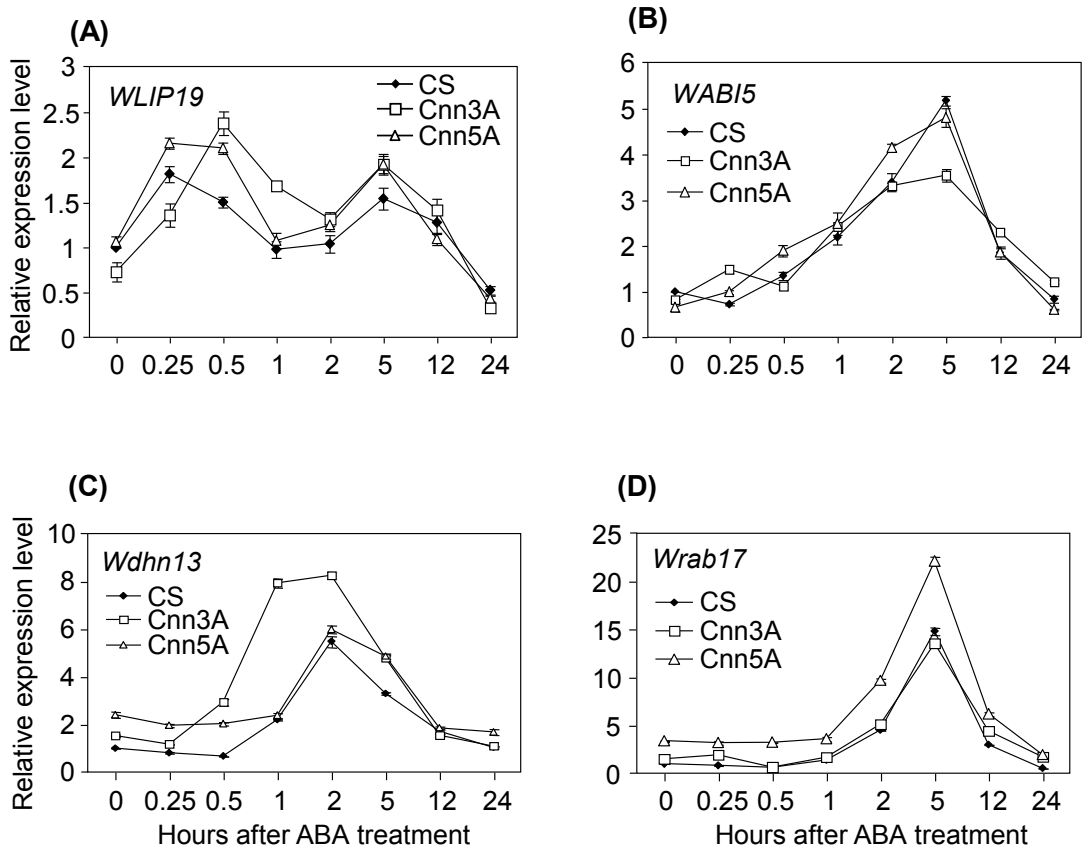

Figure II-6. Time course analysis of ABA-inducible genes in CS and in CS-Cnn 3A and 5A substitution lines after 20 μM ABA treatment. Transcript accumulation levels of the transcription factors *WLIP19* (A) and *WABI5* (B), and *Cor-Lea* genes *Wdhn13* (C) and *Wrab17* (D) were estimated by quantitative RT-PCR analysis. Each transcript level was represented as the value relative to that of the CS level at 0 h. CS is indicated by filled diamonds, the CS-Cnn 3A line by open squares, and the CS-Cnn 5A line by open triangles.

treatment in CS-Cnn 3A and was more than 1.5-fold higher than CS during this period. For *Wrab17*, the expression level of CS-Cnn 3A was identical to that of CS, but in CS-Cnn 5A it was 1.49-fold higher 5 h after ABA treatment (Figure II-6D). The accumulation of *Wrab17* transcripts increased from 1 to 5 h after treatment in CS-Cnn 5A, and the level of expression of *Wrab17* was more than 2-fold higher than in CS from 1 to 2 h after ABA treatment. These observations confirmed that both the CS-Cnn 3A and 5A lines control the expression of *Cor-Lea* genes.

Abiotic stress tolerance also was examined in these two chromosome substitution lines. Since ABA induces stomatal closure during drought stress to limit water loss, the stomatal response during leaf dehydration was evaluated based on the rate of water loss from detached leaves. Both CS-Cnn 3A and 5A lines lost water more slowly than CS (Figure II-7A). The water loss rate in CS-Cnn 5A was similar to that in Cnn and slightly lower than that in CS-Cnn 3A. On the other hand, salt stress tolerance was evaluated by weight gain of seedlings grown under stress conditions (0.2% Hyponex solution [Hyponex Japan] containing 0.1 M NaCl). Since the growth rate among lines differed slightly, the percentage of weight gain was calculated by dividing weight gain under salt stressed conditions with fresh weight before the salt stress treatment. The salt stress tolerance of Cnn was higher than CS (Figure II-7B). The CS-Cnn 3A and 5A lines exhibited salt tolerance similar to that of CS, indicating that factors present on chromosomes 3A and 5A of Cnn did not improve the salt tolerance of CS.

5. Discussion

5-1. Relationship between ABA responsiveness and regulation of *Cor-Lea* gene expression

ABA regulates many agronomically important aspects of plant development, and plays important roles in plant adaptation to abiotic stress (Finkelstein et al. 2002). ABA responsiveness at the seedling stage was correlated with freezing tolerance in common wheat (Kobayashi et al. 2006, 2008d). Genes controlling ABA responsiveness might regulate expression of *Cor-Lea* genes and abiotic stress tolerance. Although several components of ABA signaling pathways have been identified in wheat, few genes determining cultivar differences in ABA responsiveness have been reported. To elucidate the genetic factors determining these cultivar differences, ABA responsiveness of CS, Cnn and Hope was compared on the basis of seedling growth inhibition in the presence of ABA.

Cnn and Hope showed higher ABA responsiveness than CS, and the expression levels of the *Cor-Lea* genes *Wrab17* and *Wdhn13* were also higher than those in CS.

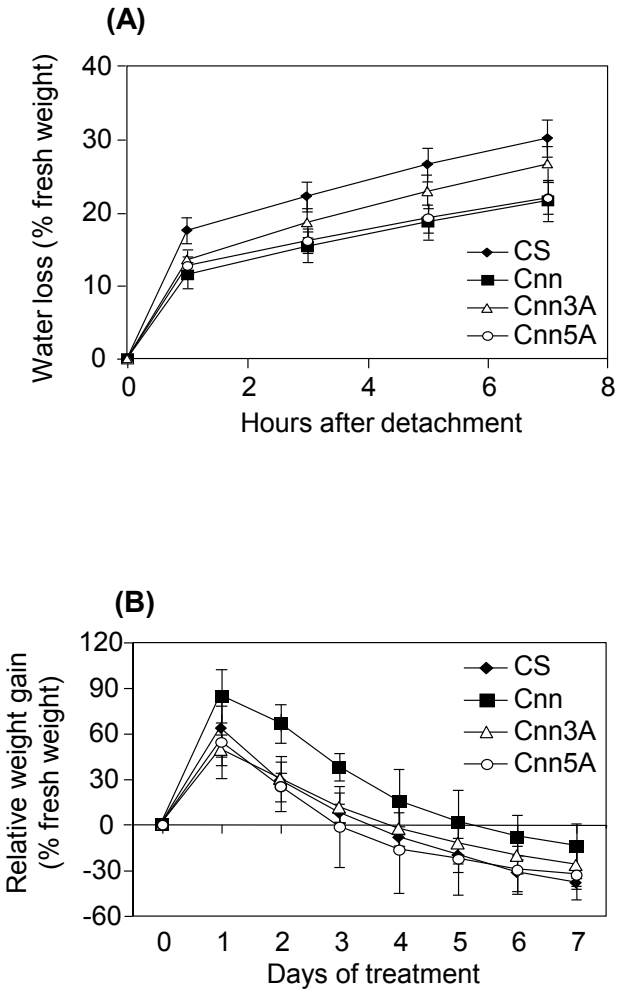

Figure II-7. Abiotic stress tolerance in CS, Cnn, and CS-Cnn 3A and 5A substitution lines. (A) Water loss from detached leaves. The first leaf of 7-day-old seedlings was detached and its fresh weight was measured at the indicated times. Mean ± SD of percentage of water loss of CS is indicated by filled diamonds, Cnn by filled squares, CS-Cnn 3A by open triangles, and CS-Cnn 5A by open circles. (B) Salt stress tolerance. Weight gain was determined in 7-day-old seedlings grown in 0.2% Hyponex solution containing 0.1 M NaCl. The percentage of weight gain of CS is indicated by filled diamonds, Cnn by filled squares, CS-Cnn 3A by open triangles, and CS-Cnn 5A by open circles. Means ± SD from three independent experiments are represented.

In a previous study, QTL analysis of ABA responsiveness at the seedling stage was performed using RILs derived from a cross between common wheat cultivars CS and M808, in which QTLs for ABA responsiveness were also shown to regulate *Cor-Lea* expression (Kobayashi et al. 2010). This demonstration suggests that ABA responsiveness and *Cor-Lea* regulation are controlled by common signaling components. Although the expression levels of *Cor-Lea* genes were different for CS and Cnn, no difference was observed in the expression of transcription factors WLIP19 and WABI5. These transcription factors directly upregulate *Cor-Lea* expression (Kobayashi et al. 2008a, 2008b, 2008c). Therefore, these observations postulate the existence of transcription factor(s) in addition to WLIP19 and WABI5 that regulate *Cor-Lea* expression through an ABA-dependent pathway.

5-2. Wheat chromosomes controlling ABA responsiveness and *Cor-Lea* gene expression

Four substituted chromosomes, 5D, 5A, 7A and 3A of Cnn, were found to significantly increase the ABA responsiveness of CS. The correlation between root and shoot growth inhibition was positive in both CS-Cnn and CS-Hope substitution lines, suggesting a similar mechanism of shoot and root growth regulation by ABA. Chromosomes 3A, 5A and 7A were also involved in regulation of *Wdhn13* expression. Although none of the six chromosomes found to regulate *Wrab17* expression appeared to be involved in root growth in the CS-Cnn lines, *Wdhn13* transcript accumulation and ABA responsiveness were positively correlated in CS-Cnn lines. Chromosomes 4D and 6D regulated not only the accumulation of *Wrab17* transcript but also that of *Wdhn13*. These results indicate that factors present on chromosomes 4D and 6D mainly regulate *Cor-Lea* expression and have little or no effect on ABA responsiveness, indicating that the ABA signaling pathway controlling root growth arrest is similar to that controlling the ABA-dependent expression of *Wdhn13*.

Analysis of ABA responsiveness and *Cor-Lea* gene expression revealed that chromosomes similar to those found in the CS-Cnn substitution lines were in the CS-Hope substitution lines. Chromosomes 3A and 5A of Cnn and Hope increased ABA responsiveness and *Wdhn13* expression in the CS background, and chromosomes 4D and 7B increased the level of *Wrab17* expression. On the M808-CS linkage map, QTLs for ABA responsiveness are located on chromosomes 1B, 2A, 3A, 6D and 7B (Kobayashi et al. 2010). Taking these observations into account, three highly ABA-responsive cultivars, Cnn, Hope and M808, appear to have at least one factor on chromosome 3A that increases the responsiveness of the less ABA-responsive cultivar

CS. Other factors present on chromosomes 5A and 6D of at least two of the highly responsive cultivars also increase the ABA responsiveness of CS. In the CS-Cnn substitution lines, chromosome 6D was involved in the regulation of *Wrab17* and *Wdhn13* expression but had no significant effect on ABA responsiveness; however, in the CS-Hope substitution lines, this chromosome had a large effect on ABA responsiveness. In addition, the transcript accumulation levels of *Wrab17* and *Wdhn13* in CS-Hope 6D were more than 1.5-fold higher than in CS. These results indicate that chromosome 6D of Hope positively regulates *Cor-Lea* expression. Similarly, it was previously reported that the QTL on chromosome 6D was involved in both ABA responsiveness and *Cor-Lea* regulation (Kobayashi et al. 2010). The genetic factor on chromosome 6D of Hope might correspond to the QTL on 6D for ABA responsiveness. Thus, chromosomes 3A, 5A and 6D play important roles in the ABA responsiveness and regulation of *Cor-Lea* expression in common wheat.

5-3. Effect of chromosomes 3A and 5A on *Cor-Lea* gene expression and abiotic stress tolerance

The substitution of 3A chromosome of CS by its homologous pair from Cnn not only affected the expression level of *Wdhn13* but also that of *WLIP19* and *WABI5*. In contrast, substitution of chromosome 5A affected the expression of *Cor-Lea* genes but not the expression of transcription factor genes. Since the expression levels of *WLIP19* and *WABI5* were similar for CS and Cnn, and the accumulation of *WLIP19* transcript at 0.5 h after treatment was similar for CS-Cnn 3A and CS-Cnn 5A, the increase in *Wdhn13* expression was independent of *WLIP19* transcript accumulation. This observation supports the idea that unknown transcription factors might participate in the ABA-dependent regulation of *Cor-Lea* expression.

The basal expression levels (0 h) of *Cor-Lea* genes were more than 2-fold higher in Cnn than in CS. In previous work, it was reported that frost-tolerant wheat cultivars start accumulating *Cor-Lea* transcripts when grown under relatively cold temperatures (9-20ºC) (reviewed in Kosová et al. 2011). If chromosome 5A of the frost-susceptible spring wheat CS is replaced by the corresponding chromosome from the frost-tolerant winter variety Cnn, the frost tolerance of CS is greatly increased (Sutka 1981). The substitution of chromosome 5A also increased the basal expression levels (grown at 23ºC) of *Cor-Lea* genes. However, the relationship between gene(s) involved in frost tolerance or ABA responsiveness on chromosome 5A and the regulation of the basal expression level of *Cor-Lea* genes is unknown.

Abiotic stress tolerance was also tested in the CS-Cnn 3A and 5A lines. The water loss rate of CS was improved when chromosome 3A or 5A was replaced by that of Cnn. CS-Cnn 5A showed higher stomatal response than CS-Cnn 3A during leaf dehydration. However, the salt stress tolerance of CS was not improved in the CS-Cnn 3A and 5A substitution lines. These results suggest that factors present on chromosomes 3A and 5A of Cnn can improve the stomatal response to dehydration but not the salt stress tolerance of CS.

Several genes and QTLs for seed dormancy, preharvest sprouting, ABA responsiveness and ABA signaling components have been mapped (Groos et al. 2002; Osa et al. 2003; Kulwal et al. 2005; Nakamura et al. 2007; Kobayashi et al. 2010). A major QTL for ABA accumulation in dehydrated leaves (Quarrie et al. 1994), one QTL for seed dormancy and preharvest sprouting (Groos et al. 2002; Nakamura et al. 2007), and many QTLs involved in plant adaptation to environment (Dubcovsky et al. 1995; Cattivelli et al. 2002) have been mapped to the long arm of chromosome 5A. The transcription factor *WABI5* is also located on homoeologous group 5 chromosomes (Kobayashi et al. 2008c), but its expression was similar in CS and Cnn. Because many other factors crosstalk with ABA signaling (such as gebberellins, ethylene, etc.), we cannot exclude the possibility that the factors present on the identified chromosomes may belong to signaling components other than those of ABA.

Chapter III

A quantitative trait locus on chromosome 5A for abscisic acid responsiveness affects dehydration tolerance in common wheat seedlings

1. Abstract

The phytohormone abscisic acid (ABA) plays an important role in responses to environmental stress as well as in seed maturation and dormancy. In common wheat, quantitative trait loci (QTLs) for ABA responsiveness at the seedling stage have been reported on chromosomes 1B, 2A, 3A, 6D and 7B. In this study, we identified a novel QTL for ABA responsiveness on chromosome 5A using a F_2 population derived from a cross between common wheat cultivar Chinese Spring (CS) and a chromosome substitution line CS-Hope5A (Hope5A). Functional analyses of the identified QTL found a small effect on dehydration tolerance. Stomatal response during leaf dehydration and transcript accumulation levels of the *cold-responsive* (*Cor*)*/late embryogenesis abundant* (*Lea*) genes *Wrab18* and *Wdhn13* tended to be higher in individuals carrying Hope allele, which showed higher ABA responsiveness. Association of the QTL for ABA responsiveness with salinity tolerance and seed dormancy could not be detected in our experimental conditions, although a QTL for seed dormancy has been reported on the same 5A chromosomal region. Our results suggest that the identified QTL for ABA responsiveness has a small effect on dehydration tolerance, and might contribute in the improvement of wheat drought tolerance by pyramiding with other genes/QTLs.

2. Introduction

The phytohormone ABA serves as an endogenous messenger to mediate responses to environmental stress as well as in plant growth and development including seed maturation and dormancy (Finkelstein et al. 2002). During abiotic stress, ABA induces expression of a variety of genes that function in the regulation of gene expression, signal transduction and stress tolerance (Yamaguchi-Shinozaki and Shinozaki 2006). One family of genes encodes the COR/LEA proteins, which promote stress tolerance by protecting cellular components from environmental stress (Thomashow 1999).

In common wheat (*Triticum aestivum* L.), C-repeat (CRT)/dehydration-responsive element (DRE), ABA-responsive element (ABRE) and other cold-responsive motifs have been identified in the promoter regions of four *Cor/Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al. 2008a). These *Cor/Lea* genes are responsive to

low temperature, drought and ABA (Ohno et al. 2003; Kobayashi et al. 2004, 2006; Egawa et al. 2006). Some of the transcription factors acting in ABA signaling are bZIP-type transcription factors WLIP19 (Kobayashi et al. 2008b) and WABI5 (Kobayashi et al. 2008c), and ethylene-responsive factor (ERF)/APETALA2 (AP2) domain-containing transcription factors WDREB2 (Egawa et al. 2006) and TaDREB1 (Shen et al. 2003). *Trans*-activation analysis of the *Cor/Lea* gene promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2, whereas *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al. 2008a, 2008b, 2008c).

In crop species, such as wheat, an adequate level of seed dormancy is required. It must be high enough to prevent pre-harvest sprouting or vivipary and low enough to promote uniform germination. The catabolism of ABA is a crucial step in dormancy release in several plant species (Gubler et al. 2005). The hydroxylation of ABA at the 8'-position, catalyzed by cytochromome P450 with ABA 8'-hydroxylase activity (ABA8'OH), is the key step of ABA inactivation. In wheat and barley, two genes encoding ABA8'OH have been reported (Millar et al. 2006; Nakamura et al. 2010; Chono et al. 2013). *TaABA8'OH1* has been located on the long arm of homoeologous group-6 chromosomes and a role in seed dormancy was suggested (Chono et al. 2013). On the other hand, *TmABA8'OH2* has been mapped on the centromeric region of chromosome $5A^m$. Using a diploid wheat mapping population of recombinant inbred lines (RILs) derived from a cross between accessions of *T. monococcum* L. and *T. boeoticum* L., *TmABA8'OH2* was found to be linked to QTL for seed dormancy (Nakamura et al. 2010).

ABA is also known to inhibit DNA replication and blocks cells at the G1/S transition (reviewed in del Pozo et al. 2005), possibly by the induction of *Kip-related protein 1* (*KRP1*) (Wang et al. 1998). KRP1 interacts with cyclin-dependent kinase A (CDKA)/Cdc2a and inhibits histone H1 kinase activity (Wang et al. 1998). In barley, dormant seeds germinate at 20°C but not at 30°C. This inability to germinate at 30°C is associated with ABA responsiveness and inability of embryo to inactivate ABA (Benech-Arnold et al. 2006). Imbibition at 30°C and at 20°C in the presence of ABA resulted in a blocking of the G2/M transition, associated with low expression of *CYCB1* and *CDKB1*, and G1/S transition likely due to low expression of *CYCD4* and *CDKB1* (Gendreau et al. 2012). Although *KRP4* was up-regulated at 30°C, this induction was not observed when incubated at 20°C in the presence of ABA (Gendreau et al. 2012).

In our previous study, the chromosomes involved in the regulation of ABA responsiveness and *Cor/Lea* expression were identified using two sets of chromosome

substitution lines (Chapter II). In these lines, a chromosome pair of common wheat cultivar CS was substituted for the corresponding homologous pair of Cheyenne or Hope. Substitution of CS chromosomes 3A or 5A with the respective homologous pair from Cheyenne or Hope enhanced ABA responsiveness and *Cor/Lea* expression. These two chromosomes were also found to be involved in the regulation of stomatal response during leaf dehydration. QTLs for ABA responsiveness have been reported on chromosomes 1B, 2A, 3A, 6D and 7B (Kobayashi et al. 2010). In this study, we identified a novel QTL for ABA responsiveness at the seedling stage on chromosome 5A using a F_2 population derived from a cross between Hope5A and CS, and studied its effect on abiotic stress tolerance and seed dormancy.

3. Materials and methods

3-1. Plant materials

A mapping population of 110 F_2 individuals derived from a cross between *T. aestivum* cv. CS and the chromosome substitution line Hope5A was used in this study. The Hope5A substitution line consisted of CS background (recipient), in which a chromosome 5A pair was substituted with the corresponding homologous pair of the Hope donor. The CS-Hope substitution lines were developed by E. R. Sears at the University of Missouri, as described in previous study (Law and Worland 1996). This substitution line was supplied by the National BioResource Project-Wheat, Japan (http://www.nbrp.jp/report/reportProject.jsp?project=wheat).

3-2. Bioassay for ABA responsiveness

After imbibition of seeds in tap water for 5 h, they were incubated overnight at 4°C. Seeds were pre-germinated for 24 h at 24°C in darkness. Five to six synchronously germinated seeds were placed in a plastic Petri dish containing two overlapping sheets of filter paper wetted with 6 mL of distilled water (control) or 20 μM (±)-ABA (98% purity) (Sigma-Aldrich, Missouri, USA), then incubated at 24°C in the dark. After 48 h of treatment, the length of shoots and primary roots were recorded in four to five independent experiments. At least 20 seeds per each of control and ABA treated groups were used. Bioassay was performed using the F_3 generation, and the average of F_3 individuals was used as the value of their respective F_2 individuals in QTL analysis. Relative growth inhibition was calculated as the difference between growth of the control group and the ABA-treated group, relative to the control. The data were statistically analyzed using JMP software ver. 5.1.2 (SAS Institute, Cary, NC, USA). The correlations among the examined traits were estimated based on Pearson's

correlation coefficient values.

3-3. Genetic map construction and QTL analysis

A total of 65 simple sequence repeat (SSR) markers were used to screen the parental lines for polymorphism. Total DNA was extracted from the parents and F_2 individuals using standard procedures. For the SSR genotyping, 40 cycles of PCR were performed using 2x Quick Taq HS DyeMix (Toyobo, Osaka, Japan) and the following conditions: 10 s at 94°C, 30 s at the annealing temperature, and 30 s at 68°C. The last step was incubation for 1 min at 68°C. Information on the SSR markers and their annealing temperatures was obtained from the National BioResource Project (NBRP) KOMUGI web site (http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp) and the GrainGenes web site (http://wheat.pw.usda.gov/GG2/maps.shtml). The PCR products were separated by electrophoresis on 2% agarose or 13% non-denaturing polyacrylamide gels and stained with ethidium bromide. For polyacrylamide gel electrophoresis, the high efficiency genome scanning system (Nippon Eido, Tokyo, Japan) of Hori et al. (2003) was used. Genetic mapping was performed using MAPMAKER/EXP version 3.0b (Lander et al. 1987). The threshold for log-likelihood scores was set at 3.0, and genetic distances were calculated with the Kosambi function (Kosambi 1944).

QTL analysis was carried out by the composite interval mapping with Windows QTL Cartographer ver. 2.5 software (Wang et al. 2011) using the forward and backward method. A log-likelihood (LOD) score threshold for each trait was determined by computing a 1,000 permutation test. The percentage of phenotypic variation explained by a QTL for a trait and any additive effects were also estimated using this software.

3-4. Expression analysis of genes involved in cell cycle regulation and ABA-inducible genes

For expression analysis at the seedling stage, seeds (F_3 generation) were pre-germinated and treated with ABA as in bioassay (section 3-2). After 48 h of ABA treatment, total RNA was extracted from root and shoot.

For expression analysis during dehydration, F_3 seedlings were grown in soil at 24°C under constant light condition. Seven-day-old seedlings were removed from soil and after washing of roots, they were kept on dry filter paper under standard conditions. Total RNA was extracted from leaves at various times after dehydration treatment using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized

from DNase I-treated mRNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse transcriptase (Toyobo).

The transcript accumulation of each gene was detected by quantitative RT-PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with the gene-specific primer sets given in Table III-1. The *Actin* gene was used as an internal control. The rate of amplification was monitored using Thunderbird SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. The relative expression level was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Actin*.

3-5. Determination of ABA content in seedlings

Seeds of CS and Hope5A were pre-germinated as in section 3-2. Extraction, purification and quantification of ABA from 1-day-old seedlings, including the endosperm, were performed as described by Tokuda et al. (2013). Quantification was performed using four seedlings in three independent experiments.

3-6. Determination of water loss

F_3 plants were grown under standard conditions as for expression analysis, and the first leaves of 7-day-old seedlings (n = 10) were detached. The leaves were kept at 24ºC (60-70% relative humidity) and their fresh weight was measured. Water loss was calculated as the weight difference between initial fresh weight and weight at the indicated time, divided by initial fresh weight. Two independent experiments were performed.

3-7. Bioassay for drought tolerance

F_3 plants were grown under short day condition (12 h light/12 h dark) at 24 ºC in soil. The soil of five-day-old seedlings was saturated with water and after that, the control group was daily irrigated but it was stopped in the drought-treated group. Soil moisture was controlled using soil moisture tester (model DM-18, Takemura Electric Works Ltd., Tokyo, Japan) every day and final moisture of ~25% was kept in the drought-treated group. Each group was composed by eleven F_3 plants and plant height was recorded every three days.

Table III-1. Primer sets used for real-time RT-PCR analysis in this study

Gene	Forward primer	Reverse primer
CycA3	AGGAGTACAAGCTCGTTGCC	CCAAGGAGCTGCAGCCTATT
CycB1	TGCTCGTGTACTCGCCATCC	CCTTCTGCTTGCTGCTCCC
CycB2	CGCAAGTACAGCACCTTCAA	AACATCGAACGTTGGTCTCC
CycD4	GCCGTCGACTGGATATGGAA	TCTTGGCAGCAAGAGACAGG
CDKA1	TCCTTCTTGGAGCAAGGCAG	AGTCAGGCAAGGAACTCACG
CDKB1	GGAATTGCACACTGCCATGG	CGTCACGATCTCATGGGTGT
CDKD1	CAGAGTGTCTGCCCCTGATG	AATGTTGGTCGAGGAGCTGG
KRP1	CAGCGCCATTGCACTATGAC	CGTCGAAGTTGTACTTGGCG
KRP5	CAGCCTGATCTACAGCTCGG	GGCGAAGAACTCGTCCATCT
Histone H2B	GAGAAGAAGGGCAAGAAGAAGT	TGGGCTTCTTGTTGTACCTG
Histone H4	CACCACCAGCAACAAAGCCA	TGGTGATGCCCTGGATGTTG
TaABA8'OH1	ACAGATGGTCCACCTCCAAG	CCTCTATCGTGCCGTTGATT
TaABA8'OH2	GGTGATTTTGGAGAGCCTGA	AAGTAGTCCGGGCTGTGATG
Wdhn13	GGCGAGAAGAAGGGCGTCAT	GTGGTGGCTGTGGTGGCAT
Wrab17	AGACGGGGAAGACCATTCAAGA	CTCCTTGGTGGCATCGGCA
Wrab18	TCGATTCATCCAAGCCAGAG	ACCAAACGAGTAAAGGAAGCAA
Wrab15	GGGATTCTTTCTTCGCGTCT	AGCCTCGGCCTTGAGTATGT
WABI5	CACCTCAGCGCCAAGAC	CTCCCATACCAACTGCCCTC
WDREB2	CTCTCTACCTGGATGGCTTG	CCGACCAAACACCATAGACA
Actin	GCCGTGCTTTCCCTCTATG	GCTTCTCCTTGATGTCCCTTA

3-8. Bioassay for salt stress tolerance

Six-day-old F_3 seedlings grown under standard conditions were removed from soil, roots were washed with tap water and then transferred to 0.2% Hyponex (Hyponex Japan, Osaka, Japan) solution. After 24 h, the solution was changed to that containing or not (control group) NaCl. In the salt-treated group, the concentration of NaCl was increased 25 mM every day until reach 125 mM. Fresh weight of plants was measured every two days, and weight gain was calculated as the percentage of weight gain (weight at the indicated time - initial fresh weight) of the salt-treated group versus weight gain of the control group. Experiments were performed using 11-12 individuals in each group.

3-9. Evaluation of seed dormancy

The degree of seed dormancy for the selected individuals was evaluated using 15 freshly harvested seeds (collected from 1st and 2nd florets of the center part of spike) per F_3 plant in three independent experiments. The seeds were sown on filter paper (8-cm diameter) wetted with 4 mL of distilled water in Petri dishes (9-cm diameter). The number of germinated seeds was counted daily during 5 days and expressed as the percentage of germination or weighted germination index (Walker-Simmons 1988). This index gives maximum weight to seeds that germinate rapidly and is calculated from the following formula:

$$\text{Germination index (GI)} = \frac{(5 \times N_1 + 4 \times N_2 + 3 \times N_3 + 2 \times N_4 + 1 \times N_5)}{(total\ days\ of\ test \times total\ seeds)}$$

where N_1, N_2, N_3, N_4 and N_5 are the number of seeds that had germinated from day 1 to 5. The maximum index is 1.0 if all seeds germinate by day 1 whilst lower indices are indicative of increasing level of seed dormancy. The results are presented as the mean GI of three replicates.

3-10. *In silico* mapping of SSR markers on physical map of barley

Because of the extensive conserved synteny between barley and wheat chromosomes (Mayer et al. 2011), SSR markers located on the QTL region for ABA responsiveness were mapped on physical map of barley. Primer sequences of the SSR markers were obtained from the GrainGenes web site (http://wheat.pw.usda.gov/GG2/maps.shtml). First, blastn search of the primer sequences of SSR markers was performed against the draft genomic sequence of *T. urartu* (Ling et al. 2013), the A genome progenitor of common wheat (word size = 11, *E*-value < 10). Then, protein-coding sequences present on the obtained hit were used as query to perform blastn search against barley genomic

sequence (International Barley Genomic Sequencing Consortium, IBGSC 2012). *WABI5* (accession number: AB193553), *TmERA3* (accession number: AB239134) and *TmABA8'OH2* (accession number: AB455561) were also *in silico* mapped using as query the sequences obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI) against the genomic sequence of barley (*E*-value < 10^{-5}).

4. Results

4-1. ABA responsiveness in the F_2 population

ABA responsiveness was evaluated based on inhibition of root and shoot growth by exogenous ABA. In Hope5A, root and shoot length were significantly short in the presence of ABA (RL-ABA and SL-ABA) and the relative root growth inhibition (RRGI) was significantly higher (Table III-2), indicating that ABA responsiveness is significantly higher in Hope5A than in CS as in our previous study (Chapter II). Shoot length in control group (SL) also differed significantly between the parents. No significant difference in relative shoot growth inhibition (RSGI) and root length in control group (RL) were observed between these two lines. The values of these traits in most of F_2 plants ranged within that of parents, suggesting the involvement of multiple loci.

A significant ($P < 0.001$) negative correlation was observed between RRGI and RL-ABA in F_2 population while no correlation was found between RRGI and RL (Table III-3). Significant ($P < 0.01$) but low positive correlation was also observed between RL and RL-ABA. These observations indicate that the difference in RL has little or no effect on RRGI and RL-ABA in F_2 plants. Similarly, RSGI negatively correlated with SL-ABA, but significant correlations ($P < 0.001$) were also observed between SL and SL-ABA and between SL and RSGI. This indicates that the difference in shoot growth is also reflected in the response of shoot to ABA.

4-2. Linkage map construction and QTL analysis for ABA responsiveness

A total of 65 SSR markers known to be located on chromosome 5A were tested for polymorphism between CS and Hope5A, and 21 markers (32.31%) were available for linkage map construction. The map length was 178.8 centiMorgans (cM) with an average marker interval of 8.51 cM.

QTL analysis was performed for all the six traits, detecting one QTL for each of RL-ABA (*QRla.kpg-5A.1*), RRGI (*QRgi.kpg-5A.1*) and SL-ABA (*QSla.kpg-5A.1*) (Figure III-1). All these QTLs were located on the same centromeric region; the LOD

Table III-2. Parental and F_2 population means for ABA responsiveness

	CS	Hope5A	F_2 population
RL-ABA (mm)	19.00 ± 2.17***	15.58 ± 2.73	19.49 ± 2.47
RL (mm)	59.18 ± 4.62	59.36 ± 4.25	61.14 ± 2.78
RRGI (%)	67.90 ± 3.66***	73.76 ± 4.60	68.09 ± 3.94
SL-ABA (mm)	5.50 ± 0.67***	4.59 ± 0.48	5.37 ± 0.38
SL (mm)	26.82 ± 2.28***	22.57 ± 3.80	25.41 ± 1.51
RSGI (%)	79.49 ± 2.51	79.65 ± 2.12	78.79 ± 1.51

Data are expressed as mean ± standard deviation

Student's *t*-test was used for statistical significance (***$P < 0.001$).

Table III-3. Correlation coefficient (*r*) matrices for ABA responsiveness in F_2 population

	RL	RRGI	SL-ABA	SL	RSGI
RL-ABA	0.2500**	-0.9305***	0.6294***	0.1688	-0.5031***
RL		0.1192	0.3416***	0.5555***	0.1298
RRGI			-0.5100***	0.0450	0.5661***
SL-ABA				0.4301***	-0.6235***
SL					0.4246***

Levels of significance are indicated by asterisks, ** $P < 0.01$, *** $P < 0.001$

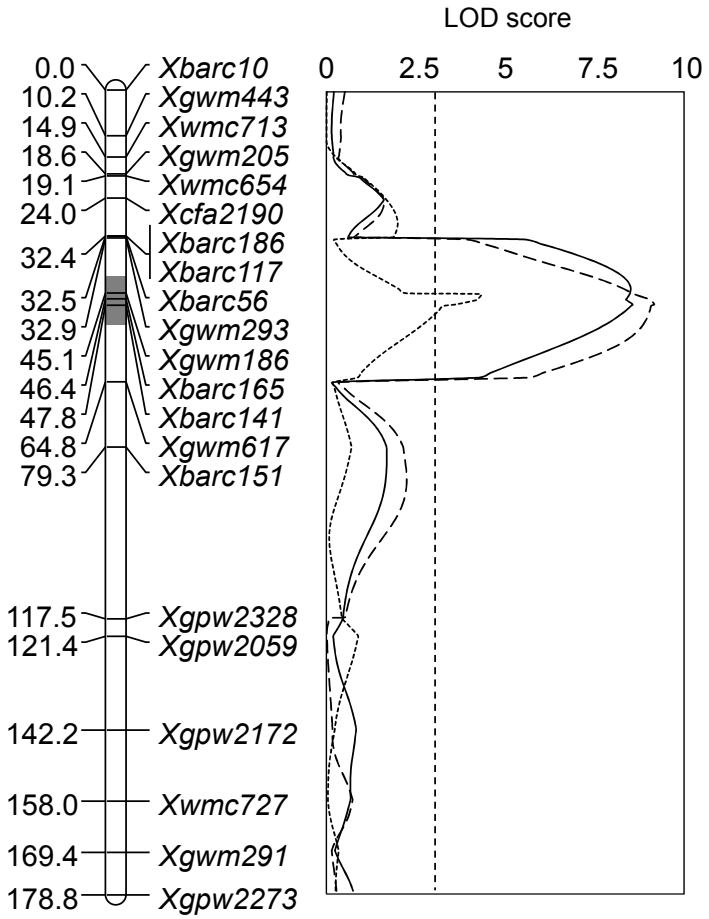

Figure III-1. Linkage map and QTL-likelihood curves of LOD scores showing the locations of QTL for ABA responsiveness on chromosome 5A. LOD curve for RL-ABA is indicated by broken line, that for RRGI by solid line and that for SL-ABA by dotted line. The 2.70 LOD threshold is indicated by a vertical dashed line. Genetic distances are given in centiMorgan. Gray bar indicates the putative position of centromere.

scores ranged from 4.31–9.16, and explained 17.50–32.01% of the phenotypic variation of F_2 plants (Table III-4).

In QTLs identified for RL-ABA and SL-ABA, allele from the lower ABA-responsive parent showed negative values of additive affect (Table III-4), indicating that the allele derived from CS produced low ABA-responsive phenotype (longer root and shoot length in the presence of exogenous ABA). There was a significant difference ($P < 0.05$) between F_2 individuals carrying alleles from the highly responsive and low responsive parents at *QRla.kpg-5A.1* but not at *QSla.kpg-5A.1* (Figure III-2A and III-2B). *QRgi.kpg-5A.1* showed a positive additive effect, indicating that the allele for high ABA responsiveness was derived from Hope. The genotype effect at this QTL also differed significantly ($P < 0.05$) in F_2 individuals (Figure III-2C). We defined QTL for ABA responsiveness as the chromosome 5A region flanked by SSR markers *Xbarc186* and *Xgwm617* hereafter.

4-3. Expression analysis during post-germination growth

For functional analysis of the QTLs, eight F_2 individuals were selected based on the genotypes at SSR markers flanking the QTL, and F_3 plants derived from each F_2 individuals were bulked for further analysis. Because ABA regulates genes involved in cell-cycle control (Wang et al. 1998; Gendreau et al. 2012), we performed gene expression analysis of four *Cyclins* (*CYCA3*, *CYCB1*, *CYCB2 and CYCD4*), three *CDKs* (*CDKA1*, *CDKB1* and *CDKD1*), two *KRPs* (*KRP1* and *KRP5*) and two *Histones* (*Histone H2B* and *Histone H4*) during post-germination growth. As in ABA bioassay, pre-germinated seeds were grown in the presence or absence of 20 μM ABA and total RNA was extracted after 48 h of incubation. In general, expression levels of *CYCB2*, *Histone H2B* and *Histone H4* was down-regulated more than 2-fold by ABA treatment in root (Figures III-3C, III-3J and III-3K), compared with the control group, and *CYCD4* and *KRP1* was up-regulated more than 2-fold (Figure III-3D and III-3H). However, no significant differences were observed between the two genotypes. In shoot, the expression pattern of the analysed genes was similar to that in root except for *KRP1*, which tended to be down-regulated (Figure III-4). Expression level of *CYCA3* and *CDKB1* was significantly ($P < 0.05$) higher in individuals with Hope allele (mean expression level of 1.45 and 1.14, respectively) than those with CS allele (1.05 and 0.74, respectively). Conversely, transcript accumulation of *KRP1* was significantly higher ($P < 0.05$) in individuals carrying CS allele (average of 0.89 vs. 0.61).

Because *ABA8'OH2* and *WABI5* are known to be located on the centromeric region of chromosome 5A (Nakamura et al. 2010; Motomura et al. 2013), we also evaluated

Table III-4. Summary of identified QTLs for ABA responsiveness

Traits	Locus	Map location	LOD score	LOD threshold	Contribution (%)	Additive effect
RL-ABA	*QRla.kpg-5A.1*	*Xbarc186-Xgwm617*	9.16	2.60	32.01	-2.31
RRGI	*QRgi.kpg-5A.1*	*Xbarc186-Xgwm617*	8.56	2.70	30.33	3.02
SL-ABA	*QSla.kpg-5A.1*	*Xgwm186-Xbarc141*	4.31	2.50	17.50	-0.34

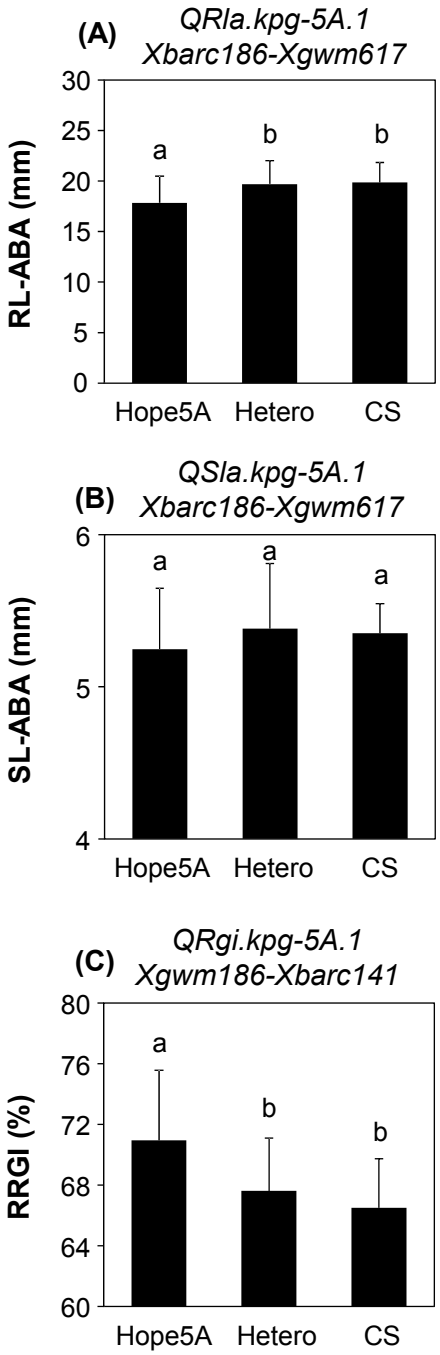

Figure III-2. The genotype effect at identified QTLs for ABA responsiveness. Lower values of RL-ABA (A) and SL-ABA (B), and higher values of RRGI (C) indicates higher ABA responsiveness. The genotypes at the marker interval listed above each graph were used for analysis. Mean ± standard deviation (n = 25 for Hope5A, n = 44 for heterozygotes and n = 19 for CS) with the same letter were not significantly different ($P > 0.05$, Tukey-Krammer HSD test).

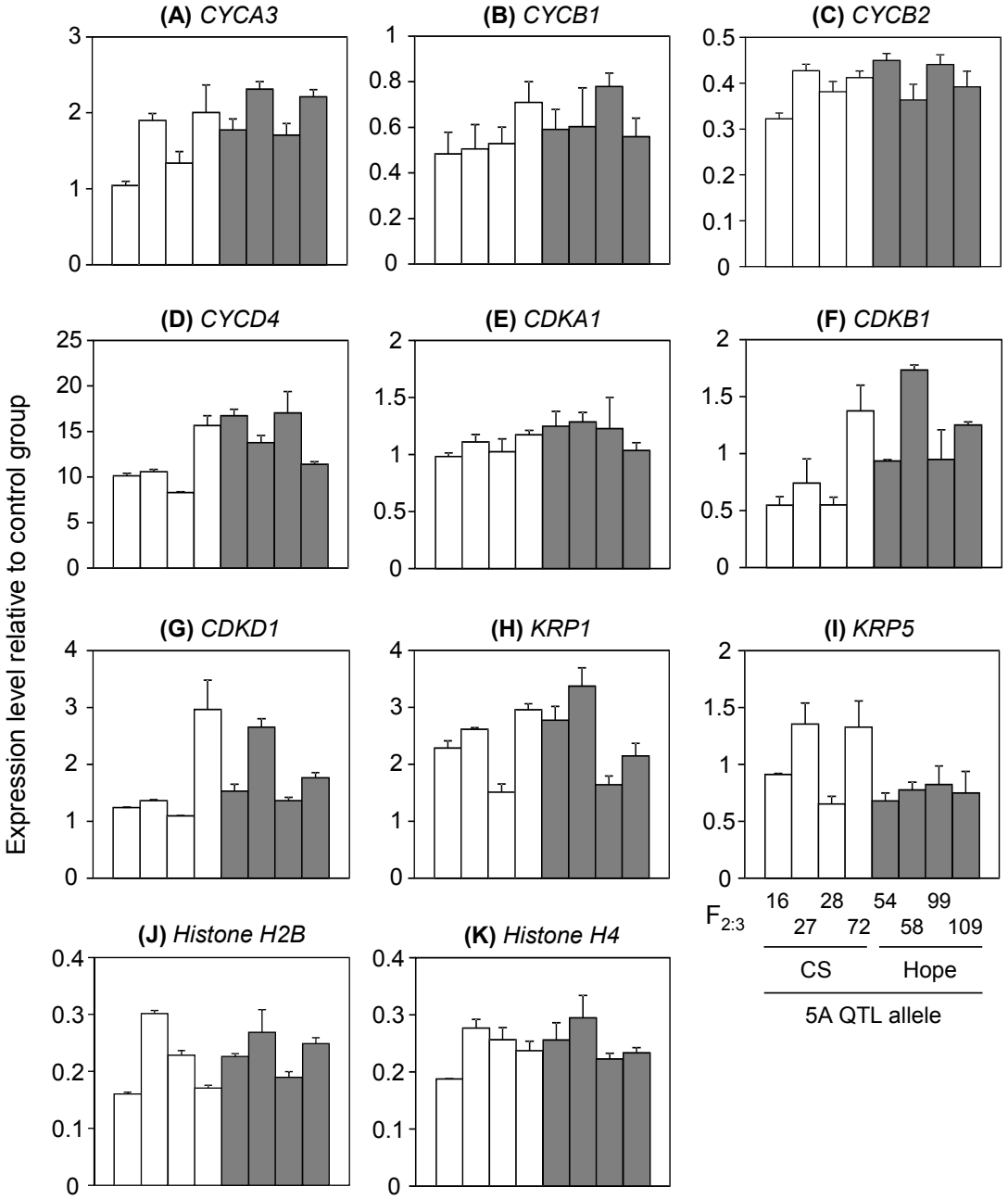

Figure III-3. Expression analysis of genes involved in cell-cycle regulation in root. Transcript accumulations of *Cyclin* (A-D), *CDK* (E-G), *KRP* (H and I) and *Histone* (J and K) genes were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 µM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying CS and Hope alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

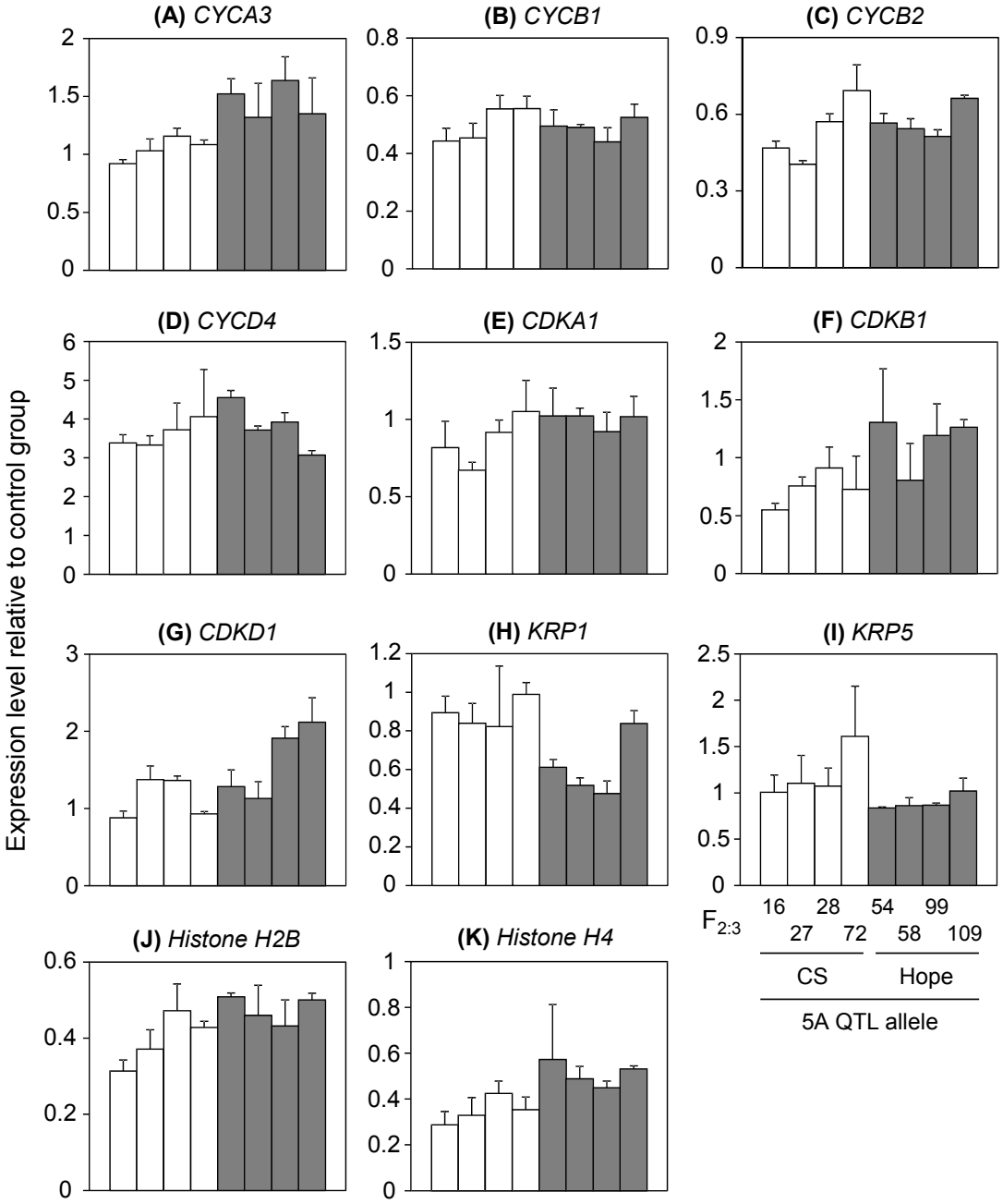

Figure III-4. Expression analysis of genes involved in cell-cycle regulation in shoot. Transcript accumulations of *Cyclin* (A-D), *CDK* (E-G), *KRP* (H and I) and *Histone* (J and K) genes were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 µM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying CS and Hope alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

the effect of the identified QTL on the expression of *TaABA8'OH1*, *TaABA8'OH2* and *WABI5*. Although the expression level of all these genes did not differed significantly between the two genotype classes in root and shoot (Figure III-5), the expression level of *TaABA8'OH2* tended to be higher in the root of individuals carrying the CS allele (average of 0.80 [CS allele] vs. 0.35 [Hope allele], Figure III-5C). This result suggests that the catabolism of ABA is higher in individuals carrying the CS allele at the 5A QTL. The ABA content of the 1-day-old seedling, just before ABA treatment, did not differed significantly between CS and Hope5A but was slightly higher in this latter (Figure III-6).

4-4. Effect of the QTL on abiotic stress tolerance

First, salinity tolerance was evaluated by weight gain of seedlings grown under stress conditions (0.2% Hyponex solution [Hyponex Japan] containing NaCl). The concentration of NaCl was increased 25 mM per day to finally keep 125 mM. Salinity tolerance did not differ between individuals carrying the CS- and Hope5A-type QTL in this experimented condition (Figure III-7).

Because ABA induces stomatal closure during drought stress to limit water loss, the stomatal response during leaf dehydration was evaluated based on the rate of water loss from detached leaves. In individuals with Hope5A-type QTL, the water loss rate tended to be lower than individuals with CS-type QTL (Figure III-8A). At 6 h after leaf detachment, the mean water loss rate in plants carrying CS allele was 32.24 ± 1.45% and those carrying Hope5A allele was 28.90 ± 1.94% but this difference was not significant ($P = 0.06$). We also tested the long-term dehydration tolerance evaluating the growth rate under limited water condition. Five day after sowing, irrigation was withheld to finally keep soil moisture of ~25%. The growth rate, evaluated based on the plant height relative to control group, decreased greatly after nine days of water withholding (Figure III-8B). In this experimental condition, we could not detect the difference in growth rate between both alleles.

To test whether the QTL for ABA responsiveness regulate stress inducible genes, expression analysis during dehydration was performed. The *Cor/Lea* gene *Wrab18* expression tended to be higher in plants carrying highly ABA-responsive parental allele, at 8 h after dehydration treatment (Figure III-9A). Expression level of *Wdhn13* was also slightly higher in these individuals from 4–8 h after dehydration (Figure III-9B). In contrast, the expression of *Wrab17* and *Wrab15* did not differ between both alleles (Figures III-9C and III-9D). The transcription factor gene *WABI5* was also differentially expressed, being significantly higher ($P < 0.05$) in individuals with Hope5A allele

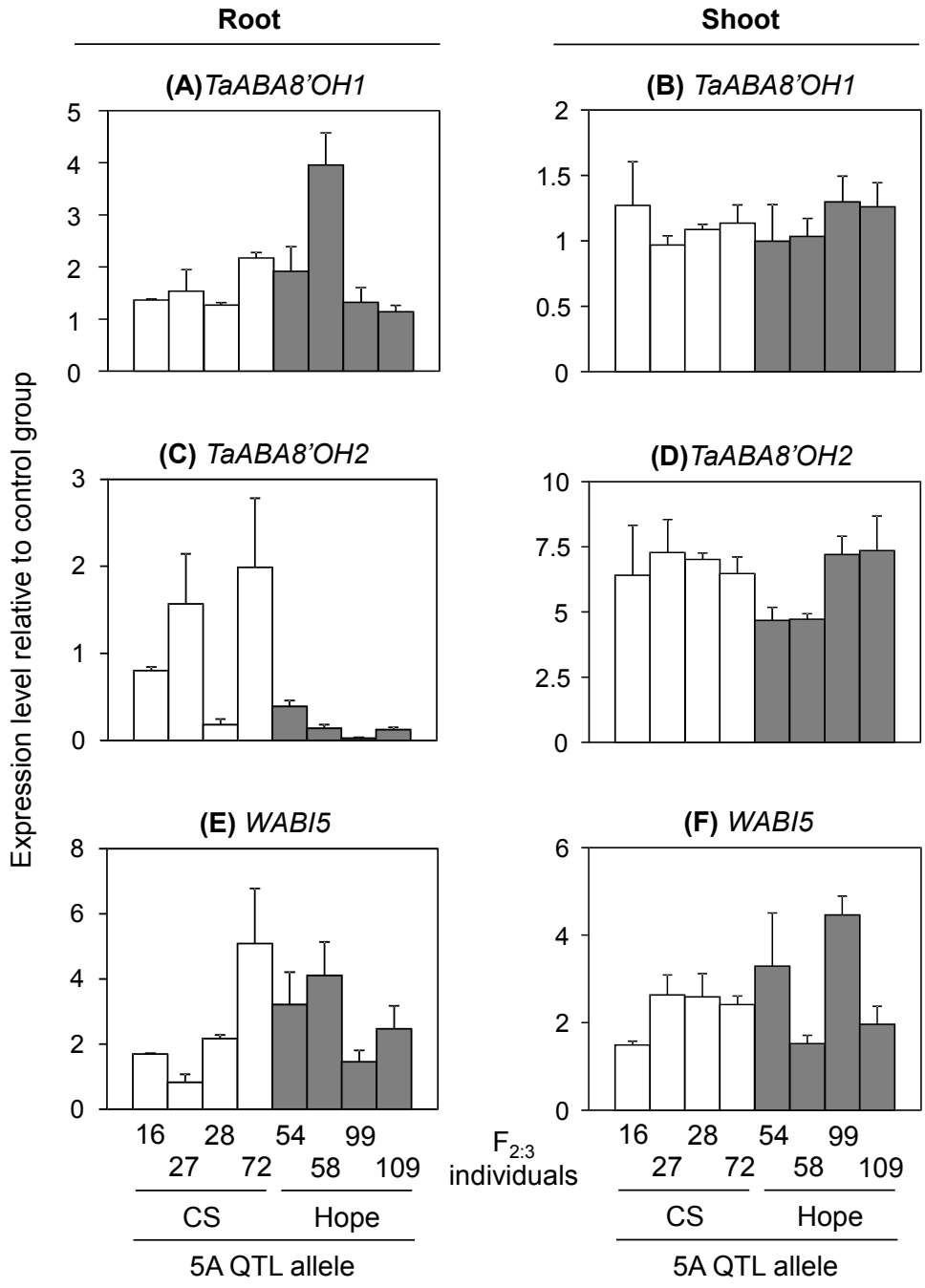

Figure III-5. Gene expression analysis of the ABA catabolic enzymes and *WABI5*. Transcript accumulations of *TaABA8'OH1* (A and B), *TaABA8'OH2* (C and D) and *WABI5* (E and F) were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 μM ABA. Total RNA was extracted 48 h after treatment from root (A, C and E) and shoot (B, D and F), and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying CS and Hope alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

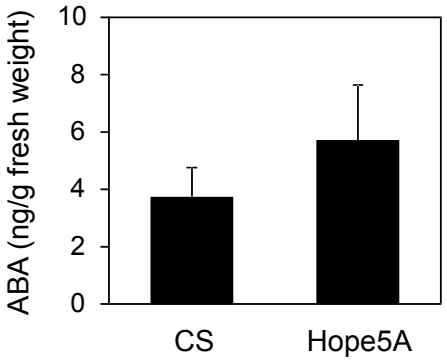

Figure III-6. ABA content in whole seedlings of CS and Hope5A. ABA was quantified using 1-day-old seedlings (n = 4), including endosperm. Data are mean ± SD of three independent experiments.

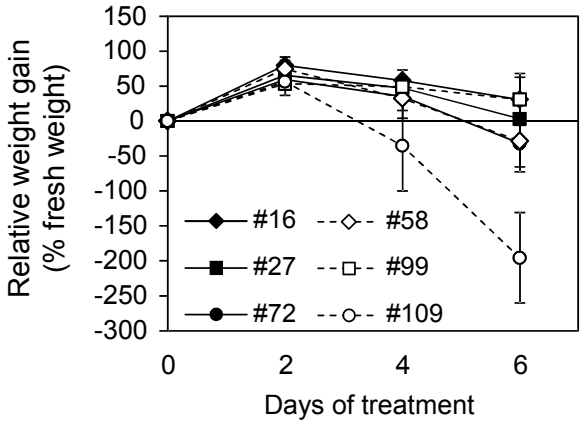

Figure III-7. Salinity tolerance of the selected F_2-derived F_3 plants. Weight gain was determined in 7-day-old seedlings grown in 0.2% Hyponex solution containing NaCl. The concentration of NaCl was increased 25 mM per day to finally keep 125 mM. The percentage of weight gain of individuals carrying CS allele is indicated by filled symbols and solid lines, and those carrying Hope allele by open symbols and broken lines. Data are means ± SD of 11–12 individuals.

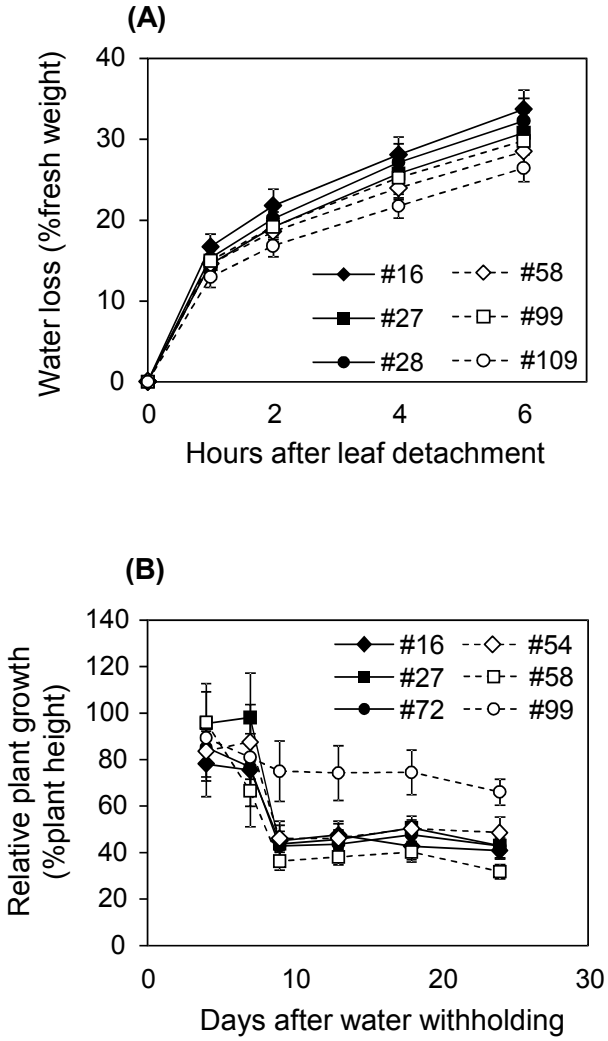

Figure III-8. Dehydration tolerance of the selected F_2-derived F_3 plants. (A) Water loss from detached leaves. The first leaf of 7-day-old seedlings (n = 10) was detached and its fresh weight was measured at the indicated times. (B) Growth rate of drought stressed plants. Irrigation of 5-day-old seedlings (n = 11) was withheld to finally keep soil moisture of ~25%. The growth rate was evaluated based on the plant height relative to control group. Mean ± SD of individuals with CS allele is indicated by filled symbols and solid line, and those with Hope allele by open symbols and broken lines.

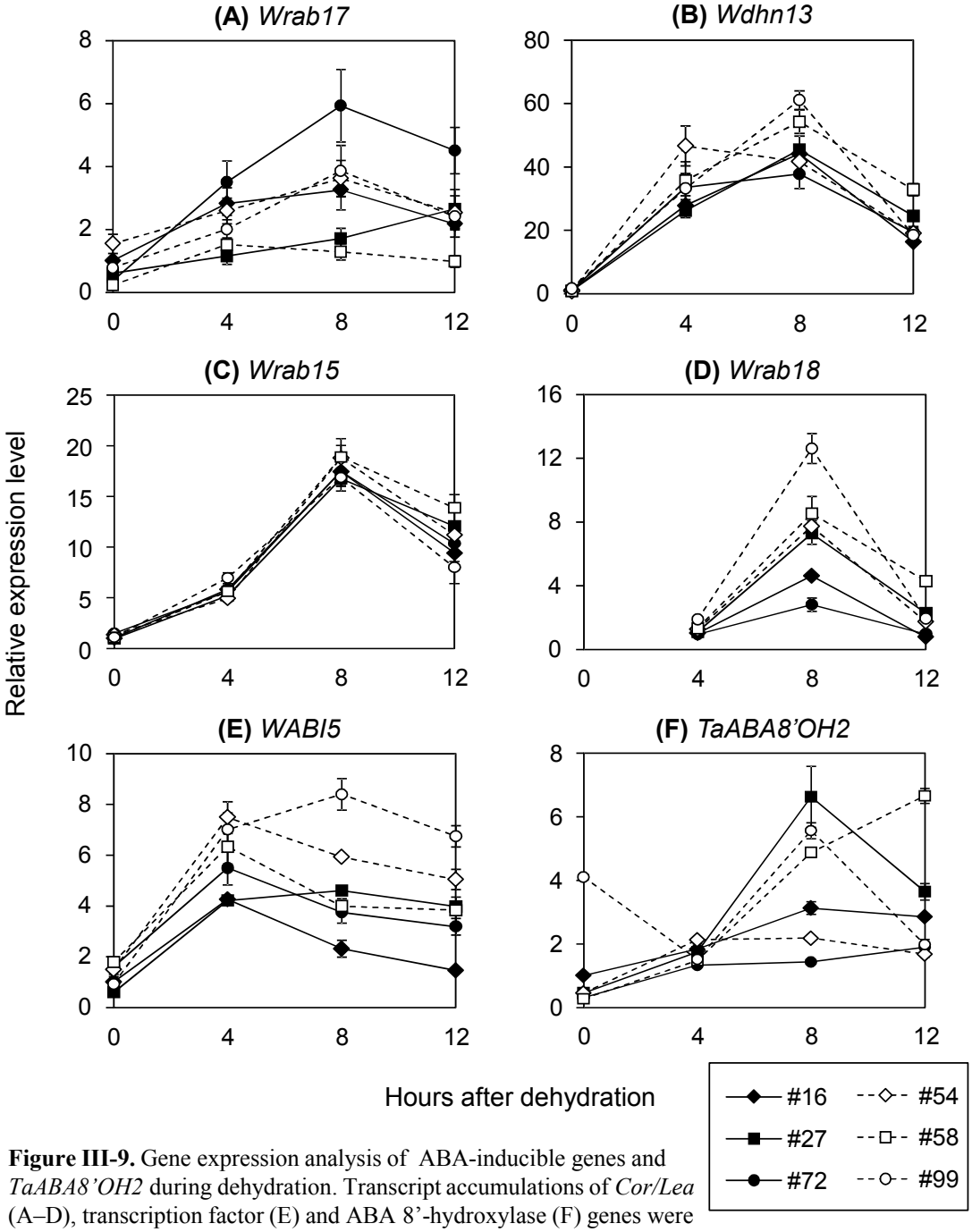

Figure III-9. Gene expression analysis of ABA-inducible genes and *TaABA8'OH2* during dehydration. Transcript accumulations of *Cor/Lea* (A–D), transcription factor (E) and ABA 8'-hydroxylase (F) genes were analyzed by real time RT-PCR. Total RNA was extracted at indicated times after dehydration treatment from leaves. Transcript accumulation of individuals carrying CS and Hope alleles are indicated by solid and broken lines, respectively. *Actin* was used as internal control. Data are mean ± SD relative to individual #16 at 0 h.

(6.94 ± 0.58) than in those with CS allele (4.65 ± 0.73) at 4 h after dehydration (Figure III-9E). On the other hand, *TaABA8'OH2* expression did not differ clearly between the two alleles (Figure III-9F).

4-5. Effect of the identified QTL on seed dormancy

ABA is also involved in the regulation of seed dormancy (Finkelstein et al. 2002). The seeds of each selected F_2-derived F_3 individuals, were imbibed at 20°C for 5 days. No germinated seeds were observed at 35 DPA, and at 45 DPA the germination percentage ranged from 0–18.33% (Figures III-10A and III-10B). At 55 and 65 DPA, germination started from 2 days after imbibition and almost all of the seeds were germinated after 5 days of imbibition in most of the individuals (Figures III-10C and III-10D). There was no significant difference in GI between the two alleles (Figure III-10E), suggesting that the 5A QTL for ABA responsiveness has little or no effect on seed dormancy.

5. Discussion

The phytohormone ABA plays an important role in responses to environmental stress as well as in seed maturation and dormancy (Finkelstein et al. 2002). In our previous studies, ABA responsiveness at the seedling stage was evaluated based on RRGI because the cultivar difference is clearer in this parameter than RSGI (Kobayashi et al. 2010; Chapter II). In this study, we analyzed the correlation among growth rates of root and shoot in F_2 plants of Hope5A/CS population. A positive and significant correlation was observed between RRGI and RSGI as in previous study (Chapter II). However, SL differed significantly between parental lines (Table III-2) and it was reflected in RSGI and SL-ABA (Table III-3). In contrast, no significant difference was observed in RL between parents and it was not reflected in RRGI and RL-ABA, indicating that RL-ABA is sufficient in the evaluation of ABA responsiveness in this mapping population. In fact, QTL was identified for RL-ABA and RRGI with similar LOD score and contribution, SL-ABA presented LOD score lower than that for RL-ABA and RRGI, and no QTL was detected for RSGI (Table III-4). Therefore, QTL for ABA responsiveness was equated to the QTL region of *QRgi.kpg-5A.1* and *QRla.kpg-5A.1*.

Post-germination growth arrest by ABA in root and shoot could be explained by the down-regulation of B-type cyclins and histones (Figure III-3 and III-4). B-type cyclins are known to accumulate at G2 and early mitosis (Menges et al. 2005), suggesting that ABA blocks the G2/M transition by down-regulating these cyclins in both tissues. In addition, a CDK inhibitory protein-coding gene, *KRP1*, was up-regulated after ABA treatment in root but not in shoot (Figure III-3H and III-4H).

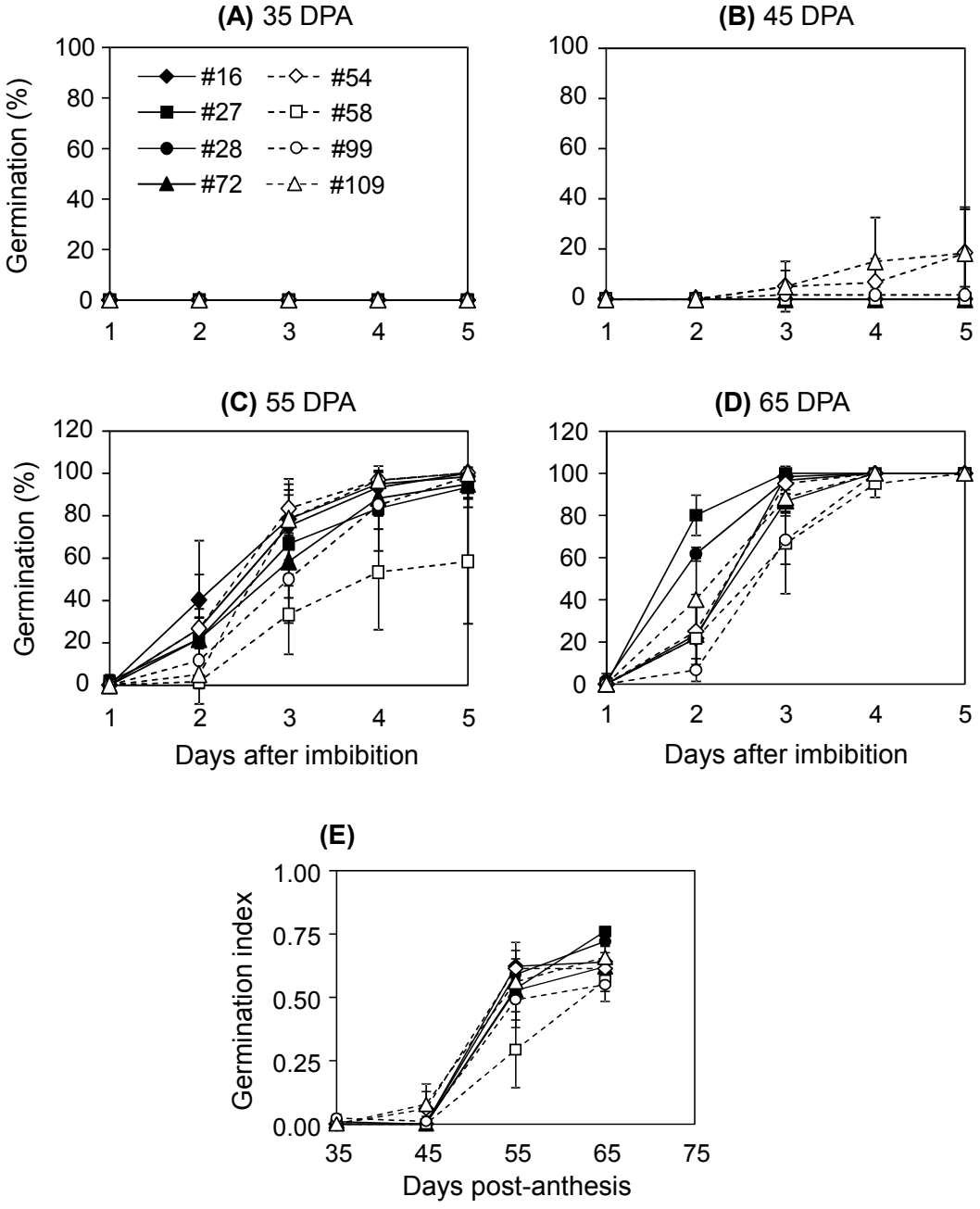

Figure III-10. Analysis of seed dormancy using the selected F_2-derived F_3 seeds. Germination rate at 35 days post-anthesis (35 DPA, A), 45 DPA (B), 55 DPA (C) and 65 DPA (D). Data are mean ± SD of four independent experiments using fifteen freshly harvested seeds. Individuals carrying CS and Hope alleles are indicated by solid and broken lines, respectively. Based on these data, GI was also calculated (E).

KRP inhibits the formation of cyclin-CDK complex at G1/S phase transition (Menges et al. 2005; Van Leene et al. 2010), suggesting a growth arrest by ABA at this phase in root. These results indicate that post-germination growth arrest by ABA between root and shoot is controlled in part by different mechanisms. We could not detect the effect of allelic difference in the expression analysis of cell cycle regulators.

During post-germination root growth, the *TaABA8'OH2* transcripts accumulation was down-regulated by ABA treatment in individuals with Hope-type 5A QTL but not in those with CS allele (Figure III-5C). Because the ABA content in 1-day-old seedlings, just before ABA treatment, was slightly higher in Hope5A than CS, it might explain in part the higher ABA responsiveness of Hope5A. Nakamura et al. (2010) reported that *TmABA8'OH2* is located on the centromeric region of chromosome $5A^m$ and is linked to the QTL for seed dormancy in diploid wheat. Comparison between the QTL maps and physical map of barley chromosome 5H (IBGSC 2012) showed that *ABA8'OH2* and *WABI5* is located around the peak of QTL for ABA responsiveness (Figure III-11). The expression level of *WABI5* at this stage was similar between the two alleles in root and shoot (Figure III-5E and III-5F). Although the QTL for ABA responsiveness was located in a similar chromosomal region to the QTL for seed dormancy, it seems not to be associated with seed dormancy (Figure III-10).

ABA is also known to induce stomatal closure during drought stress to limit water loss. In individuals carrying Hope allele at the 5A QTL, stomatal response and transcript accumulation of *Wrab18*, *Wdhn13* and *WABI5* during dehydration tended to be higher than those with CS allele (Figures III-8A and III-9). Cultivar difference in the expression levels of *Cor/Lea* genes and *WABI5* were associated with the difference in freezing tolerance (Tsuda et al. 2000; Kobayashi et al. 2004, 2008c). Of the genes found to be located on the QTL region for ABA responsiveness, *TaABA8'OH2* was regulated differentially by ABA during post-germination growth between the two alleles but not *WABI5*. Conversely, allelic difference in *WABI5* expression was observed during dehydration but not in *TaABA8'OH2* expression. One possibility to explain these observations is the mutation in the coding sequence of *TaABA8'OH2*, which results in higher catabolism of ABA in individuals with CS allele. Another possibility is the difference in the promoter region of *WABI5* causes a higher expression of this transcription factor in individuals with Hope allele. This differential expression might be visible at early stages of ABA treatment during post-germination growth and not after 48 h of treatment (Figure III-5E). We cannot discharge the possibility of other causal gene.

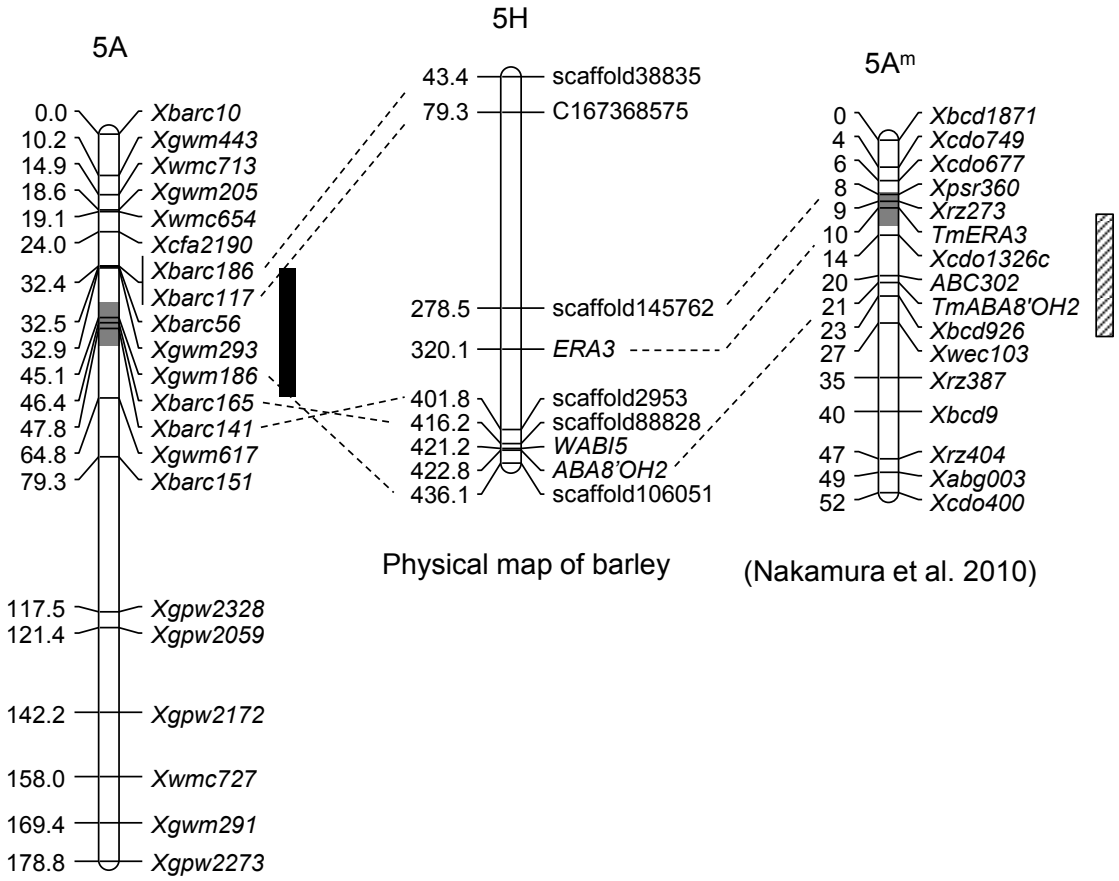

Figure III-11. Comparative map of QTL regions for ABA responsiveness and seed dormancy. Linkage map constructed in this study (left) was compared with the physical map of barley (middle) and the linkage map of diploid wheat (right). SSR markers were first aligned to the A genome of *T. urartu* and then the obtained hits were blastn searched against the genomic sequence of barley. Black bar indicates the QTL region for ABA responsiveness and striped bar that for seed dormancy. Gray bars indicate the putative centromeric region. Genetic distance are given in cM and the physical distance in megabase pair.

Because QTL for total dry matter under water-limited condition has been identified in the centromeric region of chromosome 5A in tetraploid wheat mapping population (Peleg et al. 2009), this region might be associated with dehydration tolerance as well as seed dormancy. However, we failed in the detection of association between the identified 5A QTL and long-term dehydration and salinity tolerance in our experimental conditions (Figures III-7 and III-8B). Drought tolerance has been found to be a complex quantitative trait controlled by a large number of genes or QTLs with small effects (reviewed in Mir et al. 2012). Our results suggest that the identified QTL for ABA responsiveness has a small effect on dehydration tolerance, and might contribute in the improvement of wheat drought tolerance by pyramiding with other genes/QTLs. It can be effectively performed using modern breeding approaches such as marker-assisted recurrent selection or genomic selection (Mir et al. 2012).

Chapter IV

Discovery of high-confidence single nucleotide polymorphisms from large-scale *de novo* analysis of leaf transcripts of *Aegilops tauschii*, a wild wheat progenitor

1. Abstract

Construction of high-resolution genetic maps is important for genetic and genomic research, as well as for molecular breeding. Single nucleotide polymorphisms (SNPs) are the predominant class of genetic variation and can be used as molecular markers. *Aegilops tauschii*, the D-genome donor of common wheat, is considered a valuable genetic resource for wheat improvement. In a previous study implied that *Ae. tauschii* accessions can be genealogically divided into two major lineages. In this study, the transcriptome of two *Ae. tauschii* accessions from each lineage, lineage 1 (L1) and 2 (L2), was sequenced, yielding 9435 SNPs and 739 insertion/deletion polymorphisms (indels) after *de novo* assembly of the reads. Based on 36 contig sequences, 31 SNPs and six indels were validated on 20 diverse *Ae. tauschii* accessions. Because almost all of the SNP markers were polymorphic between L1 and L2, and the D-genome donor of common wheat is presumed to belong to L2, these markers are available for D-genome typing in crosses between common wheat varieties and L1-derived synthetic wheat. Due to the conserved synteny between wheat and barley chromosomes, the high-density expressed sequence tag barley map and the hypothetical gene order in barley can be applied to develop markers on target chromosomal regions in wheat.

2. Introduction

Common wheat (*Triticum aestivum* L., genome constitution AABBDD) is an allohexaploid species that arose by natural hybridization between tetraploid wheat *Triticum turgidum* L. (AABB), including emmer and durum wheats, and a diploid wild wheat relative *Aegilops tauschii* Coss. (DD) (Kihara 1944; McFadden and Sears 1944). *Ae. tauschii* is widely distributed in Eurasia and shows abundant genetic variation (Dudnikov and Goncharov 1993; Dvorak et al. 1998; Dudnikov and Kawahara 2006; Matsuoka et al. 2007, 2008, 2009; Takumi et al. 2009a; Kajimura et al. 2011). Population structure analyses revealed two major phylogenetic lineages and an HG17 minor lineage [haplogroup lineage (HGL) 17] in *Ae. tauschii* (Mizuno et al. 2010a). In turn, the major lineages 1 (L1) and 2 (L2) can be genealogically divided into six and three sublineages, respectively. It is supposed that the *Ae. tauschii* populations involved in the origin of common wheat are limited to a narrow distribution range, apparently

restricted to L2, which has given rise to a founder effect in hexaploid wheat (Feldman 2001; Mizuno et al. 2010a). In fact, the D-genome of common wheat is less polymorphic than the A- and B-genomes (Cadalen et al. 1997; Chao et al. 2009; Allen et al. 2011). Tetraploid wheat and *Ae. tauschii* can be crossed artificially to produce synthetic hexaploid wheat (Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004). These synthetics can be used as intermediates to exploit the natural variation of *Ae. tauschii* in hexaploid wheat improvement (Trethowan and Mujeeb-Kazi 2008).

High-resolution genetic map construction is important for genetic and genomic research as well as for molecular breeding (Yano 2001). In wheat, genetic maps are commonly constructed using simple sequence repeats (SSR) markers (Röder et al. 1998; Somers et al. 2004), but the number of SSR markers reported in public databases such as the National BioResource Project (NBRP) KOMUGI web site (http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp) and GrainGene web site (http://wheat.pw.usda.gov/GG2/maps.shtml) are not sufficient for high-resolution maps. Although SSR are the most popular marker system, the constructed map resolution remains low in plant species without a known genome sequence. An alternative marker system, single nucleotide polymorphism (SNP), has received considerable attention because it is the predominant class of genetic variation (Deschamps and Campbell 2010). High-throughput SNP-typing systems can be developed for organisms with a reference genome or comprehensive expressed sequence tag (EST) database, e.g., barley (Close et al. 2009).But in many plants having large and complex genomes and insufficient reference information, genome-wide SNP discovery is insufficient because of the presence of highly repetitive regions. In *Ae. tauschii*, an organism without a reference genome and composed of highly repetitive regions, annotation-based genome-wide SNP discovery has been developed to overcome these problems. In this strategy, Roche 454 shotgun reads with low genome coverage of one genotype were annotated, and then genomic and cDNA shotgun reads of another genotype were generated on SOLiD or Solexa platforms to identify putative SNPs. However, around 56% of the sequence length, characterized as a repetitive region, was excluded from the analysis (You et al. 2011).

In the present study, RNA-seq, a next generation sequencing technology for transcripts, was used as a cost-effective, simpler means of SNP discovery than annotation. SNPs, insertion/deletion polymorphisms (indels), and SSRs were discovered from *de novo* assembly data from ESTs of two *Ae. tauschii* accessions, PI476874 (L1) and IG47182 (L2), that were mapped on an SSR linkage map using an F_2 population (Koyama et al. 2012). We show that SNP markers can be developed in a chromosomal

region of interest using the low-coverage genome sequence of wheat chromosome 7D (Berkman et al. 2011) or barley GenomeZipper results obtained from a synteny model conserved among barley, *Brachypodium*, rice and sorghum (Mayer et al. 2011).

3. Materials and methods

3-1. Plant material, RNA extraction and next generation sequencing

Twenty *Ae. tauschii* accessions from each sublineage were used (Table IV-1). Two *Ae. tauschii* accessions, PI476874 from the L1-2 sublineage and IG47182 from the L2-2 sublineage, were used for cDNA sequencing. Total RNA was isolated from leaves of 21-day-old seedlings using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). mRNA was isolated from 45 μg of RNA using an mRNA Purification Kit (Takara-Bio, Ohtsu, Japan). A 200 ng aliquot of mRNA was used to fragment the mRNA and synthesize cDNA from it using a cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany). Approximately 10^8 adapter-ligated cDNA molecules from the samples were used for library preparation using a GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics). The library was sequenced with a GS FLX Titanium Sequencing Kit on a GS FLX System (Roche Diagnostics) according to the manufacturer's instructions. Files containing raw sequence data were deposited in the sequence read archive of the DNA Data Bank of Japan (DDBJ) (accession number: DRA000536).

3-2. Assembly, SNP and indel discovery, and SSR mining

All reads from both accessions were pooled and *de novo* assembled with the GS *de novo* assembler algorithm (Newbler) version 2.6 (Roche Diagnostics) to generate reference contig sequences. During assembly, primer sequences and poly-A tails were trimmed from raw reads; parameters of a minimum overlap length of 40 bp and minimum overlap identity of 90% were used. SNPs and indels were discovered by aligning all individual reads to the reference contig sequences using GS Reference Mapper version 2.6 (Roche Diagnostics). Only the accession-specific sequence variants (supported by at least two reads) were extracted as true polymorphisms from "All" and "High-Confidence" (HC) sequence differences produced by GS Reference Mapper. SSR motifs within the reference contig sequences were identified by Sputnik software (http://espressosoftware.com/sputnik/index.html), and indels were searched for within these motifs to extract polymorphic SSRs.

Table IV-1. Lineage, sublineage, accession number and origin of *Ae. tauschii* accessions used in this study

Lineage – sublineage	Accession number (country)
L1 – 1	IG48508 (Turkmenistan), KU-2627 (Afghanistan)
L1 – 2	PI476874 (Afghanistan), IG126387 (Turkmenistan)
L1 – 3	KU-2826 (Georgia), KU-2087 (Iran)
L1 – 4	IG131606 (Kyrgyzstan), IG48559 (Tajikistan)
L1 – 5	IG48747 (Armenia), KU-2144 (Iran)
L1 – 6	AT47 (China), AT76 (China)
L2 – 1	KU-2069 (Iran), KU-2811 (Armenia)
L2 – 2	IG47182 (Azerbaijan), KU-2100 (Iran)
L2 – 3	KU-2159 (Iran), KU-2093 (Iran)
HGL17	AE454 (Georgia), AE929 (Georgia)

Underlining indicates the accessions used for RNA-seq.

HGL: haplogroup lineage.

3-3. Gene annotation and analysis of synonymous and non-synonymous mutations

The reference sequences were aligned with the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database and the *Brachypodium distachyon* (version 1.2, ftp://ftpmips.helmholtz-muenchen.de/plants/brachypodium/v1.2), rice (RAP-DB, http://rapdb.dna.affrc.go.jp/) and wheat TriFLDB protein databases (http://trifldb.psc.riken.jp/index.pl) using BlastX with an *E*-value cut-off of 10^{-3}. Gene ontology (GO) terms were assigned using Blast2GO (Conesa et al. 2005) and a locally installed database based on BlastX hits against the NCBI nr database.

To study whether the discovered SNPs and indels affect amino acid sequences, the longest open reading frames (ORFs) of the isotigs that include all the 1793 contigs with HC polymorphism were extracted using EMBOSS (Rice et al. 2000). The isotigs without any BlastX result, described above, were excluded from analysis. Only the ORFs supported by the BlastX search were selected.

3-4. SNP and SSR validation

Validation of identified SNPs was performed through cleaved amplified polymorphic sequence (CAPS), derived CAPS (dCAPS) and high-resolution melting (HRM) methods. Gene-specific primer sequences, SNP locations, product length and restriction enzymes are summarized in Table IV-2. dCAPS primers were designed using the dCAPS Finder 2.0 program available on the website http://helix.wustl.edu/dcaps/dcaps.html (Neff et al. 2002). The polymerase chain reaction(PCR) conditions for CAPS and dCAPS markers were 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 30 s, 60°C (for CAPS) or 56°C (for dCAPS) for 30 s, and 68°C for 30 s. HRM analysis was performed using a LightCycler 480 Real-Time PCR System and LightCycler 480 HRM Master 2x reagents (Roche Diagnostics) according to our previous study (Matsuda et al. 2012). For HRM analysis, all samples were spiked with 10-50% of PI476874 DNA to facilitate discrimination of the homozygous genotype. All PCR products were checked on a 1.5% agarose gel to ensure the presence of a single band.

SSR markers, depending on the indel length, were amplified following the same conditions for CAPS markers or were analyzed by HRM as described above. The polymorphic information content (PIC) was calculated using Excel Microsatellite toolkit add-in software (Park 2001).

Table IV-2. List of the 37 validated markers

Marker name	Primer sequence	Product size (bp)	Type	Restriction enzyme	Number of SNPs
Ctg01655	GGCTCCGAAGACGAACTCC CTGCCATTGCCGTCTTCT	101	HRM†	-	4
Ctg01754	GCGCAAAGAAGCAGGATAAG CGTAAAGTATGGAGGCTGTGG	112	HRM	-	1
Ctg02103	GGTGTATTTCGGCACGACTT TTGCCACCATCCATTACAAA	269	CAPS	*Msp*I	3 (1)
Ctg02121	GGGTGAGGACAAGCAGTTTT GGTGACAACAGCATTCTTAATAGTT	112	HRM	-	1
Ctg02636	CATGGAGCCCATCGTGTT CTCTTGAGTTCTGCCCATCTG	200	HRM	-	1
Ctg02664	CATTCTGCGTATTGCTTCCA CTCATCCAATGCTTCCGATT	101	HRM	-	1
Ctg02686	TCTTATAATTAACCGCGAGTGAG GAATTTGTTGTTGTTGTAAGATGTTC	101	CAPS	*Xho*I	1
Ctg02996	GGCCTTTGTTTCATCAGCAT GGGCAGCATGGTAGAAGTTG	200	CAPS	*Afa*I	2 (1)
Ctg03005	TGGCAGGTTATTAGCCCAGT CCTTGACACTCTTTGCCACA	118	HRM	-	1
Ctg03017	TCCAACTAAAGGCAACGGATA GGAGGCAATCAAGCATGTG	102	SSR	-	0
Ctg03055	AGAGGTCTCCTGTTGGCTGA TGATCCTCATCCTGGAAACC	350	CAPS	*Mbo*I	1
Ctg05103	GCCTTCATCTCGGTGGTCT ACCACCACTACCACCACCAC	113	HRM	-	1
Ctg05115	GCGGTTTCCAGGAGACAATA CGGACTTGGTTGAGTCCAAT	178	CAPS	*Msp*I	3 (2)
Ctg05139	CTTCACATGTGGGCAATCAG CCTTGCCATTGTGTTCAACTT	150	CAPS	*Acc*I	1
Ctg05151	AAGGTGGACGTGCAGAACAT TTGTAAGCGACGGAGGAGAT	257	CAPS	*Hae*III	1
Ctg05162	TCAACACCATTCGCCTCT GTTCTTCAGGTGCAATTGAC	172	CAPS†	*Hha*I	1

Table IV-2. Continued

Marker name	Primer sequence	Product size (bp)	Type	Restriction enzyme	Number of SNPs
Ctg05193	TGAGCAGCATCTGAATCTGG AAAGTTCCTCTCGGCATCCT	181	CAPS	*Hha*I	1
Ctg05203	GAAAGCACACAGCGACAAGA CACAGGCACTTCCACTGAGA	178	CAPS	*Msp*I	1
Ctg05208	GCCACCTAAAATCCAGCTCA TTTGACAAAGTTGAGGATACCG	97	HRM	-	1
Ctg05233	AAACTGAACCAACAATTTGC GTACAACATCAGCCGTCATT	130	CAPS	*Afa*I	4 (1)
Ctg05247	TCTGCTTGACAACCTTCTTGG GCGTAGGAAGACAGGAGGAA	101	SSR	-	0
Ctg05251	GCACGGTCCAGGAATGTAGT CCGGGCGGTAGTACTGGTAG	175	CAPS	*Hha*I	2 (1)
Ctg05253	TGCCGTAACGGGCTAGTG TTAGTGGTGCCAGCATCTTCT	200	CAPS	*Afa*I	1
Ctg05313	ACCAAACTGTTGTCCCCTTG GCTGCTGGACACAAAAGACA	200	CAPS	*Hha*I	1
Ctg05381	CCAGGTAGGAAGATGCTTCAAG GCTGAGAGTTGTACGTTTGTGC	202	CAPS	*Bci*T130I	2 (1)
Ctg05398	CCTTTCCAGAGAAGTCGGATC TAAGTGGAAGCAACTCGAGG	175	dCAPS	*Bam*HI	1
Ctg05611	CCTCATTCATAATAGAGTCAAATGGA TGGACAGATTGTCACATGGATTA	101	HRM	-	1
Ctg06225	ATCGCTGGCTCGAGTGTAGT CCATCAAGCTAGCCCAGAAA	146	SSR	-	0
Ctg06571	CCTGAAGCCAAACCAATAAGAC TCTAAGGACAAAGCACATTTCAGAT	83	HRM	-	1
Ctg06577	ATGATCCTGCTGGTGAAGATGT GAGGAAGATGCAGACGGAGTAG	93	HRM†	-	1
Ctg06721	TGCTACTGATCATAACTAGTGCAATTA ACGTAGTAGCGTGGCACTCC	135	SSR†	-	0
Ctg06827	AATGCTTTTTGGCCATTAATTGTA TTCTTACACCTAAGTACACCCCAAG	93	HRM	-	1

Table IV-2. Continued

Marker name	Primer sequence	Product size (bp)	Type	Restriction enzyme	Number of SNPs
Ctg06827	GGCGAGGAGGACGACTAGAT CCCAAGGTCTCACGTACACA	102	SSR*	-	1
Ctg07225	CAGCAGCAAGGTGTTCTTGC AATAACTGCGGATTCTTTGG	157	dCAPS	*Hha*I	1
Ctg07326	GTTTCCTTTTCCTGCGTCAA ACGACGACGACATCTTTGC	107	SSR*†	-	0
Ctg07532	ACACTCTTATTGAGGAAGTGCTTTG GCAGGTGGGAGAAAAAGATTCTA	106	HRM	-	2
Ctg07833	TCAGTTGCCTGGATCTCGTAGT AGATGCTGTACTCCACCTCCAA	76	HRM	-	1

The name of validated makers, primer sequence (from 5' to 3'), PCR product size, marker type, restriction enzyme used for CAPS and dCAPS markers and the number of SNPs in the amplicon is indicated. Number in the parenthesis indicates the number of SNPs recognized by restriction enzyme. Underlining indicates the mismatches introduced in the dCAPS primers. *HRM, †Lower confidence polymorphic site.

3-5. Comparison of HC SNPs and previously reported SNP dataset

For all of the 4337 HC SNPs, 100 bp sequences were extracted from 1748 contigs, positioning SNPs in the middle of the sequence. These sequences were BlastN searched against *Ae. tauschii* gene sequences with SNPs (*E*-value cutoff of 10 and gap extension penalty of 0) reported previously and published at http://avena.pw.usda.gov/wheatD/agsnp.shtml (You et al. 2011).

3-6. Linkage map construction

A set of 104 F_2 individuals derived from a cross between PI476874 and IG47182 was used as the mapping population. A total of 19 cfd, 21 barc, 17 wmc, 20 gwm, 8 gdm and 2 hbg SSR markers (http://wheat.pw.usda.gov/GG2/index.shtml, http://nics.naro.affrc.go.jp/team/dna_marker/) were assigned to each chromosome as anchor markers. A genetic map was constructed using MAPMAKER/EXP version 3.0b (Lander et al. 1987). The genetic distances were calculated with the Kosambi function (Kosambi 1944).

3-7. *In silico* mapping of contigs with HC SNPs on virtual barley chromosomes

Shotgun barley genomic reads mapped on the virtual barley chromosomes were extracted from flow-sorted chromosome reads submitted by Mayer et al. (2011) (accession number ERP000445). The tBlastX algorithm was used to align the 1793 contigs with extracted barley genomic reads and against a set of 5006 full-length cDNAs (accession numbers AK248134 to AK253139) (Sato et al. 2009) and a set of 23623 full-length cDNAs (accession numbers AK353559 to AK377172) (Matsumoto et al. 2011), with an *E*-value cutoff of 10^{-1}. The Blast search results obtained against *Brachypodium* and rice database were also used.

4. Results and discussion

4-1. Sequencing and assembly of *Ae. tauschii* ESTs

To see how many SNPs could be discovered in one RNA-seq experiment between two phylogenetically distinct *Ae. tauschii* accessions, PI476874 and IG47182 (respectively from L1 and L2) were selected. The sequencing of leaf cDNA libraries produced 669383 and 700124 reads corresponding, respectively, to 247 Mb and 254 Mb per accession after trimming. All reads from both accession-derived libraries were assembled using Newbler 2.6. Newbler assembled reads into a contig representing transcript regions, isotigs (sets of contigs representing a partial or entire transcript) and isogroups (sets of isotigs). During the assembly process, Newbler constructs multiple

alignments of overlapping reads and divides them into consistent sequences, i.e. contigs. Alignments that cannot be divided are collected as isogroups, and branching structures between them are searched by traversal paths through connected branches, i.e. isotigs (isotigs and isogroups are Newbler-specific terms). Different isotigs within the same isogroup represent alternative splicing variants. Thus, an isogroup represents genes, isoforms or gene families. In this study, sequences shorter than 100 bp were excluded from the following analyses. A total of 10224 contigs were assembled into 9145 isotigs (including some contigs that were not combined into isotigs), with a total of 7753 isogroups obtained. The length of the majority of contigs and isotigs ranged between 500 and 1000 bp, with an average of 786 and 1021 bp, respectively (Table IV-3). However, among isotigs, a sequence length as long as 11328 bp was found (isotig02315), which showed sequence similarity to a *Brachypodium distachyon* E3 ubiquitin-protein ligase UPL1-like protein (XP_003575554.1, *E*-value = 0).

For functional gene annotation, isotigs were reassembled to remove redundancy and to obtain longer transcripts using CAP3 with default parameter settings (90% identity and overlap of 40 bp) (Huang and Madan 1999) according to Ewen-Campen et al. (2011). Overall, 8895 assembled sequences were produced, and sequence similarity searches were conducted against the NCBI nr, rice, *Brachypodium* and wheat protein databases using the BlastX algorithm. Of the query sequences, 90% had BlastX hits in the NCBI nr protein database. Similarly, 89% and 87% of the query sequences showed homology to annotated *Brachypodium* proteins and annotated rice proteins, respectively. On the other hand, 68% of the contig sequences had homology to wheat proteins, which might be due to the lower number of proteins deposited in the wheat database (8590 proteins) than the *Brachypodium* (31029 proteins) and rice (40353 proteins) databases.

To see whether the sequenced transcripts are functionally diverse or not, GO annotation was performed based on the BlastX hits against the NCBI nr database. In total, 7740 sequences were assigned to one or more GO annotations. Of the assigned GO terms, 13260 were under the biological process domain, 10280 under molecular function, and 9750 under cellular component (Figure IV-1). A large functional diversity of genes was found in the transcriptomic data. Because the total RNA for the transcriptome analysis was extracted from leaves, a large proportion of the assignments fell into the plastids category of the cellular component subontology.

4-2. SNP and indel discovery

Polymorphic sites were discovered using GS Reference Mapper to align individual reads from both accessions against the reference contig sequences. Among the 10351

Table IV-3. Contig and isotig sequence length distribution

Sequence length (bp)	Number of contig	Number of isotig
100–500	3300	1162
501–1000	4350	4612
1001–1500	1622	1934
1501–2000	594	828
2001–2500	199	305
>2500	159	304
Total	10224	9145
Average length (bp)	786	1021

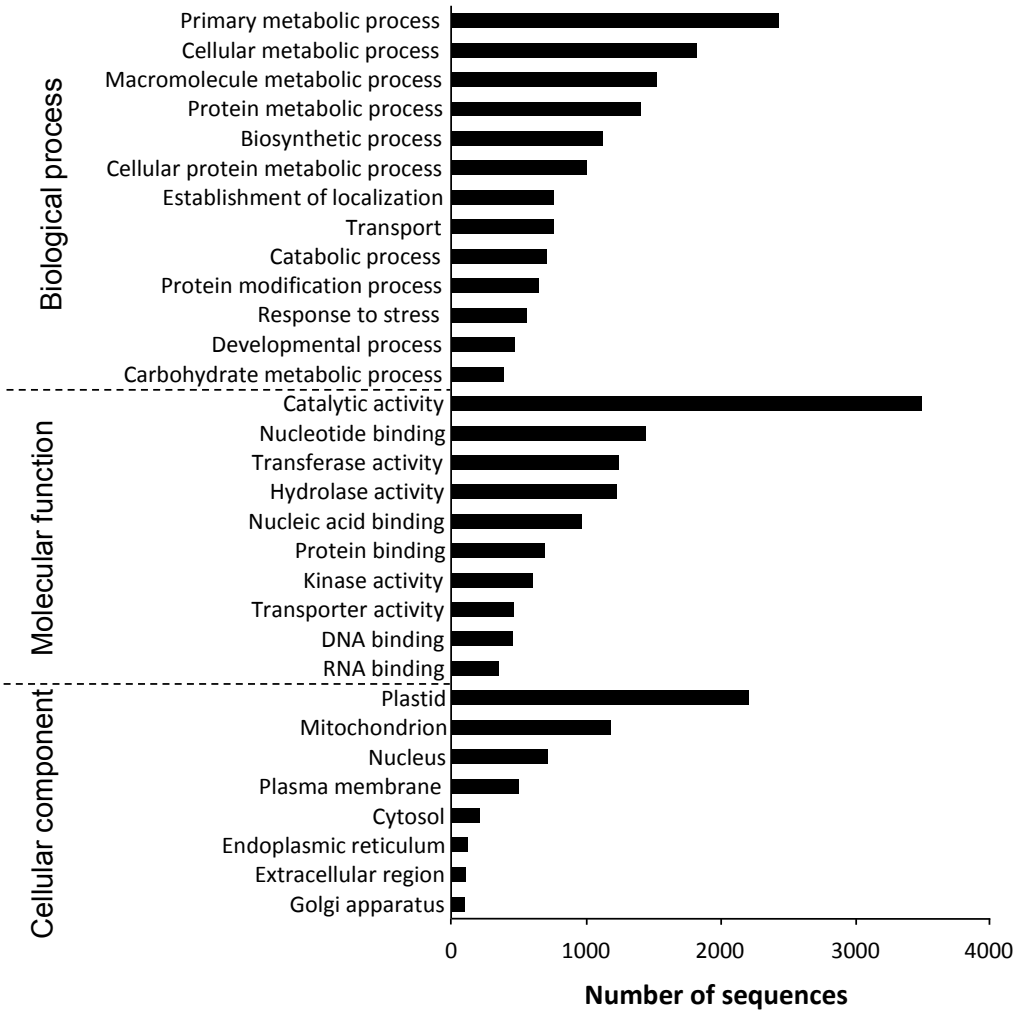

Figure IV-1. GO classification of assembled sequences. The results are summarized into three main subontologies: biological process, molecular function and cellular component. Based on BlastX results against the NCBI nr protein database, 7740 sequences were assigned to one or more GO annotations.

total polymorphic sites, 9435 were SNPs and 739 were indels (Table IV-4). Further, according to the read depth (≥ 3 nonduplicate reads) and quality (QV ≥ 20), 4578 polymorphic sites were classified as HC polymorphisms, including 4337 SNPs (in 1748 contigs), 198 indels (in 153 contigs), and 44 variants with two or more nucleotide changes. HC SNP frequency was one SNP per 1854 bp, and when considering all the discovered SNPs the frequency it was 852 bp per SNP (Table IV-4).

To study how many HC SNPs are also present in the polymorphism dataset reported by You et al. (2011), a BlastN search was performed using as query 4337 100-bp sequences, in which each of the HC SNPs were positioned in the middle. Hits were obtained for all the 4337 sequences with an *E*-value cutoff of 10, of which 546 SNPs were common to our dataset (Table IV-5). With an *E*-value cutoff of 10^{-5}, 1943 hits were obtained but the number of common SNPs between the two datasets did not differ greatly (538 SNPs). These results indicate that the majority of HC SNPs (around 3800) are only present in our dataset. Since the genome of *Ae. tauschii* is composed of large repetitive regions, only 44% of the total genomic read length has been available for SNP discovery (You et al. 2011). Our results indicate that SNP discovery by RNA-seq is cost-effective in organisms with complex genomes, as reported in maize (Hansey et al. 2012).

Although most contigs with HC polymorphisms contained one or two HC SNPs, 41 contigs had a large number of SNPs, ranging from 11 to 44 (Figure IV-2A). The number of SNPs per contig was not completely dependent on sequence length, indicated by the low coefficient of determination (R^2 = 0.05) (Figure IV-2B). To study whether these nucleotide variations affect amino acid sequences, the longest ORFs from isotigs that include contigs with HC polymorphisms were extracted. The longest ORFs supported by BlastX search were found in 1538 out of the 2214 isotigs. Synonymous and non-synonymous mutations were analyzed on contig sequences. In about 1100 out of the 1379 selected contigs, nucleotide variations were found mainly within the ORF, whereas the number of SNPs was similar between the ORF and untranslated region (UTR) in around 80 contigs. Although the number of SNPs was higher in ORFs than in UTRs, synonymous mutations predominated in most cases. Non-synonymous mutations outnumbered synonymous ones in 370 contigs. Nonsense mutations were observed in four contigs. However, we cannot discharge the possibility that the reads of paralogous genes are included in these highly polymorphic contigs.

The predominant length of indels was 3 bp, followed by 2 bp and 1 bp (Figure IV-2C). The longest indel mutation was 11 bp in contig04102 and contig05280. Indels were found in 90 out of the 1379 analyzed contigs, of which 45 contigs included indel

Table IV-4. Polymorphisms detected between two *Ae. tauschii* accessions

	Number of polymorphic sites	
	Total polymorphic sites	HC polymorphic sites
Total	10351 (3578 contigs)	4578 (1793 contigs)
SNPs	9435 (3444 contigs)	4337 (1748 contigs)
Indel	739 (539 contigs)	198 (153 contigs)
Involving two or more nucleotides	112 (100contigs)	44 (42 contigs)
Average bp per polymorphic site*	777	1756
Average bp per SNP*	852	1854

*Calculated by dividing the total number of (HC) polymorphic sites or SNPs by the total length of contigs (8039509 bp).

Table IV-5. BlastN hits against *Ae. tauschii* genomic contigs of You's dataset

E-value cutoff	Number of Blast hits	Number of common SNPs*
10	4337	546
1	3518	543
0.5	2971	540
0.1	2527	538
10^{-3}	2065	538
10^{-5}	1943	538

*Number of common SNP between the two datasets.

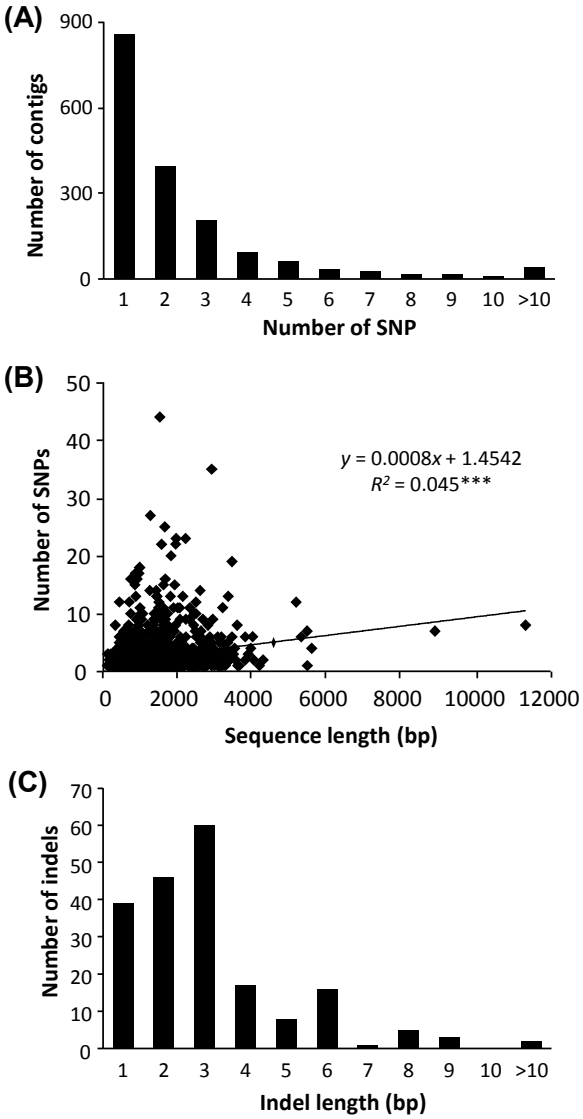

Figure IV-2. Detection of SNPs and indels in the contigs. (A) Frequency distribution of HC SNPs per contig (n = 1748 contigs). (B) Relationship between the number of SNPs per contig and the contig length; data are from 1748 contigs containing SNPs. Significant correlation is indicated by asterisks (***$P < 0.001$). (C) Frequency distribution of HC indel length in base pairs per contig in a total of 153 contigs.

mutations within ORF, and frame-shift mutations occurred in 16 contigs. These results indicate that, despite the high number of polymorphic sites, the effect on protein sequence is not necessarily high.

4-3. SNP validation

To validate the detected SNPs, 33 polymorphic contigs were randomly selected. Of the 33 contigs, 30 contained HC SNPs and the remaining 3 contained lower confidence (LC) SNPs. The validation was performed by genotyping 20 diverse *Ae. tauschii* accessions chosen based on the intraspecific diversification patterns of *Ae. tauschii* (Mizuno et al. 2010a). The selected accessions consisted of two accessions from each of the nine *Ae. tauschii* sublineages (six of L1 and three of L2) and HGL17, excluding any admixtures (Figure IV-3A).

First, all 33 contigs were searched for restriction enzyme recognition sites, with at least 14 SNPs found and converted into CAPS markers. All 14 CAPS markers were polymorphic among the examined accessions (Figure IV-4A). An alternative to a CAPS marker is a dCAPS marker, which consists of the introduction of one or more mismatches into the primer, creating a restriction site dependent on the presence or absence of the SNP (Neff et al. 1998). Five of the SNPs that were not converted into CAPS markers were used to develop dCAPS markers by introducing one or two mismatches in the primer sequence. Two dCAPS markers could not be used for genotyping because unexpected bands were amplified by PCR, and a dCAPS marker for contig05220 was unavailable due to generation of digestion-resistant PCR products in some accessions (Figure IV-4B). In dCAPS markers, a single mismatch at position 1, 2, or 3 from the 3' end of the primer is preferred to avoid digestion-resistant products (Michaels and Amasino 1998). However, in some cases, a mismatch at position 1 or 2 may also lead to undigested products, if the polymerase has proofreading exonuclease activity (Komori and Nitta 2005). The digestion-resistant products were generated for unknown reasons, because in the dCAPS analysis of the contig05220 marker, a single mismatch was introduced at position 1 and amplification was performed using a *Taq* DNA polymerase lacking proofreading activity. The remaining two dCAPS markers were successfully used in genotyping (Figure IV-4C).

SNP genotyping based on HRM analysis has also been reported in hexaploid wheat and *Ae. tauschii* (Matsuda et al. 2012). Primers for HRM analysis were designed for the remaining 17 contigs (including the three contigs that failed in the dCAPS analysis) to obtain PCR products of ~100 bp. Only two of the 17 HRM primer sets failed in polymorphism detection, although both amplified genomic regions contained HC SNPs

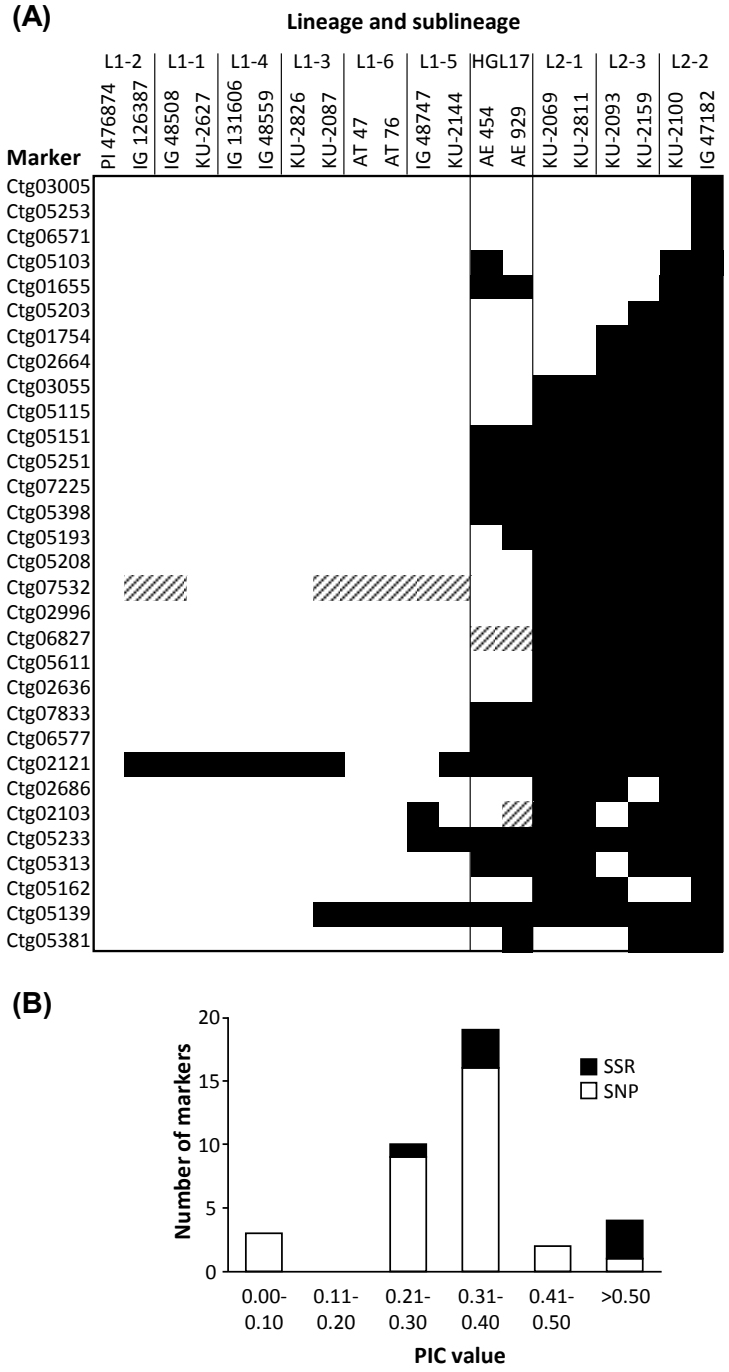

Figure IV-3. Validation of identified polymorphisms in the contigs. (A) Summary of genotyping results of the 31 polymorphic SNP markers in the 20 *Ae. tauschii* accessions. Genotypes of the two accessions used in transcriptome sequencing, PI476874 and IG47182, are shown in the first and last columns, respectively. White, black and shaded regions respectively indicate the PI476874, IG47182 and other alleles. (B) Frequency distribution of PIC values. PIC values of SNP markers (open bars) and SSR markers (black bars) among the 20 *Ae. tauschii* accessions. A total of 31 SNP and 6 SSR markers were used.

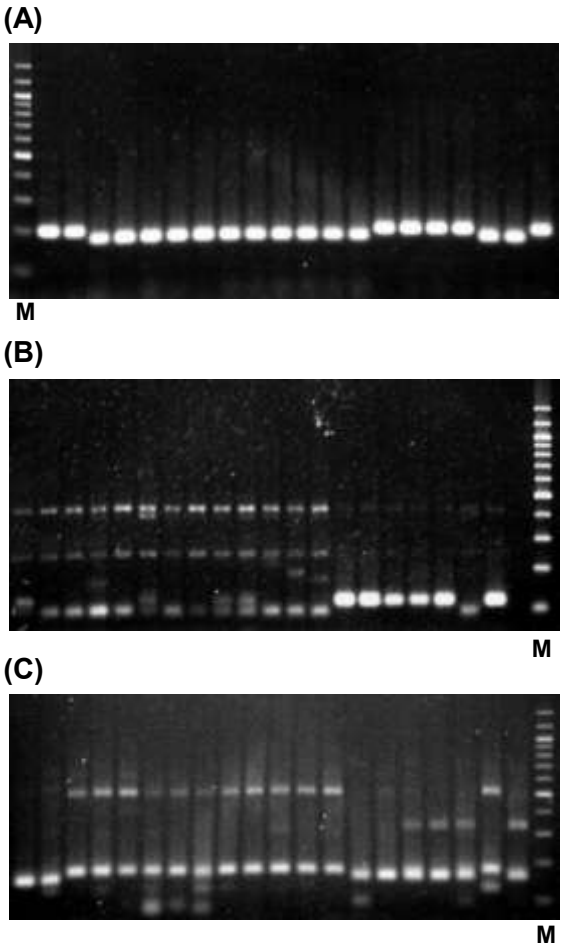

Figure IV-4. Examples of CAPS and dCAPS fragment pattern of the 20 *Ae. tauschii* accessions. PCR products after digestion of Ctg05313 CAPS marker (A), Ctg05220 dCAPS marker (B) and Ctg05398 dCAPS marker (C). From left to right: fragment pattern of two accessions of HGL17, 11 accessions of L1, five accessions of L2, PI476874 (L1) and IG47182 (L2). Digestion resistant products were observed in Ctg05398 dCAPS markers in four accessions of HGL17 and L1. M; 100-bp ladder marker.

between PI476874 and IG47182. HRM analysis is useful as an alternative to SNP-typing because it is cost-effective when the restriction enzyme in CAPS/dCAPS markers is expensive. Another advantage of HRM is the possibility of detecting more than two alleles (Figure IV-5), which is not possible with CAPS/dCAPS. In addition, the relatively short length of the PCR products required for HRM analyses increases the probability that primer pairs are located within a single exon because more than half of the exons in plant genomes are less than 145-156 bp (Table IV-6). Our results indicate that almost all of these SNPs can be converted to PCR-based molecular markers, at least in diploid wheat species.

To evaluate the allelic diversity within the *Ae. tauschii* population, PIC values were calculated for each validated SNP marker. PIC values ranged from 0.091 to 0.591 (average, 0.313) in the 20 *Ae. tauschii* accessions (Figure IV-3B). Among the 31 markers, three showed IG47182-specific SNP alleles, and 15 clearly distinguished the L1 accessions from L2 (Figure IV-3A). Because many of the validated SNPs were polymorphic between L1 and L2 accessions, the markers developed using information about these SNPs can be used in genotyping of mapping populations obtained from interlineage crosses (L1 x L2). Moreover, considering that the subspecies *strangulata* is a possible major D-genome donor and the evolutionary birthplace of hexaploid common wheat, the D-genome donor has been thought to belong to L2 (Mizuno et al. 2010a), indicating that these markers might be useful for genotyping the progeny of common wheat cultivars crossed with L1-derived hexaploid wheat synthetics. Further studies are needed to confirm this hypothesis. The HGL17 accessions, considered one of the ancestral lineages of *Ae. tauschii*, are not included in either L1 or L2, but rather are located genealogically between these two lineages (Mizuno et al. 2010a). Consistently, the genotype patterns of the two HGL17 accessions analyzed (AE454 and AE929) were intermediates between L1 and L2 accessions.

4-4. EST-SSR mining and validation

Among all 10224 contigs, a total of 2778 SSR motifs were found in 2072 contigs and were composed mainly of trinucleotide motifs (Table IV-7). Indel polymorphisms between PI476874 and IG47182 were searched as polymorphic SSRs in the 2778 motifs. In total, 54 polymorphic SSRs were found in 50 contigs, with 20 of them HC SSRs. Based on these data, 18 EST-SSR markers, including 14 HC and 4 LC polymorphic SSRs, were developed for validation in the 20 *Ae. tauschii* accessions. Seven EST-SSR markers generated multiple bands or amplified products with a length much longer than expected (probably due to the presence of introns) and were excluded for further

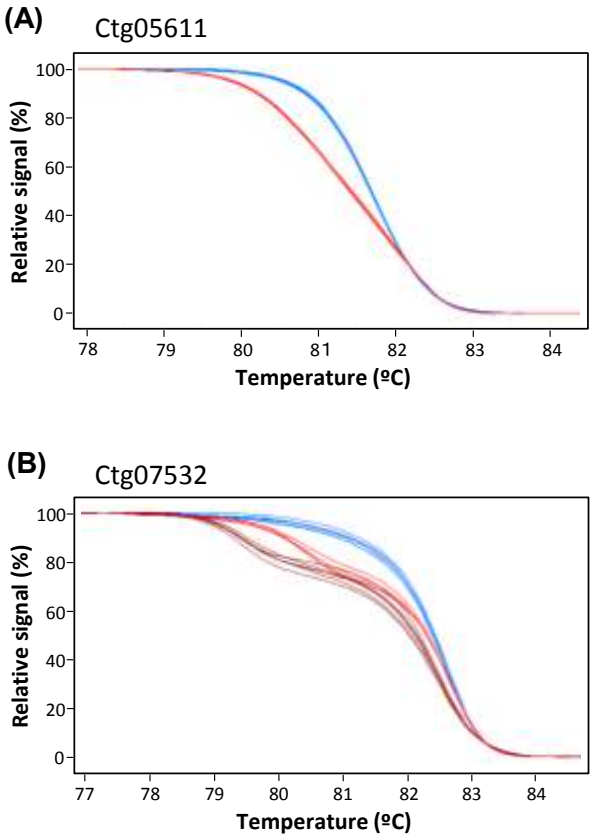

Figure IV-5. High Resolution Melting analysis of the PCR products of the 20 *Ae. tauschii* accessions. Normalized and shifted melting curves of Ctg05611 (A) with two alleles and Ctg07532 (B) with three alleles. Melting curves of accessions with IG47182 (L2) allele are indicated in red and those with PI476874 (L1) allele in blue. A third allele was observed in Ctg07532, indicated by brown lines. All samples were spiked with 10% (v/v) of PI476874 DNA to facilitate the discrimination of genotypes.

Table IV-6. Average and median of exon length among model plants

	A. thaliana	*O. sativa*	*Z. mays*
No. of mRNA	40745	42088	136770
No. of exon	215909	252918	487924
Average of exon length(bp)	296	258	288
Median of exon length (bp)	150	145	156

Data are extracted from GFF files as follows, *A. thaliana* (TAIR10, ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release), *O. sativa* (IRGSP/RAP build 5 data set, http://rapdb.dna.affrc.go.jp), and *Z. mayes* (ZmB73_5a_WGS.gff , http://ftp.maizsequence.org/current/working-set/).

Table IV-7. SSR motifs found in 10224 contigs

	Number of motifs
Total motifs	2778 (2072 contigs)
Dinucleotide motifs	201
Trinucleotide motifs	1896
Tetranucleotide motifs	380
Pentanucleotide motifs	301
Polymorphic SSRs	54 (50 contigs)
HC polymorphic SSRs	20 (19 contigs)

analyses. Four out of the eight EST-SSR markers with more than a 4-bp difference were able to distinguish genotypes of samples in 13% non-denaturing polyacrylamide gels. HRM analysis was conducted for the other EST-SSR markers, including indels with less than a 5 bp difference, and this detected polymorphisms in only two of the seven EST-SSR markers. PIC values of these six markers ranged from 0.305 to 0.686, with an average of 0.424 (Figure IV-3B). The number of alleles in the EST-SSR markers ranged from two to six (average, 3.0), which was higher compared to that in SNP markers with two to three alleles (average, 2.1). This difference might reflect the higher average PIC value of the EST-SSR markers.

4-5. Genetic map construction

We developed 37 polymorphic markers, including 31 SNPs and 6 EST-SSRs. To study the proportion of these markers that can be assigned to a genetic map, genotypes of 104 F_2 individuals obtained from a cross between PI476874 and IG47182 were determined. All these markers were successfully mapped onto a linkage map constructed using 87 publicly available SSR markers (Koyama et al. 2012; Matsuda et al. 2012). The total map length was 1282.1 cM, with an average interval of 9.5 cM between markers. In total, 12 markers were distorted on chromosome 1D, 24 markers on chromosome 2D, 6 markers on chromosome 4D and 10 markers on chromosome 5D. Segregation ratios of the newly mapped markers are presented in Table IV-8. The distribution of the 37 marker positions ranged from one on chromosome 1D to 12 on chromosome 5D (Figure IV-6). Two markers, one SNP marker and an SSR marker, were derived from different sites of contig06827, and both mapped to the same location.

We performed a BlastN search of the 36 mapped contigs against the deletion-mapped ESTs of common wheat (Qi et al. 2004), obtaining 17 matches (Table IV-9). Of the 17 contigs, 14 (82%) were located on the same homoeologous group chromosomes as the deletion-mapped ESTs. Recently, a genome survey sequence of common wheat chromosome 7D was published (Berkman et al. 2011). To compare the order of the six contigs that mapped to chromosome 7D of *Ae. tauschii*, a BlastN search was performed against the 7D Synthetic Build v2.0 (Berkman et al. 2011). Four contigs were assigned to the short arm and two to the long arm of chromosome 7D, as expected based on the *Ae. tauschii* genetic map (Table IV-10). The contig order in the survey sequence data was similar to that in the 7D linkage map, though the position of contig02996 differed slightly.

In barley, a hypothetical gene order has been proposed for each of the seven chromosomes based on conserved synteny among barley, *Brachypodium*, rice and

Table IV-8. Segregation data of mapped markers

Marker name	Segregation *	χ^2 test *P*-value
Chromosome 1D		
Xctg06721	34 / 53 / 15	0.03
Chromosome 2D		
Xctg07532	45 / 45 / 8	6.2E-07
Xctg06577	51 / 46 / 5	6.0E-10
Xctg02636	51 / 47 / 5	8.1E-10
Xctg05611	45 / 48 / 8	1.1E-06
Xctg06571	47 / 49 / 5	2.5E-08
Xctg05208	39 / 51 / 9	1.1E-04
Xctg05233	35 / 48 / 20	0.09
Chromosome 3D		
Xctg05151	24 / 52 / 26	0.94
Xctg06827_SSR	22 / 51 / 28	0.70
Xctg06827_SNP	23 / 51 / 27	0.85
Xctg01655	28 / 44 / 32	0.25
Chromosome 4D		
Xctg05253	42 / 48 / 14	3.9E-04
Xctg02664	43 / 45 / 14	1.3E-04
Xctg05247	34 / 45 / 23	0.15
Chromosome 5D		
Xctg05103	26 / 48 / 28	0.81
Xctg05115	33 / 48 / 22	0.24
Xctg01754	45 / 45 / 13	2.1E-05
Xctg07326	45 / 42 / 15	3.0E-05
Xctg03055	46 / 43 / 14	1.2E-05
Xctg05251	46 / 42 / 15	1.5E-05
Xctg05398	39 / 46 / 14	1.4E-03
Xctg05381	82 / 21	0.56
Xctg05203	34 / 48 / 21	0.15
Xctg07833	28 / 56 / 19	0.31
Xctg05193	35 / 47 / 21	0.10
Xctg02121	28 / 53 / 21	0.57
Xctg05139	28 / 74	0.85

*Number of IG47182 homozygotes/heterozygotes/PI476874 homozygotes. †Red: distorted markers.

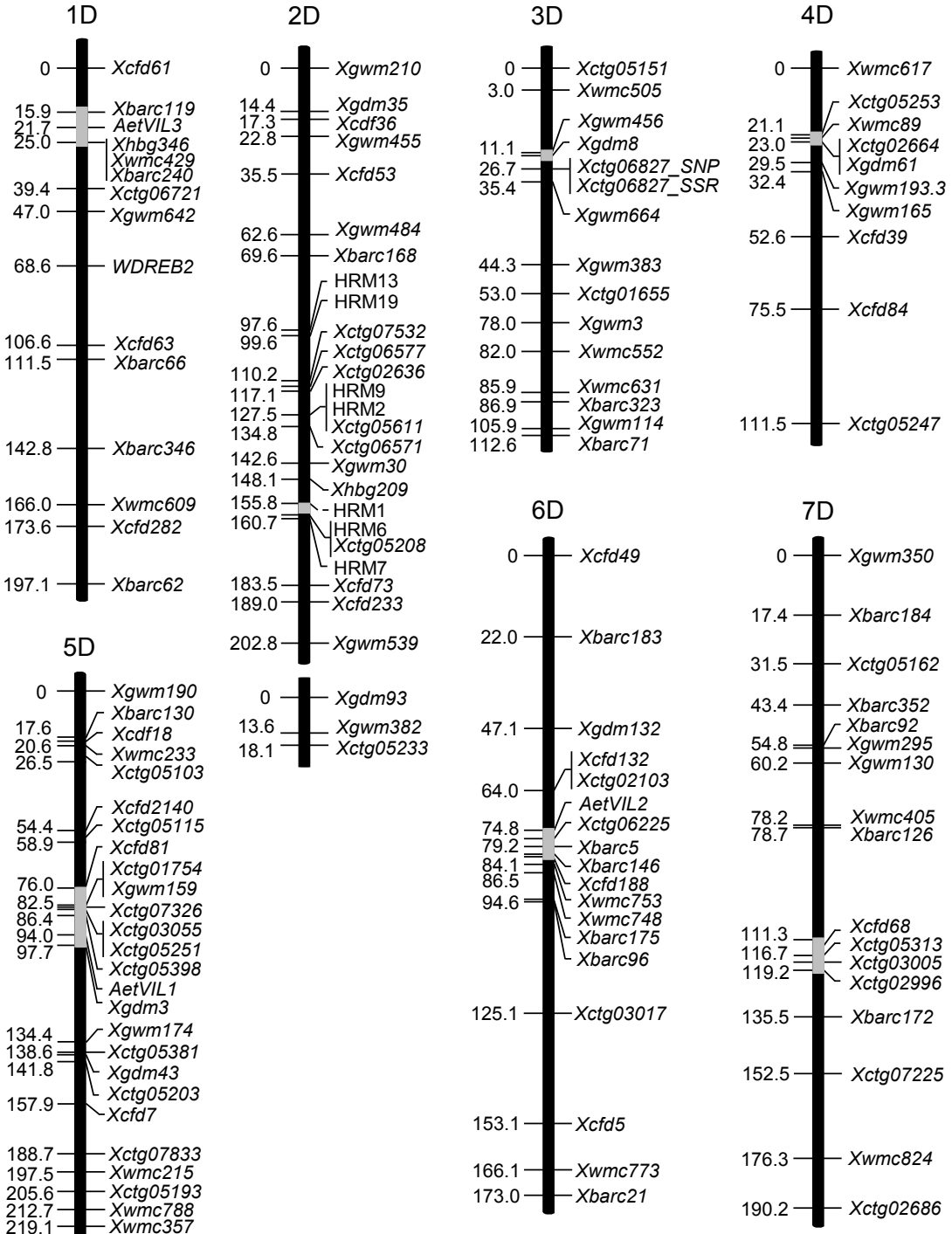

Figure IV-6. A genetic map of *Ae. tauschii* constructed using an F_2 population of PI476874 x IG47182. Gray bars indicate the putative centromere positions. SNP markers and SSR markers developed in this study were designated *Xctg* and were placed on a genetic map constructed using 87 publicly available SSR markers. Map distances are shown in centimorgans.

Table IV-9. BlastN hits against wheat bin-mapped ESTs

Contig number	Bin-mapped EST	*E*-value	Similarity (%)	Location on bin map	Location on genetic map
contig06721	BG262826	3.99E-58	81	5DL	1DL
contig02636	BE422913	0	99	2BS	2DS
contig05611	CD453463	0	96	2DS	2DS
contig06571	BG264051	2.43E-75	90	2D	2DS
contig07532	BQ172229	0	96	3D	2DS
contig05233	CD492093	0	94	2AL	2DL
contig05253	BM135436	0	100	4DS	4D
contig02664	BE442811	0	94	4DL	4D
contig05247	BE404665	0	96	4DL	4DL
contig05103	BE490728	0	99	5DS/7DS	5DS
contig05115	BQ171728	0	92	5DS	5DS
contig05193	BF478849	4.48E-160	91	5DL	5DL
contig05251	BQ167220	0	98	5D	5D
contig05398	BE493050	0	97	5DL	5D
contig07326	BE446457	0	96	3DL	5D
contig02996	BG262748	1.66E-149	96	7DS	7D
contig02686	BG274853	0	97	7DL	7DL

Locations of ESTs on bin map (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi) and contigs on genetic map were compared. In the contigs indicated in red, the location on genetic map was different to that on bin map.

Table IV-10. Position of contigs on 7D chromosome synthetic build

Contig number	Position on map	Position on 7D chromosome synthetic build	*E*-value
contig05162	31.5 cM	7D_SynBuild_v2.0:1289763..1297826 (1.2 Mb)	0
contig05313	115.8 cM	7D_SynBuild_v2.0:5087934..5091390 (5.0 Mb)	0
contig03005	117.7 cM	7D_SynBuild_v2.0:5719379..5730096 (5.7 Mb)	0
contig02996	120.1 cM	7D_SynBuild_v2.0:4605431..4611363 (4.6 Mb)	0
contig07225	150.0 cM	7D_SynBuild_v2.0:17679123..17680068 (17.6 Mb)	0
contig02686	188.9 cM	7D_SynBuild_v2.0:21462678..21465038 (21.5 Mb)	0

Number in the parenthesis indicates the approximate number of base pairs from the first nucleotide of the synthetic build sequence. In the contig indicated in red, the order in genetic map was slightly different compared to the order inferred from the 7D chromosome draft sequence. Approximated centromere position: 9.2 Mb.

sorghum (Mayer et al. 2011). Due to the conserved synteny between wheat and barley (Carollo et al. 2005), the contigs with HC SNPs were mapped on the virtual barley chromosomes, aligning with the shotgun barley genomic sequences (Mayer et al. 2011). A total of 1450 contigs (*E*-value cutoff of 10^{-20}) were mapped with a distribution of around 200 contigs per chromosome (Table IV-11). The order of contigs on the genetic map of chromosomes 2D and 5D was also compared, respectively, with the virtual barley chromosomes 2H and 5H. These two chromosomes were selected because the number of mapped contigs was higher than that for the remaining chromosomes. Of the seven contigs that mapped to chromosome 2D, one failed to be assigned to any barley chromosomes, four were assigned to chromosome 2H, and two to other chromosomes (Table IV-12). To confirm this result, tBlastX search was performed against 5006 and 23614 barley full-length cDNAs (Sato et al. 2009; Matsumoto et al. 2011), and BlastX search was performed against *Brachypodium* and rice protein database. Based on these Blast hits, six out of the seven contigs were located on chromosome 2H (Table IV-12), indicating that some homologous genes on the chromosome other than 2H were hit in the former analysis. Contig06577 failed to be assigned to any barley chromosome. The contig order on chromosome 2D of *Ae. tauschii* corresponded to the gene order on chromosome 2H of barley (Figure IV-7A). Similarly, 12 out of 13 contigs of *Ae. tauschii* chromosome 5D were assigned to chromosome 5H of barley based on tBlastX hits against barley shotgun genomic reads (Table IV-13). Contig05381 was assigned to chromosome 2H, and this result was confirmed performing a Blast search against barley cDNA and *Brachypodium* and rice protein sequences (Table IV-13). The contigs on chromosome 5D of *Ae. tauschii*, except for contig05398, showed an order identical to that on barley chromosome 5H, while contig05398 was located in a slightly different region (Figure IV-7B). Previously, ~14% of barley genes could not be assigned to virtual chromosomes by the GenomeZipper approach (Mayer et al. 2011). Therefore, contig06577 that failed to be assigned to the virtual barley chromosomes might correspond to the unassigned 14% of barley genes. Indeed, this contig showed sequence similarity with *Brachypodium* and rice genes Bradi1g19250 and Os07g0656700 that are probably located on the short arm of chromosome 2H, according to the synteny with barley (Table IV-12). Furthermore, contig05381 (mapped on chromosome 5DL but assigned to barley 2HS) also showed sequence similarity with Bradi4g34390 and Os09g0502200 that probably map to chromosome 5HL of barley (Table IV-13). The chromosomal locations of the remaining mapped contigs were similarly confirmed (Table IV-14). These results suggest that the contigs should be assigned to virtual barley chromosomes not only by alignment with the shotgun genomic reads, but also by

Table IV-11. Number of contigs with HC SNPs mapped on the virtual barley chromosome

Chromosome number	*E*-value cutoff		
	< 0.1	$< 10^{-5}$	$< 10^{-20}$
1H	229	209	197
2H	247	235	219
3H	234	214	205
4H	203	187	182
5H	275	254	241
6H	211	199	192
7H	259	234	214
Total	1658	1532	1450

Table IV-12. Blast hit against barley shotgun genomic reads, barley full-length cDNAs, and *Brachypodium* and rice protein database of contigs mapped on chromosome 2D

Contig number	*Ae. tauschii*'s genetic map	Barley shotgun genomic reads	*E*-value	Barley chromosome	Barley full-length cDNA	*E*-value	Barley chromosome
contig07532	2DS	CUST_22008_PI390587928_591	0	2HS	AK248510	$2.00E^{-158}$	-
contig06577	2DS	-	-	-	NIASHv2094A13	$9.00E^{-166}$	-
contig02636	2DS	CUST_30712_PI390587928_1038	0	4HL	AK249924	0	-
contig05611	2DS	CUST_16351_PI390587928_15607	0	2HS	AK250341	0	2HS
contig06571	2DS	CUST_21576_PI390587928_15723	0	2HS	NIASHv1087P11	0	2HS
contig05208	2DS	CUST_12214_PI390587928_32909	$9.00E^{-28}$	7HS	NIASHv3143H01	0	2HS
contig05233	2DL	FTR0EHA01BS8PC	$1.00E^{-108}$	2HL	NIASHv2018L18	0	2HL

Contig number	*Ae. tauschii*'s genetic map	*Brachypodium* gene	*E*-value	Barley chromosome	Rice gene	*E*-value	Barley chromosome
contig07532	2DS	Bradi1g18710	$6.00E^{-80}$	2HS	Os07g0662500	$8.00E^{-75}$	2HS
contig06577	2DS	Bradi1g19250	$3.00E^{-97}$	2HS?	Os07g0656700	$8.00E^{-94}$	2HS?
contig02636	2DS	Bradi1g19220	0	2HS	Os03g0323200	0	4HL
					Os07g0656500	0	2HS?
contig05611	2DS	Bradi1g21330	0	2HS	Os07g0622200	0	2HS
contig06571	2DS	Bradi1g21490	$2.00E^{-90}$	2HS	Os07g0615200	$2.00E^{-81}$	2HS
contig05208	2DS	Bradi5g11750	0	-	Os04g0448900	0	-
contig05233	2DL	Bradi5g27110	0	2HL	Os04g0687900	0	2HL

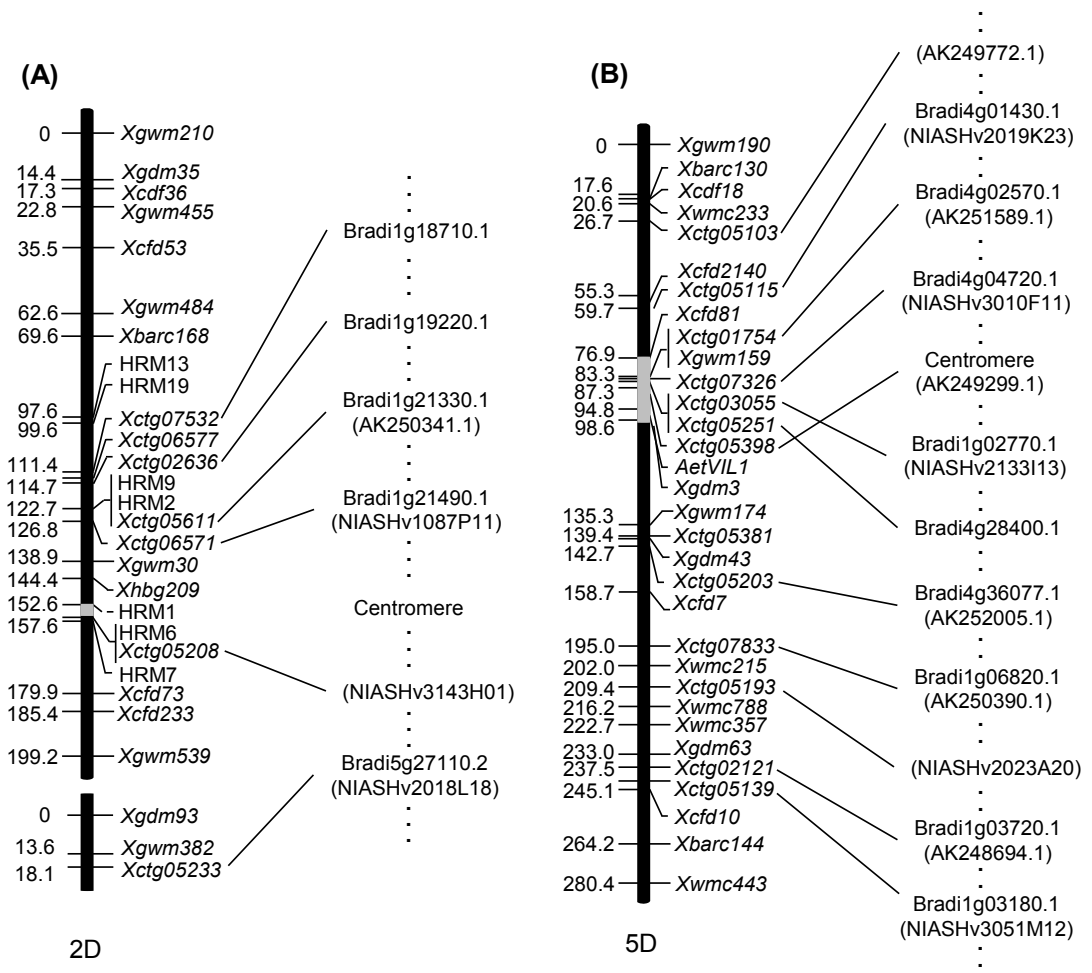

Figure IV-7. Synteny between chromosomes 2D and 5D of *Ae. tauschii* and 2H and 5H of barley. Based on tBlastX hits against barley full-length cDNA and *Brachypodium* protein database, the contig orders on chromosomes 2D (A) and 5D (B) were compared with those on the barley virtual chromosome in the genome zipper. The clone names and accession numbers of barley cDNAs are indicated in parentheses.

Table IV-13. Blast hit against barley shotgun genomic reads, barley full-length cDNAs, and *Brachypodium* and rice protein database of contigs mapped on chromosome 5D

Contig number	*Ae. tauschii*'s genetic map	Barley shotgun genomic reads	*E*-value	Barley chromosome	Barley full-length cDNA	*E*-value	Barley chromosome
contig05103	5DS	CUST_10901_PI390587928_14644	0	5HS	AK249772	0	5HS
contig05115	5DS	FXV4SVK02I834V	$1.00E^{-119}$	5HS	NIASHv2019K23	0	5HS
contig01754	5DS	CUST_29232_PI390587928_17927	0	5HS	AK251589	$1.00E^{-107}$	5HS
contig07326	5DS	CUST_16330_PI390587928_15629	0	5HS	NIASHv3010F11	$2.00E^{-108}$	5HS
contig03055	5DL	FXBQQIN01C3CON	$5.00E^{-97}$	5HL	NIASHv2133I13	0	5HL
contig05251	5DL	FXBQQIN02FGLPG	$1.00E^{-178}$	5HL	AK252199	0	-
contig05398	5DL	FXBQQIN01ATS3J	0	5HL	AK249299	0	5HL
contig05381	5DL	CUST_17970_PI390587928_21595	$4.00E^{-26}$	2HS	AK249411	0	-
contig05203	5DL	CUST_25285_PI390587928_2598	0	5HL	AK252005	0	5HL
contig07833	5DL	CUST_16540_PI390587928_251	0	5HL	AK250390	$9.00E^{-156}$	5HL
contig05193	5DL	CUST_26722_PI390587928_41045	$1.00E^{-142}$	5HL	NIASHv2023A20	0	5HL
contig02121	5DL	CUST_10593_PI390587928_59	0	5HL	AK248694	0	5HL
contig05139	5DL	CUST_39046_PI390587928_3096	0	5HL	NIASHv3051M12	0	5HL

Table IV-13. Continued

Contig number	*Ae. tauschii*'s genetic map	*Brachypodium* gene	*E*-value	Barley chromosome	Rice gene	*E*-value	Barley chromosome
contig05103	5DS	Bradi3g07010	0	6HS	Os12g0559200	0	5HS
contig05115	5DS	Bradi4g01430	0	5HS	Os12g0617800	0	5HS
contig01754	5DS	Bradi4g02570	$2.00E^{-29}$	5HS	Os12g0601800	$8.00E^{-09}$	-
contig07326	5DS	Bradi4g04720	$2.00E^{-55}$	5HS	Os05g0129300	$9.00E^{-27}$	1HS
contig03055	5DL	Bradi1g02770	0	5HL	Os09g0250700	0	5HL
contig05251	5DL	Bradi4g28400	0	5HL	Os09g0346400	0	5HL
contig05398	5DL	Bradi4g08780	0	-	Os09g0248300	0	5HL
contig05381	5DL	Bradi1g25517	$6.00E^{-156}$	2HS	Os07g0538000	$1.00E^{-157}$	-
		Bradi4g34390	$3.00E^{-71}$	5HL?	Os09g0502200	$8.00E^{-73}$	5HL?
contig05203	5DL	Bradi4g36077	0	5HL	Os09g0532400	0	5HL
contig07833	5DL	Bradi1g06820	$6.00E^{-38}$	5HL	Os03g0780400	$3.00E^{-38}$	5HL
contig05193	5DL	Bradi1g06670	$2.00E^{-109}$	5HL	Os03g0782500	$3.00E^{-109}$	5HL
contig02121	5DL	Bradi1g03720	0	5HL	Os03g0821100	0	5HL
contig05139	5DL	Bradi1g03180	0	5HL	Os03g0827700	0	5HL

Question mark indicates the putative chromosomal location inferred based on synteny between barley and *Brachypodium* and rice

Table IV-14. Blast hit against barley shotgun genomic reads, barley full-length cDNAs, and *Brachypodium* and rice protein database of contigs mapped on chromosome 1, 3, 4, 6 and 7D

Contig number	*Ae. tauschii*'s genetic map	Barley shotgun genomic reads	*E*-value	Barley chromosome	Barley full-length cDNA	*E*-value	Barley chromosome
contig06721	1DL	-	-	-	NIASHv3001H07	0	1HS
contig05151	3DS	-	-	-	NIASHv2043G08	0	7HS
contig06827	3DL	FSHS8SA01A9XQJ	$1.00E^{-101}$	3HL	AK250617	$5.00E^{-165}$	3HL
contig01655	3DL	FSSWHZR02JOQN4	$3.00E^{-36}$	3HL	AK248786	$4.00E^{-16}$	3HL
contig05253	4DS	FWLU4E402JBX5Y	$1.00E^{-72}$	4HS	NIASHv2010K11	0	4HS
contig02664	4DL	CUST_16287_PI390587928_3587	0	4HL	AK252403	0	4HL
contig05247	4DL	CUST_9033_PI390587928_14423	0	4HL	AK248753	0	4HL
contig02103	6DS	FX84EFC02FVYBS	$1.00E^{-154}$	6HS	NIASHv3108F04	0	-
contig06225	6DS	CUST_28931_PI390587928_18039	$1.00E^{-28}$	2HL	NIASHv2053C02	$6.00E^{-179}$	-
contig03017	6DL	FS30M0W02HNQLU	0.002	3HS	-	-	-
contig05162	7DS	CUST_28233_PI390587928_28688	$1.00E^{-141}$	7HS	AK251676	0	-
contig05313	7DS	FULMQYZ01CRAQR	$1.00E^{-108}$	7HS	AK253130	0	7HS
contig02996	7DS	CUST_26227_PI390587928_39112	0	7HS	NIASHv2076D06	0	7HS
contig03005	7DS	CUST_24302_PI390587928_40232	0	7HS	AK250992	$4.00E^{-35}$	7HS
contig07225	7DL	CUST_3798_PI390587928_14358	0	7HL	NIASHv2150D03	0	7HL
contig02686	7DL	CUST_6655_PI390587928_755	0	7HL	AK248704	0	7HL

Table IV-14. Continued

Contig number	*Ae. tauschii*'s genetic map	*Brachypodium* gene	*E*-value	Barley chromosome	Rice gene	*E*-value	Barley chromosome
contig06721	1DL	Bradi2g22290	$2.00E^{-122}$	-	Os05g0477600	$2.00E^{-118}$	1HL
contig05151	3DS	Bradi3g39220	0	7HS	Os08g0495800	0	7HS
		Bradi2g04310	$5.00E^{-162}$	3HS?	Os01g0170000	$2.00E^{-66}$	3DS?
contig06827	3DL	Bradi2g56260	$3.00E^{-104}$	3HL	Os01g0869800	$2.00E^{-89}$	3HL
contig01655	3DL	Bradi2g59650	$2.00E^{-04}$	3HL	Os01g0813700	0.004	-
contig05253	4DS	Bradi4g22760	0	4HS	Os11g0207200	$4.00E^{-167}$	4HS
contig02664	4DL	Bradi1g65910	$2.00E^{-171}$	4HL	Os03g0284100	$8.00E^{-141}$	4HL
contig05247	4DL	Bradi1g78220	0	4HL	Os08g0480100	0	-
contig02103	6DS	Bradi3g35680	0	-	Os03g0314500	0	4HL
		Bradi3g01460	$5.00E^{-147}$	6HS			
contig06225	6DS	Bradi3g05800	$1.00E^{-76}$	6HS?	Os02g0178100	$3.00E^{-72}$	6HS?
contig03017	6DL	-	-	-	-	-	-
contig05162	7DS	Bradi1g50590	0	7HS	Os02g0700600	$2.00E^{-145}$	6HL
contig05313	7DS	Bradi1g43220	0	7HS	Os06g0298200	$4.00E^{-161}$	7HS
contig02996	7DS	Bradi1g44177	0	7HS	Os06g0255200	0	7HS
contig03005	7DS	Bradi1g41907	0	7HS	Os06g0498400	0	7HS
contig07225	7DL	Bradi1g36050	$2.00E^{-78}$	7HL	Os06g0612800	$1.00E^{-78}$	7HL
contig02686	7DL	Bradi1g32310	$1.00E^{-98}$	7HL	Os06g0669400	$6.00E^{-98}$	7HL

Question mark indicates the putative chromosomal location inferred based on synteny between barley and *Brachypodium* and rice

alignment with *Brachypodium* and rice protein sequences. The chromosomal locations of unassigned contigs might be searched based on the synteny among barley, *Brachypodium*, and rice. Our results indicate that information on hypothetical gene order in barley may be useful for development of molecular markers in target chromosomal regions of wheat.

5. Conclusion

Marker development in targeted chromosomal regions is important for map-based gene cloning and for molecular breeding in wheat and other crops. In this study, transcriptomes of two *Ae. tauschii* accessions belonging to different major lineages were compared, yielding 4578 HC-polymorphic sites from 1793 contigs. Thirty-one SNPs were validated in the 20 diverse *Ae. tauschii* accessions through conversion into CAPS, dCAPS and HRM markers. Development of CAPS markers is dependent on the availability of appropriate restriction enzymes and their cost. In some dCAPS markers, generation of digestion-resistant PCR products interferes with precise genotyping. In this study, we showed that HRM analysis is available for SNP typing. The SNP markers developed in this study could be applied to construct an F_2 map between L1 and L2 accessions of *Ae. tauschii*, and probably between the D genomes of common wheat and L1-derived synthetic wheat. One next generation sequencing experiment on transcriptomes of parental accessions leading to *de novo*-generated SNP markers was able to identify the chromosomal location of more than 1700 genes in the wheat D genome. RNA-seq is a more cost-effective method for SNP discovery than the annotation-based one in organisms with large and complex genomes. In addition, due to the conserved synteny between wheat and barley chromosomes, the barley high-density EST map and linear gene order can be applied to develop markers on the target chromosomal regions of wheat. Accompanied by further progress on the wheat genome project coordinated by the International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org/), more information about genomic sequence data of each chromosome will become available for wheat marker development.

Chapter V

Genome-wide marker development for the wheat D genome based on single nucleotide polymorphisms identified from transcripts in the wild wheat progenitor *Aegilops tauschii*

1. Abstract

In organisms with large and complex genomes, such as wheat, RNA-seq analysis is cost effective for discovery of genome-wide single nucleotide polymorphisms (SNPs). In this study, deep sequencing of the spike transcriptome from two *Aegilops tauschii* accessions representing two major lineages led to discovery of 13,347 high-confidence (HC) SNPs in 4,872 contigs. After removing redundant SNPs detected in the leaf transcriptome from the same accessions in an earlier study, 10,589 new SNPs were discovered. In total, 5,642 out of 5,808 contigs with HC SNPs were assigned to the *Ae. tauschii* draft genome sequence. On average, 732 HC polymorphic contigs were mapped *in silico* to each *Ae. tauschii* chromosome. Based on the polymorphic data, we developed markers to target the short arm of chromosome 2D and validated the polymorphisms using 20 *Ae. tauschii* accessions. Of the 29 polymorphic markers, 28 were successfully mapped to 2DS in the diploid F_2 population of *Ae. tauschii*. Among 10 hexaploid wheat lines, which included wheat synthetics and common wheat cultivars, 25 of the 43 markers were polymorphic. In the hexaploid F_2 population between a common wheat cultivar and a synthetic wheat line, 23 of the 25 polymorphic markers between the parents were available for genotyping of the F_2 plants, and 22 markers mapped to chromosome 2DS. These results indicate that molecular markers developed from polymorphisms between two distinct lineages of *Ae. tauschii* are useful for analysis not only of the diploid but also of the hexaploid wheat genome.

2. Introduction

Common wheat (*Triticum aestivum* L.) is an allohexaploid species that originated by natural hybridization between tetraploid wheat (*Triticum turgidum* L.), containing the A and B genomes, and the wild diploid relative *Aegilops tauschii* Coss., containing the D genome (Kihara 1944; McFadden and Sears 1944). *Ae. tauschii* is widely distributed in Eurasia and shows abundant genetic variation (Dvorak et al. 1998; Dudnikov and Kawahara 2006; Matsuoka et al. 2007, 2008, 2009; Takumi et al. 2009a). Based on population structure analyses, *Ae. tauschii* has been divided into two major genealogical lineages, lineage 1 (L1) and lineage 2 (L2), and a minor lineage, HGL17 (Mizuno et al.

2010a; Wang et al. 2013). The gene pool of *Ae. tauschii* can be easily accessed in wheat breeding, but remains largely unexplored. Synthetic hexaploid wheat can be obtained through interspecific hybridization between tetraploid wheat and *Ae. tauschii* (Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004), and the resulting lines can be used as intermediates to exploit the natural variation in *Ae. tauschii* for improvement of common wheat (Trethowan and Mujeeb-Kazi 2008; Jones et al. 2013). It is believed that the *Ae. tauschii* populations involved in the origin of common wheat are limited to a narrow distribution range and restricted to L2, which has given rise to a founder effect in hexaploid wheat (Feldman 2001; Mizuno et al. 2010a; Wang et al. 2013). Therefore, *Ae. tauschii*, especially L1, has large genetic diversity that is not represented in common wheat (Feldman 2001; Mizuno et al. 2010a).

Development of molecular markers is an important step for molecular breeding and map-based cloning. RNA-seq, a next generation sequencing technology for transcripts, is cost effective for single nucleotide polymorphism (SNP) discovery in organisms having large and complex genomes and insufficient reference information (Hansey et al. 2012; Chapter IV). Although a draft genome sequence of *Ae. tauschii* based on a whole genome shotgun strategy has been published, only 1.72 Gb out of the 4.36 Gb genome was anchored to chromosomes (Jia et al. 2013). A physical map of *Ae. tauschii* covering 4 Gb has also been developed (Luo et al. 2013), while the availability of bacterial artificial chromosome sequences is limited. We previously sequenced the leaf transcriptome of two *Ae. tauschii* accessions each from L1 and L2 (Chapter IV). After *de novo* assembly of the reads, 4,337 SNPs were discovered in at least 1,700 contigs, and around 200 polymorphic contigs per chromosome were mapped *in silico* to barley virtual chromosomes by the GenomeZipper approach (Mayer et al. 2011). More recent draft sequence information on the barley genome could be more helpful for *in silico* mapping (International Barley Genome Sequencing Consortium (IBSC) 2012). Because almost all of the validated SNP markers were polymorphic between L1 and L2, it was assumed that these markers would be available for wheat D-genome genotyping (Chapter IV). Objectives of the present study were to discover new SNPs from RNA-seq data from spikes of *Ae. tauschii*, and to assess the *Ae. tauschii* SNP libraries for D-genome analysis of diploid and hexaploid wheat. We also evaluated the D-genome SNP markers using genomic information on *Ae. tauschii* and barley.

3. Materials and methods

3-1. Plant material and cDNA library construction

In total, 20 *Ae. tauschii* accessions from each sublineage and 10 hexaploid wheat lines

were used (Table V-1). Synthetic hexaploid wheats were obtained through crosses of tetraploid wheat cultivar Langdon (Ldn) and different *Ae. tauschii* accessions, followed by chromosome doubling of the interspecific ABD hybrids (Takumi et al. 2009b; Kajimura et al. 2011). For RNA-seq, the *Ae. tauschii* accessions PI476874 from the L1-2 sublineage and IG47182 from the L2-2 sublineage were selected. Total RNA was isolated from spikes (about 3–6 cm long) before heading stage using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). mRNA was purified from 48 μg of RNA using an *Oligotex-dT30* <*Super*> mRNA Purification Kit (Takara Bio, Ohtsu, Japan). A cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany) was used to fragment a 200 ng aliquot of mRNA and synthesize cDNA from it. Approximately 10^8 adapter-ligated cDNA molecules from the samples were used for library preparation using a GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics).

3-2. RNA-seq, sequence assembly and discovery of polymorphic sites

The cDNA libraries were sequenced with a GS FLX Titanium Sequencing Kit on a GS FLX System (Roche Diagnostics) according to the manufacturer's instructions. Files containing raw sequence data were deposited in the sequence read archive of the DNA Data Bank of Japan (DDBJ) (accession number DRA001014). Before assembly, raw sequence reads were trimmed to remove primer and adapter sequences and poly-A tails. All reads from both accessions were merged and assembled *de novo* with the GS *de novo* assembler algorithm (Newbler) version 2.6 (Roche Diagnostics) to generate reference contig sequences (minimum-overlap length of 40 bp and minimum-overlap identity of 90%). During assembly, the Newbler algorithm constructs multiple alignments of overlapping reads and divides them into consistent sequences; i.e., contigs. When contig graphs contain branching structures, Newbler traverses paths through connected branches, generating isotigs that may represent splicing variants. SNPs and insertions/deletions (indels) were discovered by aligning all individual reads to the reference contig sequences using GS Reference Mapper version 2.6 software (Roche Diagnostics). Only the accession-specific sequence variants (supported by at least two reads) were extracted as true polymorphisms from "All" and "High-Confidence" sequence differences produced by GS Reference Mapper.

3-3. Generation of non-redundant (NR) contig sequences

To obtain NR contig sequences from all the leaf- and spike-derived contigs, sequence clustering using the CD-HIT-EST web server (Li and Godzik 2006) with 95% identity (parameters: -c 0.95, -n 8) was performed for each of the two tissues. Next, the NR

Table V-1. Lineage, accession number and origin of *Ae. tauschii* and hexaploid wheat accessions used in this study

Lineage – sublineage	Accession number (country)
	Ae. tauschii accessions
L1 – 1	IG48508 (Turkmenistan), KU-2627 (Afghanistan)
L1 – 2	PI476874 (Afghanistan), IG126387 (Turkmenistan)
L1 – 3	KU-2826 (Georgia), KU-2087 (Iran)
L1 – 4	IG131606 (Kyrgyzstan), IG48559 (Tajikistan)
L1 – 5	IG48747 (Armenia), KU-2144 (Iran)
L1 – 6	AT47 (China), AT76 (China)
L2 – 1	KU-2069 (Iran), KU-2811 (Armenia)
L2 – 2	IG47182 (Azerbaijan), KU-2100 (Iran)
L2 – 3	KU-2159 (Iran), KU-2093 (Iran)
HGL17	AE454 (Georgia), AE929 (Georgia)
	Hexaploid wheat accessions
L1-derived synthetics	Ldn/IG131606 (Kyrgyzstan), Ldn/IG126387 (Turkmenistan), Ldn/PI476874 (Afghanistan)
L2-derived synthetics	Ldn/KU-2090 (Iran), Ldn/KU-2069 (Iran), Ldn/KU-2159 (Iran), Ldn/KU-2097 (Iran)
Common wheat cultivars	Norin 61 (Japan), Kitanokaori (Japan), Chinese Spring (China)

Underlining indicates the accessions used for RNA-seq.

HGL: haplogroup lineage.

contigs from both tissues were merged and sequence clustering was performed using CD-HIT-EST with 95% identity (-c 0.95, -n 8).

3-4. Gene annotation and comparison of SNPs between the spike and leaf datasets

The reference sequences were searched against the National Center for Biotechnology Information (NCBI) NR protein database using the blastx algorithm with an *E*-value cut-off of 10^{-3}. Gene ontology (GO) terms were assigned using Blast2GO software (Conesa et al. 2005) based on blastx hits against the NCBI NR database.

For all the SNPs discovered in leaves (Chapter IV) and spikes, 50 bp sequences were extracted from contigs, positioning the SNPs in the middle of the sequence. The sequences generated from the spike libraries were searched against those generated from the leaf libraries using the blastn algorithm with an *E*-value cutoff of 10 and hit length ≥ 25 bp. A SNP was classified as "common" when the 26th nucleotide (SNP) from the 5′ end of the query sequence matched the SNP of the subject sequence. For this, the BLAST output was parsed to extract SNPs in common between the two datasets using an in-house Perl script.

3-5. *In silico* mapping of new SNPs and NR contigs to genome sequences of *Ae. tauschii* and barley

Contigs with high confidence (HC), lower confidence (LC) SNPs, and NR contigs were blastn searched against *Ae. tauschii* genome sequences (Jia et al. 2013) and barley genome sequences, among which fingerprinted contigs, whole genome shotgun assemblies and HC genes were included (IBSC 2012), with an *E*-value threshold of 10^{-5} and hit length ≥ 50 bp. The LC SNPs were here defined as all detected SNPs except the HC SNPs. Based on the blastn data, the SNPs of *Ae. tauschii* were plotted onto the barley genome using Circos version 0.63 software (Krzywinski et al. 2009).

3-6. Development of SNP and indel markers

The identified SNPs were converted to cleaved amplified polymorphic sequence (CAPS) or high resolution melting (HRM) markers. The primer sequences of SNP and indel markers, product lengths, and restriction enzymes are summarized in Table V-2. PCR and analysis were performed according to our previous studies (Matsuda et al. 2012; Chapter IV). The polymorphic information content (PIC) was calculated using Excel Microsatellite toolkit add-in software (Park 2001).

Table V-2. List of primers developed in this study

Marker name	Primer sequences	Type	Restriction enzyme
Xctg02576	ATGCACCACGAGTCTCTCCT GTCGTCCTTGGAGTTGGAAG	HRM	-
Xctg03043	GGAAGTGCCATTCAGACACA TGAGTGCCACTGCCATTATC	HRM	-
Xctg03577	GAACCCTTGAGGAAGGAGAGACT AAAATGCTCTTTATCGTATGCCTTT	HRM	-
Xctg05053	CGGCATTCATCTCAAACCTT TTTTCGCGGTCTTCTCTGTT	Indel	-
Xctg05205	TACGCTCCTCTGGTTTCCTC GAAGAGTTGCCAAAGCAAGG	CAPS	*Msp*I
Xctg05269	TTACCATGAGCGGTTGACCC GGCTACTGATCCCCGTGATG	HRM	-
Xctg05312	GGAGAAGGCGAGGACAATGT GCTGATGAAATATCCCGCAGC	HRM	-
Xctg05593	GCTTTCTAGCATGAGCAAACG CACATTCTTCCGATGCACAA	HRM	-
Xctg05637	AGCAAGAGAGTGGCCTCTGA GAACAACCTATGGGCAATCG	HRM	-
Xctg05753	AGAAACCCAGTCCACAATGG CCCAAGGGGTGTAGCAGTAG	HRM	-
Xctg05841	CTCGACTCGATCTCACCACA AATCCGCGACCGATACATAC	CAPS	*Hae*III
Xctg05879	TAAAACCGTGTGCGATGTGT CCCTCTCCTCACTCTCTCCA	HRM	-
Xctg06648	CAAGTACAAATTGCATCTTAAGCAC ATATGCAGATATTGTTGCTGTTCAC	HRM	-
Xctg06982	GGGCTTTTGTAATTGCAGAGT CTGGTTCATGAGGAGTGCAT	HRM	-
Xctg07643	CAGGGTTGATGAATTCTCGTG ACAAGTTCCCCAACGTCAAG	Indel	-
Xctg10285	AATGAATGCACTCAGAGTAGGGTAG GCTTCCCTTTTGTTCAGCTTGTA	HRM	-

Table V-2. Continued

Marker name	Primer sequences	Type	Restriction enzyme
Xctg210866	CTTGGCAAGTTTAGCAGCCG TTCCGCTCCCCTCCTGAATA	CAPS	*Xba*I
Xctg210917	AGGATCTGGCGCAGCTAAT CCCAGCATGCTTGATTGAG	CAPS	*Hinf*I
Xctg211209	CCCCTGCTCTTGGATTGTT GCGCATCACACTGGAGAAGT	CAPS	*Sal*I
Xctg211320	GTGCCACGTGGATTGATGAT AAAGAAGGACACTGACGACGA	CAPS	*Msp*I
Xctg213397	AGAAGTAGCTGCATTCGGTG GGGTGGGCTAGATGCTTCTG	HRM	-
Xctg213881	CCTTCCTCGAGGTCATCAGA TATAGCTGGGCCATGGAGTC	Indel	-
Xctg214283	TGTTCGAAATGTGCTTTCCT TGAAGTTCTTGGGGTCATCA	CAPS	*Hae*III
Xctg215026	TGCGGTCGATCCTACAACAG ATGTGGCGACTTGGGACATT	HRM	-
Xctg216249	GGCATGACAATCTGAAGGTCC TGCAGCAATGGTATCTCCTGA	HRM	-
Xctg216700	TGTCTTCTGTGCCGTCTTCC GTACGTTCCAGAGCTGCAGA	CAPS	*Hinf*I
Xctg216781	CGTTTGCTTGTGGGCATTGA CCAGTGGCATCCTTGAATGC	HRM	-
Xctg217654	GCGACGTCTCTTTATTCTCATC CTACTCCTGCCATTGGTCTTG	CAPS	*Dde*I
Xctg221573	AAAGATGCAAGAACACCCGTG GAGGGTGAAGGAGAGGCAAC	HRM	-

3-7. Linkage map construction

Three F_2 mapping populations with different ploidy levels were used for construction of D-genome linkage maps. The diploid mapping population consisted of 97 F_2 individuals derived from a cross between PI476874 (L1) and IG47182 (L2) (Chapter IV); the synthetic hexaploid wheat population consisted of 117 F_2 individuals derived from a cross between Ldn/KU-2075 (L2) and Ldn/KU-2025 (L1) (Mizuno et al. 2011; Matsuda et al. 2012) and a set of 108 F_2 individuals derived from a cross between the common wheat cultivar Norin 61 (N61) and an L1-derived synthetic wheat (Ldn/PI476874) (Okamoto et al. 2012). The new markers were assigned to chromosomes 2D of the established genetic maps. Linkage maps were constructed using the MAPMAKER/EXP version 3.0b package (Lander et al. 1987) and drawn in MapChart version 2.2 software (Voorrips 2002). The genetic distances were calculated with the Kosambi function (Kosambi 1944).

4. Results

4-1. Sequencing and assembly of expressed sequence tags (ESTs)

The sequencing of spike cDNA libraries from PI476874 and IG47182 respectively produced 848,953 and 893,917 reads, which corresponded to 361 and 386 Mb per accession after trimming. Both libraries were merged and assembled *de novo* to generate the reference sequences. In total, 21,208 contigs (length ≥ 100 bp) and 18,530 isotigs were generated. The nucleotide length of the majority of contigs and isotigs ranged between 500 and 1,000 bp, with an average of 903 bp for contigs and 1,231 bp for isotigs (Table V-3).

In order to estimate how many of the contigs were expressed in spikes rather than leaves, NR contig sequences were first obtained for each of the two tissues, and then the number of spike contigs was reduced from 21,208 to 20,518 and the number of leaf contigs from 10,224 to 9,896. Next, both tissue-derived NR contigs were merged and second sequence clustering was performed again. Out of the 30,414 contigs, 24,875 NR contigs were obtained, and 15,739 contigs were found in spikes but not in leaves (Figure V-1).

For gene annotation, isotigs were reassembled using the CAP3 program (Huang and Madan 1999) with default parameters (identity ≥ 90% and overlap of 40 bp), and longer NR sequences were obtained. As a result, the 18,530 isotigs from both tissues were reassembled into 17,598 NR sequences. These NR sequences were blastx searched against the NCBI NR protein database, and significant hits were obtained for 88.4% of the query sequences. GO annotation was performed based on these blastx results,

Table V-3. Contig and isotig sequence length distribution

Sequence length (bp)	Number of contig	Number of isotig
100–500	6,559	1,356
501–1,000	7,840	8,217
1,001–1,500	3,576	4,289
1,501–2,000	1,705	2,244
2,001–2,500	759	1,071
>2,500	769	1,353
Total	21,208	18,530
Average length (bp)	903	1,231

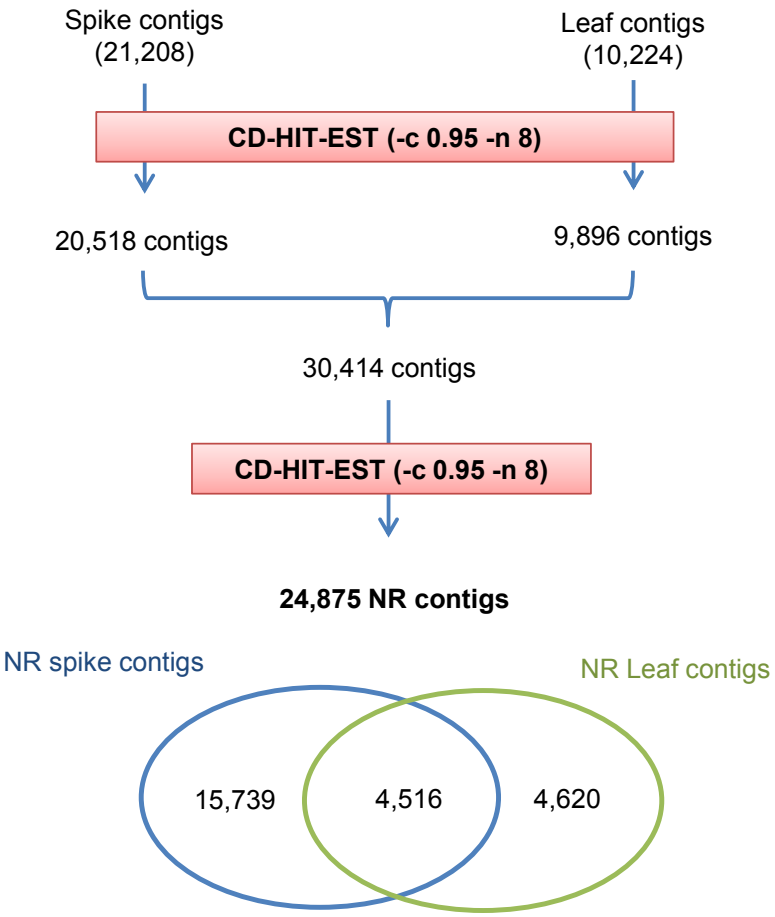

Figure V-1. Schematic showing NR contigs obtained from *de novo* assembled sequences of leaves and young spikes. Two rounds of sequence clustering were performed to obtain 28,875 NR contigs. The Venn diagram shows the number of NR contigs specific to leaves, specific to spikes, and found in both tissues.

assigning one or more GO terms to 13,309 sequences. Of the assigned GO terms, 34,500 were under the biological process domain, 15,851 under molecular function and 19,017 under cellular component. Compared with the leaf isotigs, GO term enrichment was observed in the developmental process, cellular component organization, nucleus, plasma membrane and cytosol categories (Figure V-2).

4-2. SNP and indel detection

To search for polymorphic sites, individual reads from the spike libraries of two accessions were aligned to the reference sequence using GS Reference Mapper. Of the 28,268 total polymorphic sites detected in contigs longer than 100 bp, 25,837 were SNPs, 2,108 indels, and 323 variants with two or more nucleotide changes (Table V-4). According to the read depth (≥ 3 non-duplicated reads) and quality (QV ≥ 20), 13,347 polymorphic sites were classified as HC SNPs, which were detected in 4,872 contigs. On average, one SNP was found for every 741 bp of sequence, and an HC SNP appeared once per 1,435 bp of sequence.

The SNP dataset from the published leaf libraries contained SNPs derived from contigs shorter than 100 bp (Chapter IV). Thus, we selected only SNPs detected in contigs ≥ 100 bp to compare the SNPs detected in the spike libraries with those in the leaf libraries (Table V-4). Out of the SNPs found in both tissues, SNPs considered as HC in either of the tissues were counted as HC SNPs. In total, 31,454 NR SNPs were detected in 10,054 contigs of both tissues (Figure V-3), and 16,148 SNPs (51%) in 5,808 contigs were classified as HC. Consequently, SNPs were detected in 23% of the NR contigs (5,808/24,875), and 10,589 of the 13,347 HC SNPs found in spikes were new.

4-3. *In silico* mapping of polymorphic contigs

A draft genome sequence was recently reported in *Ae. tauschii*, and around 40% scaffolds of the genomic sequence was anchored to the *Ae. tauschii* chromosomes (Jia et al. 2013). In addition, a 4.03 Gb physical map of *Ae. tauschii* has been published, and approximately 61 Mb of the genomic sequences obtained from the extended SNP marker sequences, which were used for anchoring bacterial artificial chromosome contigs to the linkage map, is now available (Luo et al. 2013). We first integrated both sets of genomic information by performing a blastn search of the extended marker sequences against the draft genome sequence of *Ae. tauschii*. Hits were obtained for all extended markers with an *E*-value ≤ 2×10^{-152}. In addition to 13,688 scaffolds (a total of 1.28 Gb) anchored to the Y2280/AL8/78 linkage map of *Ae. tauschii* (Jia et al. 2013),

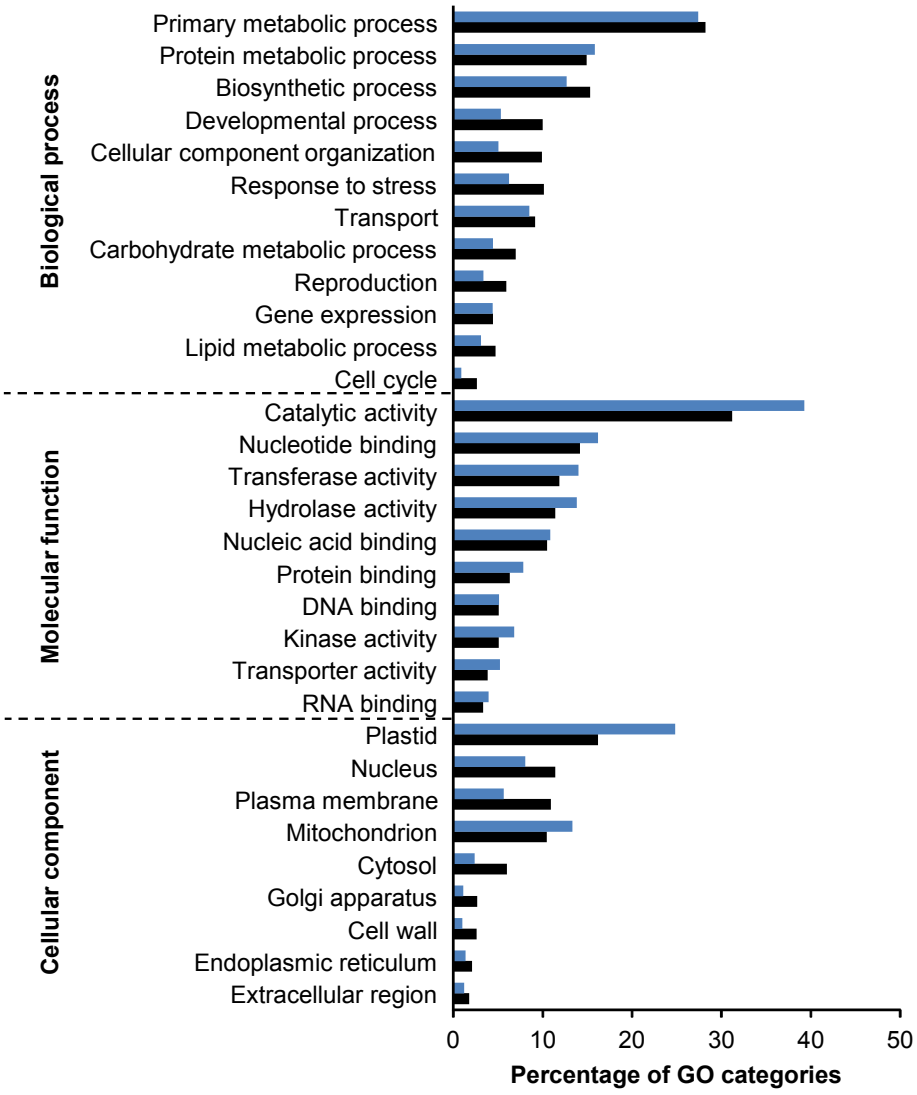

Figure V-2. GO annotation of the leaf and spike isotigs. The percentage of isotigs is summarized into three main subontologies: biological process, molecular function and cellular component. Based on blastx hits against the NCBI NR protein database, one or more GO annotations were assigned to 13,309 isotigs of spikes. Blue bars: leaf isotigs, black bars: spike isotigs. Percentage was determined based on the number of isotigs assigned to a specific GO category divided by the total number of isotigs.

Table V-4. Polymorphisms detected between the two *Ae. tauschii* accessions

	Number of polymorphic sites in contigs ≥ 100 bp	
	Spike	Leaf*
Total	28,268 (8,592 contigs)	10,355 (3,571 contigs)
All SNPs	25,837 (8,325 contigs)	9,429 (3,441 contigs)
HC SNPs	13,347 (4,872 contigs)	4,335 (1,747 contigs)
Indel	2,108 (1,444 contigs)	794 (542 contigs)
Multiple nucleotide polymorphisms	323 (298 contigs)	112 (100 contigs)
Average bp per HC SNP	1,435	1,854

*Obtained after filtering the data from Chapter IV

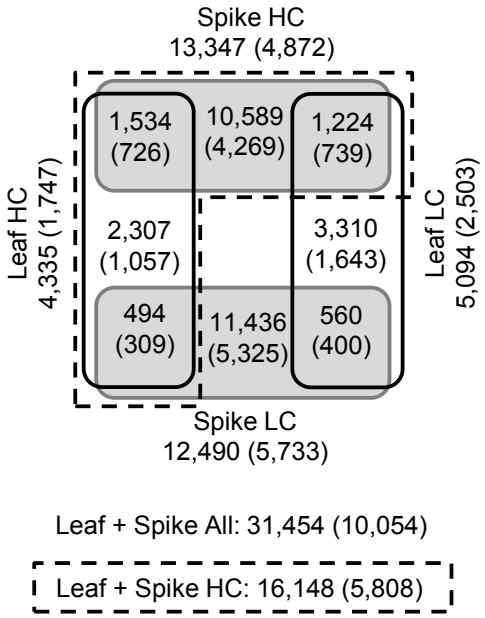

Figure V-3. Comparison of SNPs found in contigs of leaf and spike transcripts. The number of SNPs in leaves is indicated inside the black continuous lines, that of spikes in shaded boxes and HC SNPs of both tissues inside the broken black line. The number of contigs is indicated in parentheses. In the case of common SNPs, the number of contigs is represented by the number of spike contigs because of their longer sequences.

3,188 scaffolds were anchored to the AL8/78/AS75 linkage map (Luo et al. 2013), resulting in a total of 1.49 Gb of sequence anchored to the *Ae. tauschii* maps. Next, the polymorphic and NR contigs obtained in the present study were mapped *in silico* to the *Ae. tauschii* draft genome sequence based on blastn searches. Hits were obtained for 24,633 (99.0%) of the 24,875 NR contigs. For the contigs with SNPs, 10,022 (99.7%) out of 10,054 were confirmed to be present in the D-genome draft sequence, and 6,179 (61.5%) were successfully mapped to the anchored scaffolds. Additionally, it has been reported that genomic scaffolds can be anchored to known wheat linkage maps using simple sequence repeat marker and EST sequences (Jia et al. 2013). Using this information, the number of contigs with SNPs anchored to chromosomes of the D genome increased to 8,848 (Tables V-5 and Table V-6). Similarly, 5,642 of the contigs with HC SNPs were mapped to the *Ae. tauschii* genome, and 5,122 were anchored *in silico* to the *Ae. tauschii* maps. On average, 732 contigs with HC polymorphisms were mapped to each *Ae. tauschii* chromosome (Table V-7).

The polymorphic and NR contigs were also aligned to the barley draft genome because of the extensive conserved synteny between barley and wheat chromosomes (Mayer et al. 2011). blastn hits were obtained for 22,711 NR contigs, of which 22,549 were assigned to the barley chromosomes. In total, 9,516 polymorphic contigs were assigned to the barley chromosomes, which included 5,407 contigs with HC SNPs (Table V-5). On average, 772 HC polymorphic contigs were mapped to each barley chromosome (Table V-7). Because the genomic sequences anchored to the linkage map are longer in barley (> 3.09 Gb) than in *Ae. tauschii*, and physical map information is available in barley (IBSC 2012), the HC SNPs of *Ae. tauschii* were plotted onto the barley chromosomes (Figure V-4). The number of both SNPs and NR contigs was higher in telomeric regions than in centromeric regions.

4-4. Marker development and mapping to chromosome 2DS of *Ae. tauschii*

To assess the usefulness of the SNP dataset for construction of wheat linkage maps, 2DS-specific markers were designed based on the *in silico* mapping of polymorphic contigs. In total, 40 markers were new and eight were discarded because the PCR products were longer than expected. Of the remaining primer sets, three were indel markers, nine were CAPS and 20 were HRM markers. Polymorphisms using the 32 markers were examined in 20 accessions of *Ae. tauschii*, which were selected from each of the sublineages reported by Mizuno et al. (2010a) to cover the species' genetic diversity. Two HRM markers and one CAPS marker were excluded from further study because we failed to detect any polymorphism in these markers. Based on the allelic

Table V-5. Number of contigs mapped to the draft genome of *Ae. tauschii* and barley

	Ae. tauschii genome	Barley genome
NR contigs with SNPs	10,054 (5,808)	
Aligned to the genome	10,022 (5,642)	9,569 (5,435)
Spike	7,621 (4,262)	7,292 (4,112)
Leaf*	2,253 (1,053)	2,142 (1,009)
Common	1,646 (1,260)	1,595 (1,227)
Anchored to the genetic map	8,848 (5,122)	9,516 (5,407)
Spike	6,812 (3,871)	7,251 (4,091)
Leaf*	1,415 (705)	2,131 (1,003)
Common	1,522 (1,170)	1,589 (1,223)

Number of HC contigs is indicated in parenthesis.

*Data from Chapter IV.

Table V-6. Number of polymorphic contigs assigned to the D-genome chromosomes according to various genetic maps

Chromosome	Y2280 x AL8/78[a]	AL8/78 x AS75[b]	SSR/ESTs[a]	Total
1D	566 (364)	313 (191)	323 (169)	1,202 (724)
2D	602 (370)	420 (240)	379 (189)	1,401 (799)
3D	476 (282)	391 (220)	446 (219)	1,313 (721)
4D	447 (272)	385 (242)	296 (148)	1,128 (662)
5D	657 (405)	327 (186)	514 (284)	1,498 (875)
6D	384 (244)	314 (180)	373 (199)	1,071 (623)
7D	491 (313)	406 (239)	338 (166)	1,235 (718)
Total	3,623 (2,250)	2,556 (1,498)	2,669 (1,374)	8,848 (5,122)

Number of HC contigs is indicated in parenthesis.

[a]Jia et al. (2013)

[b]Luo et al. (2013)

Table V-7. Number of polymorphic contigs assigned to *Ae. tauschii* and barley chromosomes

Anchored to D-genome genetic map			**Barley physical map**		
	All	HC		All	HC
1D	1,202	724	1H	1,179	695
2D	1,401	799	2H	1,504	839
3D	1,313	721	3H	1,446	775
4D	1,128	662	4H	1,176	687
5D	1,498	875	5H	1,551	907
6D	1,071	623	6H	1,255	708
7D	1,235	718	7H	1,405	796
Total	**8,848**	**5,122**	**Total**	**9,516**	**5,407**

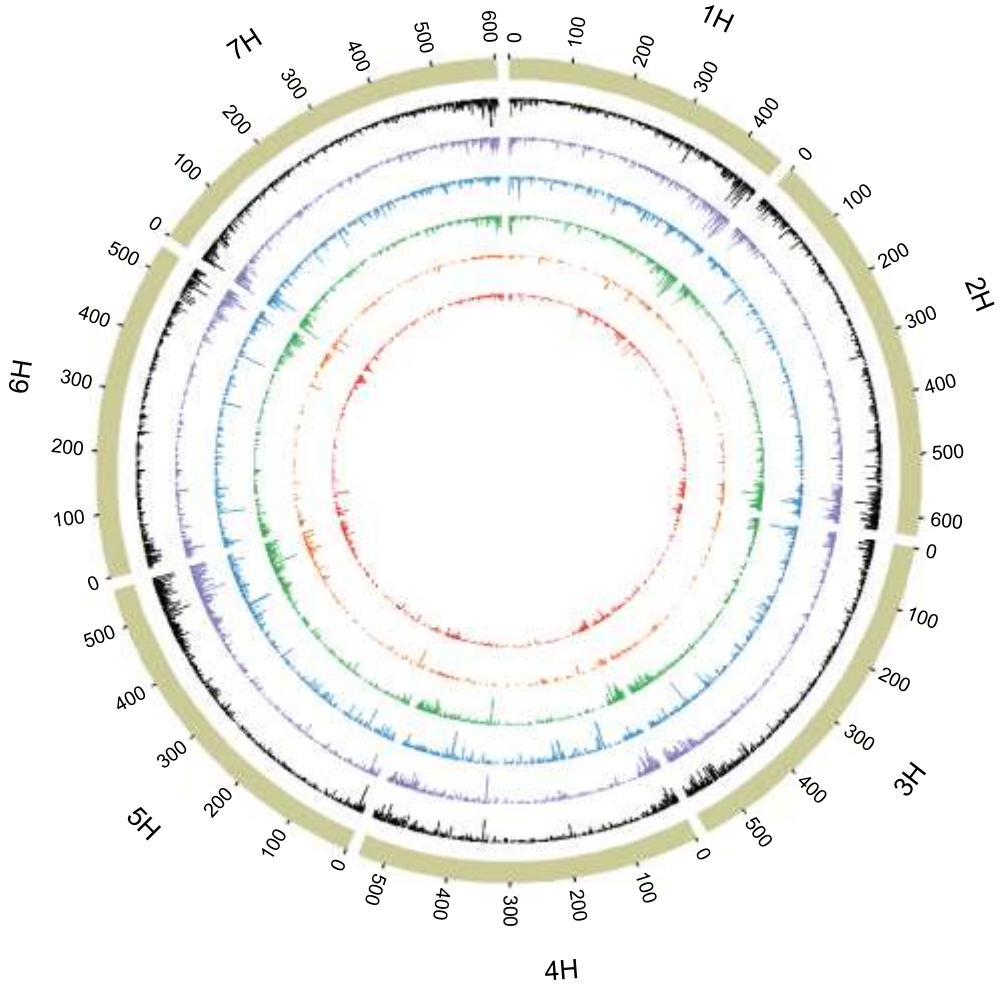

Figure V-4. Distribution of contigs and HC SNPs on the physical map of barley. From outer circle to inner: track 1 shows the physical map of barley (scale in Mb), track 2 represents a histogram of the number of mapped NR contigs, track 3 the number of all HC SNPs, track 4 the number of HC SNPs per mapped NR contig in the same region, track 5 the number of HC SNPs detected only in spikes, track 6 the number of HC SNPs detected only in leaves, and track 7 the number detected in both tissues. The distribution of contigs and SNPs are summarized in non-overlapping 250 kb intervals of the barley physical map.

diversity within the 20 accessions, PIC values were calculated, which ranged from 0.09 to 0.57, with an average of 0.29 (Figure V-5). Out of the 29 markers, two were IG47182-specific SNP alleles and one was a PI476874-specific SNP allele (PIC = 0.09) (Figure V-6). In 21 of the markers (72%), the PIC value was greater than 0.20, indicating that they were polymorphic among most of the accessions; these included six markers that clearly distinguished L1 from L2 accessions (Figure V-6).

To confirm the 2DS-specific assignment of the markers developed, an F_2 population of PI476874/IG47182 was used for genotyping. One of the HRM markers (*Xctg10285*) failed in the genotyping because of difficulty distinguishing the alleles. All of the remaining 28 markers (96.6%) were successfully assigned to chromosome 2DS (Figure V-7). Segregation distortion has been reported for chromosome 2D of this population (Chapter IV). Segregation distortion was observed in the chromosomal region between the marker bags20g23 (HRM13) (220.3 cM) and *Xgwm539* (347.1 cM). In this region, the frequency of the homozygous PI476874-allele was significantly lower than expected, whereas the homozygous IG47182-allele appeared at high frequency (Figure V-8; Table V-8).

4-5. Polymorphism and mapping of the markers in hexaploid wheat

To examine the usefulness of the markers developed for detection of polymorphisms in hexaploid wheat, three common wheat cultivars, three L1-derived synthetic wheat lines, and four L2-derived synthetics were genotyped. All the synthetic hexaploids were obtained by crossing the tetraploid wheat cultivar Ldn with the respective L1 or L2 accessions of *Ae. tauschii* (Takumi et al. 2009b; Kajimura et al. 2011). The synthetic wheat line derived from the L1 accession used in the RNA-seq analysis, Ldn/PI476874, was also genotyped, but no synthetic line derived from the L2 accession was genotyped because triploid hybrids of Ldn/IG47182 exhibited hybrid lethality (Hatano et al. 2012). In addition to the 29 markers, 14 previously developed HRM markers were also used (Table V-9). Of the 43 markers, 25 (58%) were polymorphic, defined as showing a distinct genotype in at least one of the 10 hexaploid wheat lines. Of the three marker types, 75% of the CAPS markers (six out of eight) were polymorphic, followed by 67% of indel (two out of three) and 53% of HRM markers (17 out of 32). The low polymorphism of HRM markers in hexaploid wheat might be explained by a decrease in sensitivity of HRM analysis due to the presence of the A and B genomes. PIC values ranged from 0.16 to 0.56 (average 0.36), and the markers with the lowest PIC values detected specific alleles in Ldn/PI476874 (Figure V-5). Six of the polymorphic markers clearly distinguished the L1-derived hexaploids from the L2-derived synthetics and

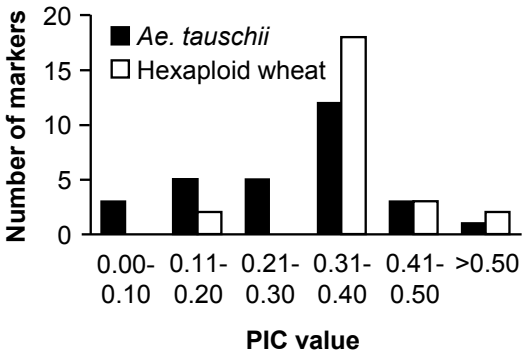

Figure V-5. Frequency distribution of PIC values of the markers developed. PIC values of 29 markers among the 20 *Ae. tauschii* accessions (black bars) and 25 markers among the 10 hexaploid wheat lines (open bars).

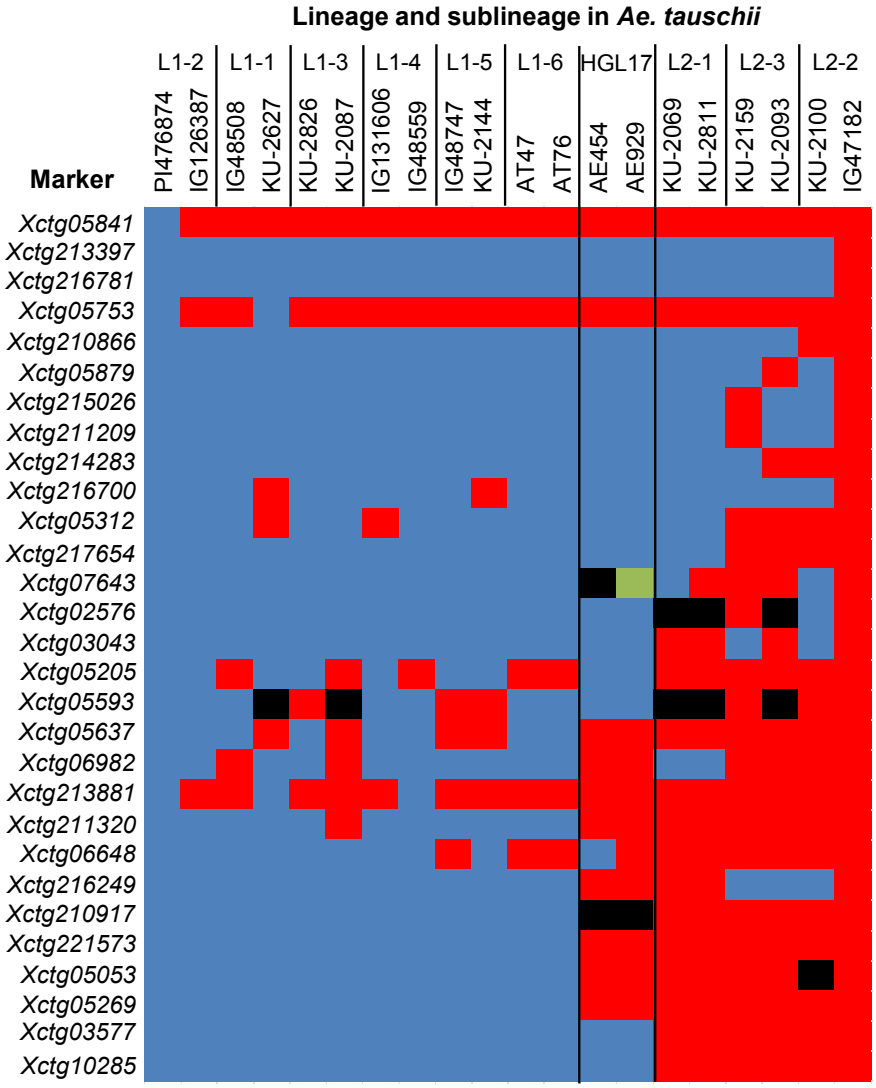

Figure V-6. Summary of genotyping results of the 29 polymorphic markers in the 20 *Ae. tauschii* accessions. The genotypes of the two accessions used in RNA-seq, PI476874 and IG47182, are shown in the first and last columns, respectively. Blue and red respectively indicate the PI476874 and IG47182 alleles. Other alleles are indicated in black and green.

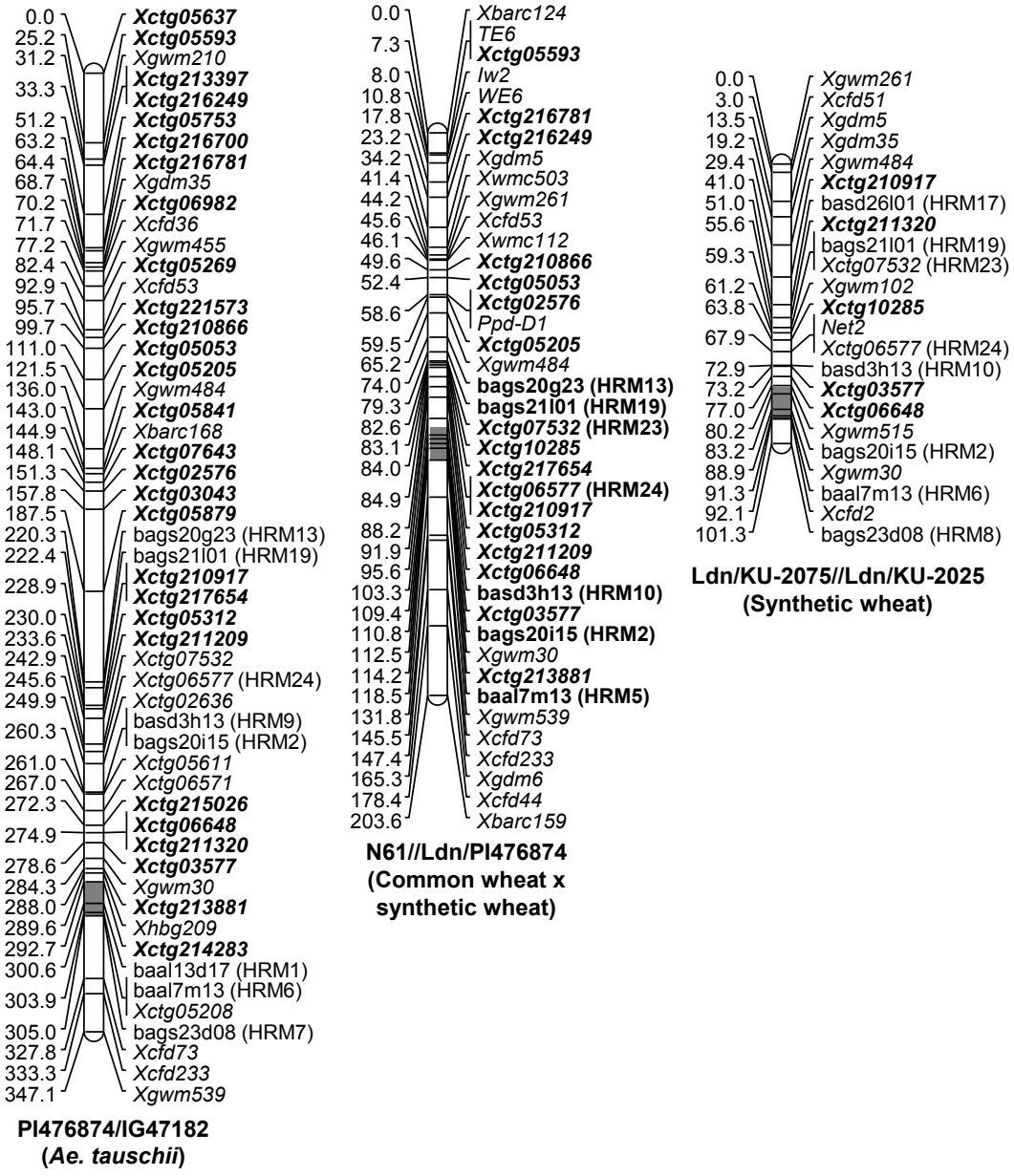

Figure V-7. Genetic maps of chromosome 2D of the *Ae. tauschii* and hexaploid wheat F_2 populations. The diploid PI476874/IG47182 map (left) and hexaploid maps of N61//Ldn/PI476874 (middle) and Ldn/KU-2075//Ldn/KU-2025 (right) populations are shown. Gray bars indicate the putative centromere positions. The markers mapped in this study (indicated in bold) were placed on previously constructed linkage maps. Map distances are shown in centimorgans (cM).

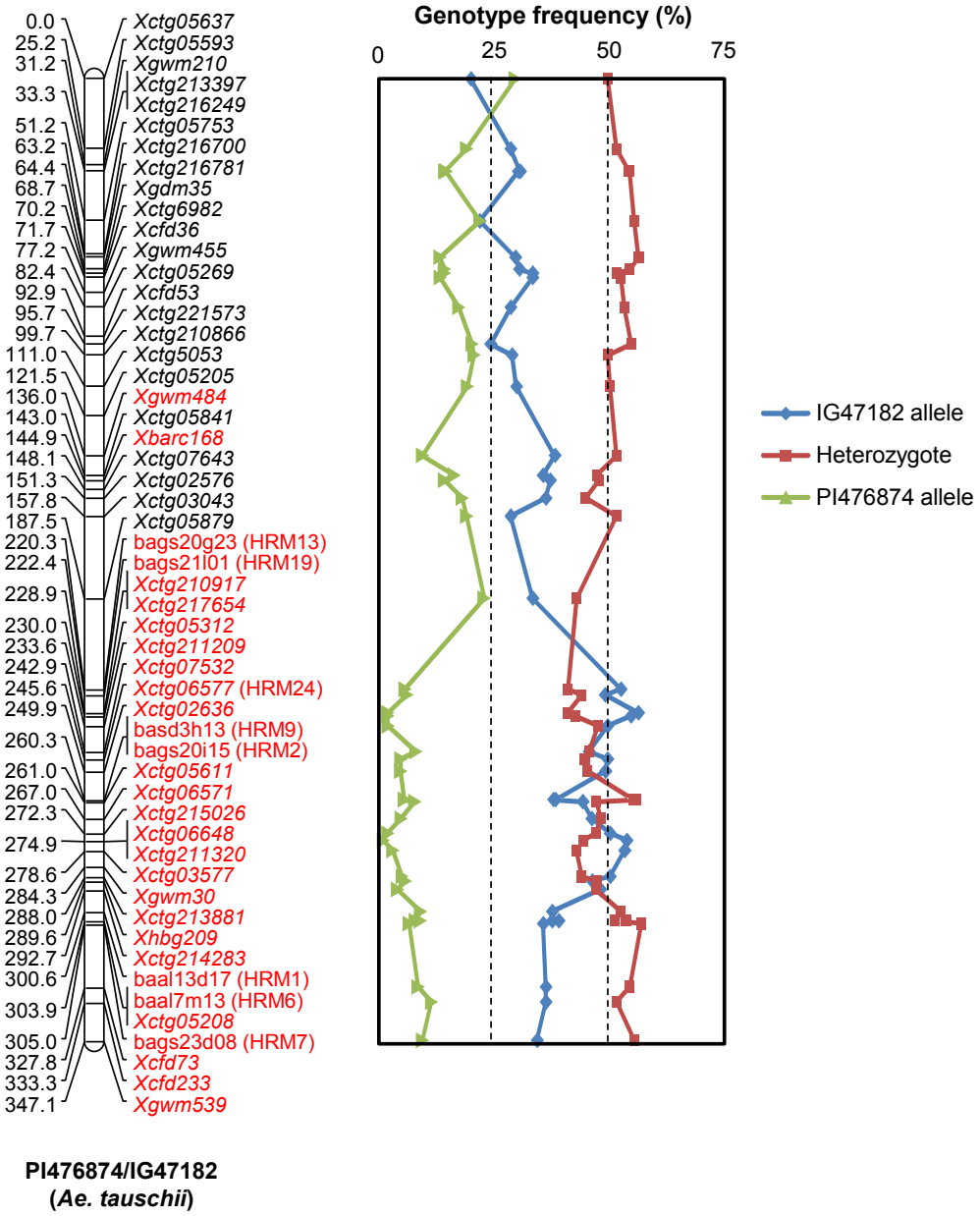

Figure V-8. Genotype frequency of the co-dominant markers mapped to chromosome 2D. The PI476874 allele frequency (green line) is lower than that of the IG47182 allele (blue line) and of heterozygotes (red line) in markers, showing significant segregation distortion (indicated in red, $P < 0.05$).

Table V-8. Segregation data for the mapped markers on 2DS of the PI476874/IG47182 map

Marker name	Position (cM)	Segregation *	*P*-value†
Xctg05637	0.0	21 / 52 / 31	0.7499
Xctg05593	25.2	30 / 54 / 20	0.7216
Xctg213397	33.3	30 / 53 / 14	0.1908
Xctg216249	33.3	29 / 52 / 14	0.2320
Xctg05753	51.2	23 / 58 / 23	0.8469
Xctg216700	63.2	31 / 64	0.5661
Xctg216781	64.4	29 / 55 / 13	0.1348
Xctg06982	70.2	35 / 54 / 15	0.0974
Xctg05269	82.4	28 / 52 / 17	0.5578
Xctg221573	95.7	24 / 54 / 20	0.8534
Xctg210866	99.7	28 / 48 / 20	0.8557
Xctg5053	111.0	31 / 52 / 20	0.6700
Xctg05205	121.5	31 / 71	0.8121
Xctg05841	143.0	37 / 49 / 17	0.0912
Xctg07643	148.1	39 / 64	0.0589
Xctg02576	151.3	38 / 47 / 19	0.0952
Xctg03043	157.8	30 / 54 / 20	0.7216
Xctg05879	187.5	35 / 45 / 24	0.3781
Xctg210917	228.9	55 / 42	< 0.001
Xctg217654	228.9	55 / 40 / 2	< 0.001
Xctg05312	230.0	54 / 42 / 2	< 0.001
Xctg211209	233.6	48 / 46 / 2	< 0.001
Xctg215026	272.3	49 / 46 / 2	< 0.001
Xctg06648	274.9	52 / 43 / 1	< 0.001
Xctg211320	274.9	53 / 44	< 0.001
Xctg03577	278.6	51 / 41 / 3	< 0.001
Xctg213881	288.0	49 / 43 / 5	< 0.001
Xctg214283	292.7	46 / 45 / 4	< 0.001

*Segregation indicates number of IG47182 homozygotes / heterozygotes / PI476874 homozygotes.

†Chi-square test probabilities for segregation distortion were calculated against the predicted 1:2:1 or 3:1 segregation. *P*-values less than 0.05 are indicated in red.

TableV-9. List of primers developed in our previous studies

Marker name	Primer sequences	Type
	From Matsuda et al. (2012)	
baal13d17 (HRM1)	ACTGGCAGGAGGCTTAACATATC GTGGAGACCGACTTCAGGAC	HRM
bags20i15 (HRM2)	AACTAGACGTGGATTGGGTAGATG AGATACATCCATTTGAAGAACAAGC	HRM
baal7m13 (HRM5)	TACATGAAGCCTTTGGAAGTTTTT TCATGTGCAGTTATGTTCTAGTGTTT	HRM
basd3h13 (HRM9)	CCTGAGAAGTCAATCCCATCA GCAGACCATGATCCATCTGA	HRM
basd3h13 (HRM10)	CTGAGAAGTCAATCCCATCATCTAT ACTACAAGACCAGTGCCCATTT	HRM
bags20g23 (HRM13)	GTTATCAACATGGCATTAAACTCGT GGTTTTCCATTACAGCAGCATAGTA	HRM
basd26l01 (HRM17)	GAGGTGGGTTGTGTCTAAATATCAT ATACATAGTTCTCACCGCCTAGGTA	HRM
bags21l01 (HRM19)	TGAAAAGGTGAGCTTTCTACTGC AGAAGGCATGCTAGAAAATATAGAGC	HRM
	From Chapter IV	
Xctg02636	CATGGAGCCCATCGTGTT CTCTTGAGTTCTGCCCATCTG	HRM
Xctg05208	GCCACCTAAAATCCAGCTCA TTTGACAAAGTTGAGGATACCG	HRM
Xctg05611	CCTCATTCATAATAGAGTCAAATGGA TGGACAGATTGTCACATGGATTA	HRM
Xctg06571	CCTGAAGCCAAACCAATAAGAC TCTAAGGACAAAGCACATTTCAGAT	HRM
Xctg06577 (HRM24)	ATGATCCTGCTGGTGAAGATGT GAGGAAGATGCAGACGGAGTAG	HRM
Xctg07532 (HRM23)	ACACTCTTATTGAGGAAGTGCTTTG GCAGGTGGGAGAAAAAGATTCTA	HRM

common wheat cultivars (Figure V-9). In most of the markers (92%), the PIC values were greater than 0.20, indicating high polymorphism, especially between L1 and L2 synthetics and between L1 and common wheat, as supposed previously (Chapter IV). The HRM marker *Xctg216781*, which recognized an allele specific to IG47182 among the 20 *Ae. tauschii* accessions, distinguished common wheat cultivars from the wheat synthetics. For three HRM markers, polymorphisms were observed even among common wheat cultivars.

To examine whether these markers could be mapped to 2DS in the allohexaploid background, F_2 individuals of two mapping populations were genotyped. In the N61//Ldn/PI476874 population, 23 of the 25 polymorphic markers between the parents were available for genotyping of the F_2 plants, and no segregation distortion was observed (Table V-10). Four of the five (80%) CAPS markers and three of the 16 (19%) HRM markers were used as dominant markers, although six of these seven markers mapped as co-dominant markers in the diploid mapping population (Figure V-10 and V-11). In total, 22 markers were assigned to 2DS (Figure V-7) and the assignment of one marker (*Xctg05269*) was ambiguous between chromosomes 2B and 5D.

The second F_2 population derived from the cross between L1 and L2 synthetics (Ldn/KU-2075//Ldn/KU-2025) varied only in the D genome, in contrast to the N61//Ldn/PI476874 population. First, we checked the polymorphism of the 29 markers developed in this study and 6 HRM markers developed in Chapter IV between the parental synthetics. Only 14% of the markers (5/35) were polymorphic and could thus be used for genotyping of the F_2 plants. The low polymorphism implied that the markers based on polymorphisms between PI476874 and IG47182 might be less useful in some cross combinations of L1 and L2 accessions. It might also be explained in part by the low sensitivity of the HRM procedure in allopolyploid species. Two CAPS markers and two HRM markers were used as dominant markers in the Ldn/KU-2075//Ldn/KU-2025 population. All five markers mapped to chromosome 2DS, and no segregation distortion was observed (Figure V-7; Table V-11).

Using the PI476874/IG47182 and N61//Ldn/PI476874 linkage maps, in which more than 20 ESTs were assigned, the relationship between genetic and physical distances was evaluated. The physical map of barley was used in this analysis because the number of genomic scaffolds aligned to the map was larger than in *Ae. tauschii*. The recombination frequency on 2DS greatly decreased at distances further than 100 Mb from the distal end of the physical map in both the *Ae. tauschii* and hexaploid wheat maps (Figure V-12). This result was consistent with previous reports in barley and *Ae. tauschii* (IBSC 2012; Luo et al. 2013).

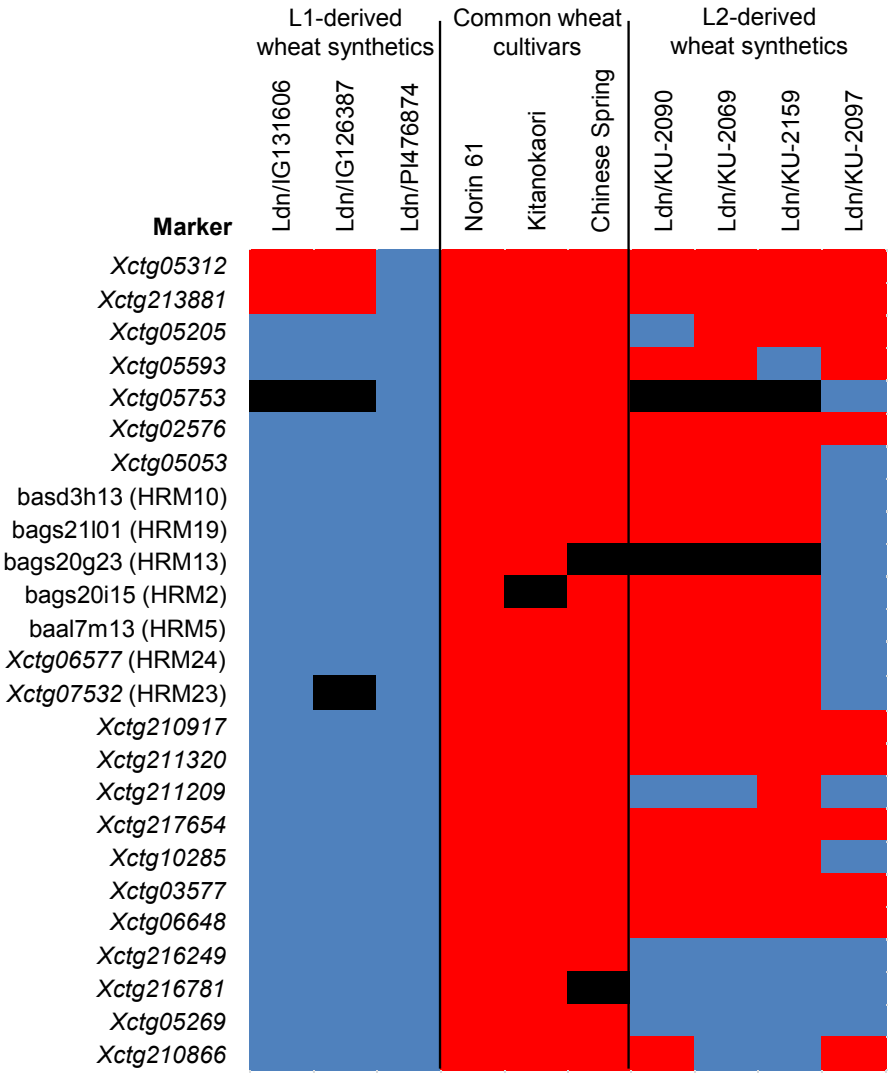

Figure V-9. Summary of genotyping results of the 25 polymorphic markers in 10 lines of hexaploid wheat. Genotypes of Ldn/PI476874 (blue) and common wheat cultivar N61 (red) were used for reference. Alleles other than these two are indicated in black.

Table V-10. Segregation data for the mapped markers on 2DS of the N61//Ldn/PI476874 map

Marker name	Position (cM)	Segregation *	*P*-value†
Xctg05593	7.3	26 / 63 / 19	0.4187
Xctg216781	17.7	29 / 58 / 17	0.3856
Xctg216249	23.1	16 / 80	0.4695
Xctg210866	49.5	25 / 82	0.9972
Xctg05053	52.3	30 / 55 / 23	0.9181
Xctg02576	58.5	25 / 82	0.9972
Xctg05205	59.4	30 / 52 / 26	0.9787
bags20g23 (HRM13)	73.9	34 / 46 / 22	0.4332
bags21l01 (HRM19)	79.2	28 / 55 / 25	0.9952
Xctg07532 (HRM23)	82.5	27 / 58 / 23	0.9261
Xctg10285	83.0	27 / 59 / 22	0.8461
Xctg217654	83.9	22 / 85	0.8903
Xctg06577 (HRM24)	84.8	26 / 59 / 22	0.8390
Xctg210917	84.8	27 / 80	1.0000
Xctg05312	88.1	25 / 58 / 22	0.8573
Xctg211209	91.8	17 / 85	0.4369
Xctg06648	95.5	28 / 56 / 23	0.9512
basd3h13 (HRM10)	103.2	27 / 51 / 19	0.8129
Xctg03577	109.3	25 / 54 / 21	0.9158
bags20i15 (HRM2)	110.7	29 / 58 / 20	0.6860
Xctg213881	114.1	28 / 61 / 19	0.5066
baal7m13 (HRM5)	118.4	29 / 60 / 18	0.4279

*Segregation indicates number of N61 homozygotes / heterozygotes / Ldn/PI476874 homozygotes.

†Chi-square test probabilities for segregation distortion.

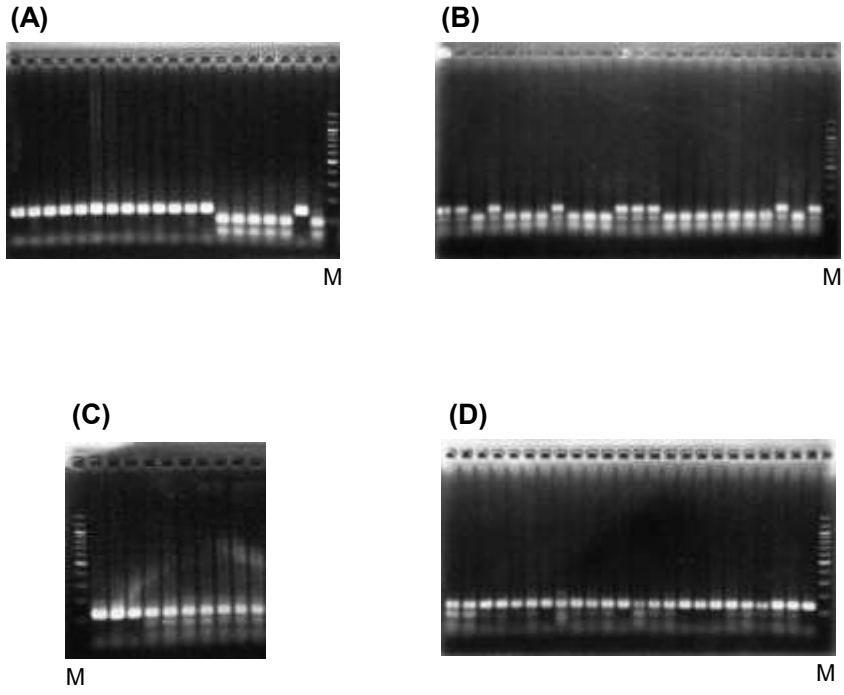

Figure V-10. Examples of CAPS markers that are co-dominant in the diploid but dominant in the hexaploid mapping population. (A–D) CAPS fragment patterns of *Xctg217654* in 20 accessions of *Ae. tauschii* (from left to right: two accessions of HGL17, 11 accessions of L1, five accessions of L2, PI476874 [L1] and IG47182 [L2]) (A), 24 F_2 individuals derived from PI476874/IG47182 (B), 10 accessions of hexaploid wheat (from left to right: three L1-derived synthetics, three common wheat cultivars and four L2-derived synthetics) (C), and 24 F_2 individuals of N61//Ldn/PI476874 (D. Due to leaky amplification of products of the A and B genomes, this marker was used as a dominant marker. M: 100-bp ladder marker.

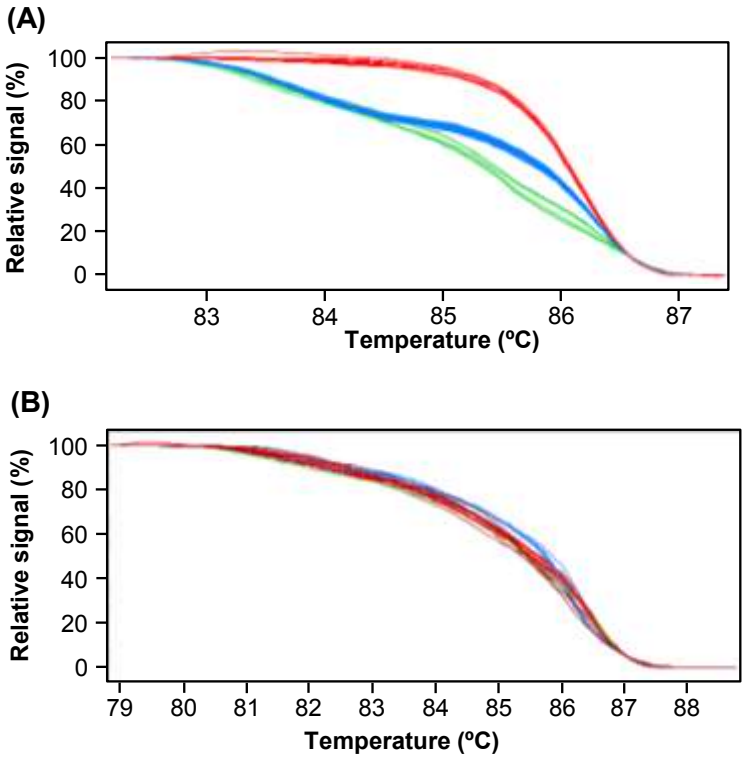

Figure V-11. Examples of HRM markers that are co-dominant in the diploid but dominant in the hexaploid mapping population. High resolution melting curves of the marker *Xctg02576* in F_2 individuals of PI476874/IG47182 (red lines, PI476874 homozygotes; blue lines, heterozygotes; green lines, IG47182 homozygotes) (A), and F_2 individuals of N61//Ldn/PI476874 (red and green lines, N61 homozygotes and heterozygotes; blue lines, Ldn/PI476874 homozygotes) (B). For HRM analysis of diploid individuals, 10% (v/v) PI476874 DNA was added to facilitate the discrimination of genotypes.

Table V-11. Segregation data for the mapped markers on 2DS of the synthetic wheat map of Ldn/KU-2075//Ldn/KU-2025

Marker name	Position (cM)	Segregation *	*P*-value†
Xctg210917	41.0	36 / 77	0.5858
Xctg211320	55.6	31 / 86	0.9977
Xctg10285	63.8	22 / 87	0.8531
Xctg03577	73.2	23 / 82	0.9699
Xctg06648	77.0	29 / 62 / 21	0.6575

*Segregation indicates number of Ldn/KU-2075 homozygotes / heterozygotes / Ldn/KU-2025 homozygotes.

†Chi-square test probabilities for segregation distortion.

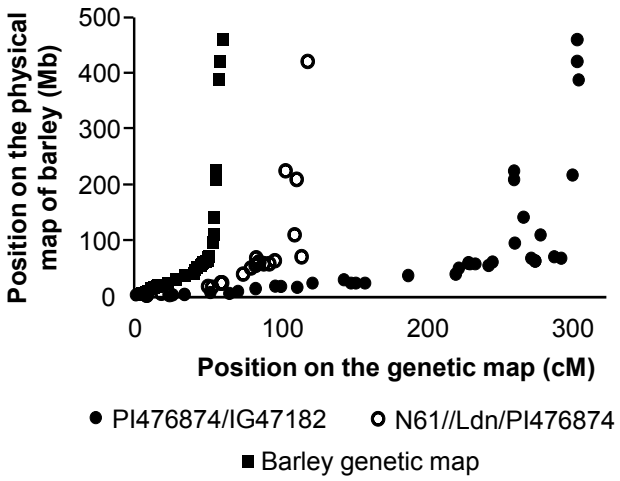

Figure V-12. Relationship between physical and genetic distances. Based on BLAST hits of the marker sequences, physical positions on the barley genome were determined. Squares indicate the positions of markers on the PI476874/IG47182 map, diamonds the positions on the N61//Ldn/PI47182 map, and triangles the positions on the barley genetic map (IBSC 2012).

5. Discussion

In the present study, RNA-seq analysis of transcripts from the spikes of two *Ae. tauschii* accessions was performed for SNP discovery and marker development. Although the number of reads and total read length did not differ greatly from values for leaves, the number of contigs and isotigs was twice as high and the number of HC SNPs was three times higher in the spike transcripts, and at least 10,589 SNPs were new (Tables V-3 and Table V-4). These results indicate that sequencing of the more diverse set of genes expressed in spikes yielded higher detection of SNPs than in leaves, and also suggest that the SNP detection rate is slightly higher for genes expressed in spikes than in leaves (Table V-4; Chapter IV).

The draft genome sequence (Jia et al. 2013) and the physical map (Luo et al. 2013) of *Ae. tauschii* will facilitate the development of molecular markers in the wheat D genome. However, the number of genomic sequences anchored to the linkage map remain small compared with barley (IBSC 2012), which is supported by the *in silico* mapping results of the present study. Although the number of polymorphic contigs assigned to the *Ae. tauschii* genome was slightly higher than that of barley, the number of contigs anchored to the linkage map was somewhat higher for barley (Table V-7). These results imply that *Ae. tauschii* genomic information may be complemented by that of barley for development of molecular markers in specified regions of the D genome.

The *in silico* mapping analysis revealed that both contigs and SNPs derived from leaves and spikes were predominantly distributed in the distal portions of chromosomes (Figure V-4), consistent with recent reports in barley (IBSC 2012) and *Ae. tauschii* (Luo et al. 2013). Genome analyses in barley and *Ae. tauschii* showed that distal portions of chromosomes are more gene-rich (IBSC 2012; Luo et al. 2013). In the case of the short arm of chromosome 2D, the number of contigs and SNPs that mapped to the barley physical map of 2HS was larger within the region of the first 100 Mb from the distal end, and this 100 Mb region coincided with high occurrence of recombination events (Figures V-4 and V-12; Luo et al. 2013). Thus, SNP discovery through RNA-seq was especially efficient in the distal portion of barley and wheat chromosomes, where gene density and recombination rate are correlated (Luo et al. 2013).

In the present study as well as in our previous report (Chapter IV), molecular markers developed using information generated through RNA-seq are generally polymorphic between L1 and L2 accessions of *Ae. tauschii* (Figure V-6). Because a restricted population within L2 appears to be the main source of the D genome of common wheat (Mizuno et al. 2010a; Wang et al. 2013), these markers were also

expected to be polymorphic between the D genomes of L1-derived synthetic hexaploid wheat and common wheat (Figure V-9). SNPs can be converted to PCR-based markers such as CAPS and HRM. In a hexaploid background, the leaky amplification of A- and B-genome sequences interferes with accurate SNP typing of the D genome. The interference results in an increase in the frequency of dominant markers, especially for conversion to a CAPS marker, and a decrease in the sensitivity of SNP detection in HRM analysis (Matsuda et al. 2012). Failure of SNP detection in CAPS markers occurs only when the fragmentation patterns of the A and B genomes mask both the digested and undigested alleles of the D genome. In such cases, the allopolyploid nature of the common wheat genome may make genotyping by CAPS and HRM markers difficult.

Many experimental platforms for SNP typing have also been applied to hexaploid wheat (Akhunov et al. 2009; Allen et al. 2011, 2013; Cavanagh et al. 2013). However, due to the comparatively low genetic diversity of the D genome of common wheat (Caldwell et al. 2004; Chao et al. 2009), the number of markers mapped to the D genome is usually three- to fivefold lower compared to the A and B genomes (Allen et al. 2011, 2013; Cavanagh et al. 2013). For wheat breeding, *Ae. tauschii* is a promising germplasm because of its abundant variation in various traits (Jones et al. 2013). Although nonadditive expression of homoeologous genes is often observed during wheat synthetic formation (Pumphrey et al. 2009), the natural variation found in *Ae. tauschii* is significantly expressed in many traits under the allohexaploid genetic background of wheat synthetics (Kajimura et al. 2011; Okamoto et al. 2012). Thus, the wide natural variation found in *Ae. tauschii*, especially in the L1 accessions, can be exploited to introduce agronomically beneficial alleles, which may have been left behind during the allopolyploidization event (Ogbonnaya et al. 2005). Our results demonstrated the potential usefulness of the SNPs discovered between the L1 and L2 accessions of *Ae. tauschii* for genome analysis and molecular breeding of hexaploid wheat. SNPs will be very valuable for marker development of the D genome, particularly in the progeny obtained through crosses between common wheat cultivars and L1-derived synthetics. On the other hand, the low polymorphism of the markers between Ldn/KU-2075 and Ldn/KU-2025 suggests that finding more SNPs in the D genome between and within the two lineages will be necessary to apply them widely to *Ae. tauschii* and hexaploid wheat lines.

Chapter VI

Variation in abscisic acid responsiveness of *Aegilops tauschii* and hexaploid wheat synthetics due to the D-genome diversity

1. Abstract

Common wheat (*Triticum aestivum* L.) is an allohexaploid that originated from natural hybridization between tetraploid wheat (*Triticum turgidum*) and diploid *Aegilops tauschii*. *Ae. tauschii* is considered one of the potential sources of new genetic variation in abiotic stress tolerance for improving common wheat. Abscisic acid (ABA) plays an important role in plant adaptation to environmental stresses. In this study, ABA responsiveness of 67 *Ae. tauschii* accessions and their synthetic hexaploid wheat lines, derived from crosses between *T. turgidum* cv. Langdon and the *Ae. tauschii* accessions, was evaluated based on growth inhibition by 20 μM ABA. Wide variation was found in ABA responsiveness for both synthetic wheat lines and their parental *Ae. tauschii* accessions. The variations due to D-genome found at the diploid level were also expressed in a hexaploid genetic background. Two pairs of synthetic wheat lines differing in ABA responsiveness were then selected for gene expression analysis and to test abiotic stress tolerance, because their parental *Ae. tauschii* accessions similarly exhibited the differential response to ABA. Gene expression of ABA inducible transcription factor, *WABI5*, and the downstream *Cor/Lea* genes (*Wrab17*, *Wdhn13* and *Wrab18*) were analysed. In one pair, the highly responsive line exhibited higher induction of *Wrab17* by ABA treatment, but no significant difference in dehydration or salinity tolerance was observed between these lines. In contrast, in the second pair, the highly ABA-responsive line showed higher levels of *Wdhn13* expression and dehydration and salinity tolerance. In synthetic wheat lines, the difference in the ABA responsiveness of the lines appeared to be determined by the different sets of D-genome genes. Our findings suggest that highly ABA-responsive *Ae. tauschii* accessions should be valuable genetic resources for improving the abiotic stress tolerance of common wheat.

2. Introduction

Higher plants have evolved complex mechanisms to rapidly sense and adapt to changing environmental conditions. ABA induces expression of the *Cor* (cold-responsive)/*Lea* (late embryogenesis abundant) gene family that promote stress tolerance by protecting cellular components from stress (Thomashow, 1999). A number

of *Cor/Lea* genes contain both CRT (C-repeat)/DRE (dehydration-responsive element) and ABRE (ABA-responsive element) motifs in their promoters, and these *cis*-acting elements are considered to function independently (Yamaguchi-Shinozaki and Shinozaki, 2006).

In common wheat, CRT/DRE, ABRE and other cold-responsive motifs have been identified in the promoter regions of many *Cor/Lea* genes such as *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al. 2008a). These *Cor/Lea* genes are responsive to low temperature, drought and ABA (Egawa et al. 2006; Kobayashi et al. 2004, 2006; Ohno et al. 2003). Some of the stress-responsive transcription factors, TaDREB1, WDREB2, WLIP19, TaOBF1 and WABI5, act through ABA signalling (Shen et al. 2003; Egawa et al. 2006; Kobayashi et al. 2008b, 2008c). *Trans*-activation analysis of these *Cor/Lea* gene promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2, whereas *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al. 2008a, 2008b, 2008c).

Common wheat (*Triticum aestivum* L., AABBDD genome) arose by natural hybridization between tetraploid wheat (*Triticum turgidum* L., AABB genome), including emmer and durum wheat, and *Aegilops tauschii* Coss. (DD genome) (Kihara 1944; McFadden and Sears 1944). *Ae. tauschii*, a wild relative of common wheat, is widely distributed in Eurasia and shows abundant genetic variation (Dudnikov and Goncharov 1993; Dvorak et al. 1998; Dudnikov and Kawahara 2006; Matsuoka et al. 2007, 2008, 2009). Common wheat is said to originate from Transcaucasia and the region along the southern coast of the Caspian Sea (Tsunewaki 1966; Dvorak et al. 1998). The narrow distribution range of the *Ae. tauschii* populations involved in the origin of common wheat, compared to the range of the entire species, suggests an expansive genetic diversity that is not represented in common wheat (Feldman 2001; Mizuno et al. 2010). *Ae. tauschii* can be crossed into tetraploid wheat artificially to produce synthetic hexaploid wheat (Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004). Agronomically important traits can be transferred to cultivated wheat by crossing with the synthetics. Therefore, *Ae. tauschii* is considered one of the potential sources of new genetic variation for improving the abiotic stress tolerance of common wheat.

In our previous study, ABA responsiveness of synthetic wheat lines could not be compared statistically with parental *Ae. tauschii* accessions because the sample number was limited (Kurahashi et al. 2009). The objectives of the present study are to elucidate natural variation in the ABA responsiveness encoded by the D-genome in the hexaploid genetic background, and to study relationships among ABA responsiveness, *Cor*/*Lea* gene expression and dehydration and salinity stress tolerance. Therefore, we first

evaluated the ABA responsiveness of a large number of synthetic hexaploid wheat lines derived from the cross between *T. turgidum* ssp. *durum* cv. Langdon (Ldn) and *Ae. tauschii* accessions (Takumi et al. 2009a; Kajimura et al. 2011), and of their parental accessions. Furthermore, two pairs of synthetic wheat lines were selected, according to their ABA responsiveness, for comparison of gene expression pattern and abiotic stress tolerance.

3. Materials and methods

3-1. Plant materials

In total, 67 synthetic wheat lines and their *Ae. tauschii* parental accessions were used in this study. The accession number and origin of 67 *Ae. tauschii* accessions are shown in Table VI-1. The passport data of *Ae. tauschii* accessions, including the geographical coordinates of the original collection sites, have been given in Matsuoka et al. (2007, 2008). The tetraploid wheat cultivar Langdon (Ldn) was used as the female parent and was crossed with each of the 67 *Ae. tauschii* accessions. The F_1 progeny were grown and selfed to produce synthetics (herein designated the F_2 generation) as previously reported (Takumi et al. 2009a; Mizuno et al. 2010b). All 67 synthetics were independently generated through unreduced gamete formation in each of the triploid F_1 hybrids (Matsuoka and Nasuda 2004). The synthetics thus contained the A and B genomes from Ldn and the diverse D genomes originating from the *Ae. tauschii* pollen parents. Some of the triploid F_1 hybrids between Ldn and *Ae. tauschii* show abnormal growth, such as hybrid necrosis, hybrid chlorosis and severe growth abortion (Matsuoka et al. 2007; Mizuno et al. 2010b). Hybrids showing necrosis, chlorosis and severely aborted growth were excluded from the 67 synthetics. The somatic chromosome number of 42 was previously confirmed using two F_3 seeds from one F_2 plant of each synthetic (Kajimura et al. 2011). In the present study, F_3 plants derived from one F_2 plant of each synthetic were used.

3-2. Bioassay for ABA responsiveness

Bioassay for ABA responsiveness and abiotic stress tolerance were evaluated as in our previous report (Chapter II). Briefly, seeds were pre-germinated for 24 h for synthetic wheat or 48 h for *Ae. tauschii* at 23°C in darkness. Four or five synchronously germinated seeds were placed in a plastic Petri dish containing two overlapping sheets of filter paper wetted with 6 mL of distilled water (control) or 20 μM (±)-ABA (Wako Pure Chemical Industries, Osaka, Japan) solution, then incubated at 23°C in the dark. After 2 days of treatment, the length of shoots and primary roots were recorded in three

Table VI-1. Origins and accession numbers of *Ae. tauschii* accessions used in this study

Origin	Accession number
Afghanistan	KU-2022, KU-2059, PI476874
Armenia	KU-2810, KU-2811, KU-2814, KU-2816, KU-2824
Azerbaijan	IG47202
China	AT47, AT55, AT76, AT80, PI499262
Georgia	AE454, AE929
India	IG48042
Iran	KU-2069, KU-2074, KU-2075, KU-2076, KU-2078, KU-2079, KU-2080, KU-20-8, KU-20-9, KU-20-10, KU-2083, KU-2088, KU-2090, KU-2092, KU-2093, KU2096, KU-2097, KU-2098, KU-2100, KU-2101, KU-2102, KU-2104, KU-2105, KU-2106, KU-2108, KU-2109, KU-2111, KU-2118, KU-2124, KU-2126, KU-2144, KU-2145, KU-2152, KU-2155, KU-2156, KU-2157, KU-2158, KU-2159, KU-2160
Kazakhstan	AE1090
Kyrgyzstan	IG131606
Pakistan	IG46663, CGN10768, CGN10770
Syria	IG46623, IG47259
Tajikistan	IG48554
Turkey	KU-2132, KU-2136
Turkmenistan	IG126387

KU: Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University, Japan.
PI: National Small Grains Research Facility, USDA-ARS, USA.
IG: International Centre for Agricultural Research in the Dry Areas (ICARDA), Syria.
CGN: Centre for Genetic Resources, The Netherlands.
AE: Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Germany.
AT: Faculty of Agriculture, Okayama University, Japan.

or four independent experiments. Relative growth inhibition was calculated as the difference between growth of the control group and the ABA-treated group, relative to the control. The data were statistically analyzed using JMP software ver. 5.1.2 (SAS Institute, Cary, NC, USA). The correlations among the examined traits were estimated based on Pearson correlation coefficient values.

3-3. Expression analysis of ABA-inducible genes

Seeds were germinated under the same conditions as in the bioassay. After 48 h, seedlings were transferred to pots containing soil and incubated at 23°C under long day conditions (16/8 h light/darkness). Seedlings that were 7-d-old (for synthetic wheat lines) or 12-d-old (for *Ae. tauschii*) were treated with 20 μM ABA solution containing 0.1% (w/v) Tween 20 by foliar spray. Total RNA was extracted from leaves at various times after ABA treatment using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized from DNase I-treated mRNA samples with oligo-dT primers using the high fidelity ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan).

The transcript accumulation of each gene was detected by quantitative RT-PCR using a Thermal Cycler Dice Real Time System (Takara-Bio, Ohtsu, Japan) with the following gene-specific primer sets: 5'-GGCGAGAAGAAGGGCGTCAT-3' and 5'-GTGGTGGCTGTGGTGGCAT-3' for *Wdhn13*, 5'-AGACGGGGAAGACCATTCAAGA-3' and 5'-CTCCTTGGTGGCATCGGCA-3' for *Wrab17*, 5'-TCGATTCATCCAAGCCAGAG-3' and 5'-ACCAAACGAGTAAAGGAAGCAA-3' for *Wrab18*, 5'-CACCTCAGCGCCAAGAC-3' and 5'-CTCCCATACCAACTGCCCTC-3' for *WABI5*, and 5'-GCCGTGCTTTCCCTCTATG-3' and 5'-GCTTCTCCTTGATGTCCCTTA-3' for *Actin*. These primers amplified the target genes derived from all A-, B- and D-genomes of common wheat. The *Actin* gene was used as an internal control. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. The relative expression level was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Actin*; representative values were expressed relative to the transcript levels in Ldn samples obtained at 0 h.

3-4. Bioassay for dehydration and salinity tolerance

Seven-d-old seedlings, grown under the same conditions as for expression analysis,

were used for evaluation of abiotic stress tolerance. Plants were removed from the soil and the roots washed with tap water. Dehydration tolerance was evaluated as percentage of water loss from the whole plant (n = 10-12) kept in a growth chamber (23°C, 40-60% relative humidity). First, the difference between initial fresh weight and weight at the indicated time was calculated, which was then divided by the initial fresh weight. Two independent experiments were performed yielding similar results.

For salinity tolerance, plants were removed from soil and grown hydroponically in a solution containing 0.2% Hyponex (HYPONEX Japan, Osaka, Japan) with (n = 12-13) or without (control group, n = 6-7) 0.15 M NaCl. Fresh weight of plants was measured every day, and weight gain was calculated as the percentage of weight gain (weight at the indicated time - initial fresh weight) of the salt-treated group versus weight gain of the control group. The results were normalized by dividing the weight gain under salt stress by the weight gain in the control group at the same day. Two independent experiments were performed yielding similar results.

4. Results

4-1. Natural variation in ABA responsiveness in *Ae. tauschii* accessions

The ABA responsiveness of 67 *Ae. tauschii* accessions was evaluated based on growth inhibition by 20 μM ABA. Wide variations were found in the relative rates of root and shoot growth inhibition. The relative rates of root growth inhibition ranged from 7.11 ± 11.90% (KU-2156) to 54.73 ± 9.68% (KU-2111), with a mean of 32.47 ± 10.34% (Figure VI-1A). The tetraploid wheat cultivar Ldn showed higher ABA responsiveness (66.38 ± 3.41%) than any of the *Ae. tauschii* accessions. Relative rates of shoot growth inhibition varied from 52.68 ± 6.07% (KU-2157) to 87.14 ± 1.20% (IG48042), with an overall mean of 72.36 ± 6.71% (Figure VI-2A). Root growth inhibition was positively correlated with shoot growth inhibition (r = 0.498, P < 0.001), suggesting that inhibition of root and shoot growth is controlled by similar mechanisms.

Ae. tauschii accessions can be classified in two major lineages (Mizuno et al. 2010a). Of 67 accessions, 24 have been classified in lineage 1 (L1) and 36 in lineage 2 (L2). The ABA responsiveness, based on root growth inhibition, was significantly higher in accessions belonging to L2 (P < 0.01) (Figure VI-3A). In turn, L1 and L2 can be divided respectively in six and three sublineages. ABA responsiveness of sublineages 2-3 (38.23 ± 6.46%) was significantly higher than that of sublineage 1-2 (24.87 ± 10.40%) (Figure VI-3B). Seven accessions were not included in this analysis. To test whether latitudinal and longitudinal clines can be recognized, we performed multiple regression analysis using latitude and longitude as independent variables and ABA

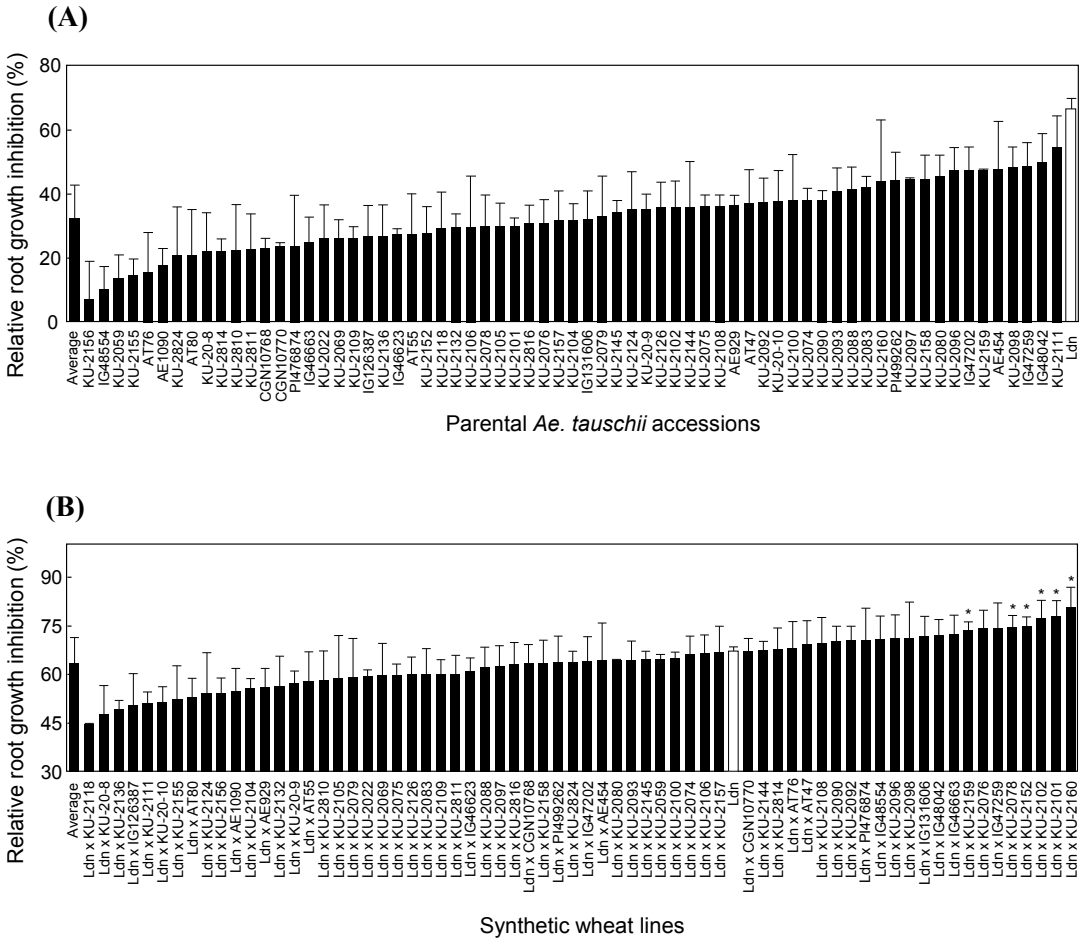

Figure VI-1. Variation in root growth inhibition by 20 μM ABA. (A) Relative root growth inhibition of 67 *Ae. tauschii* accessions. Data are means ± SD calculated from three or four independent experiments. Open bars: *T. turgidum* cv. Langdon. (B) Relative root growth inhibition of the synthetic wheat lines. Student's *t*-test was used to test for statistical significance (*$P < 0.05$).

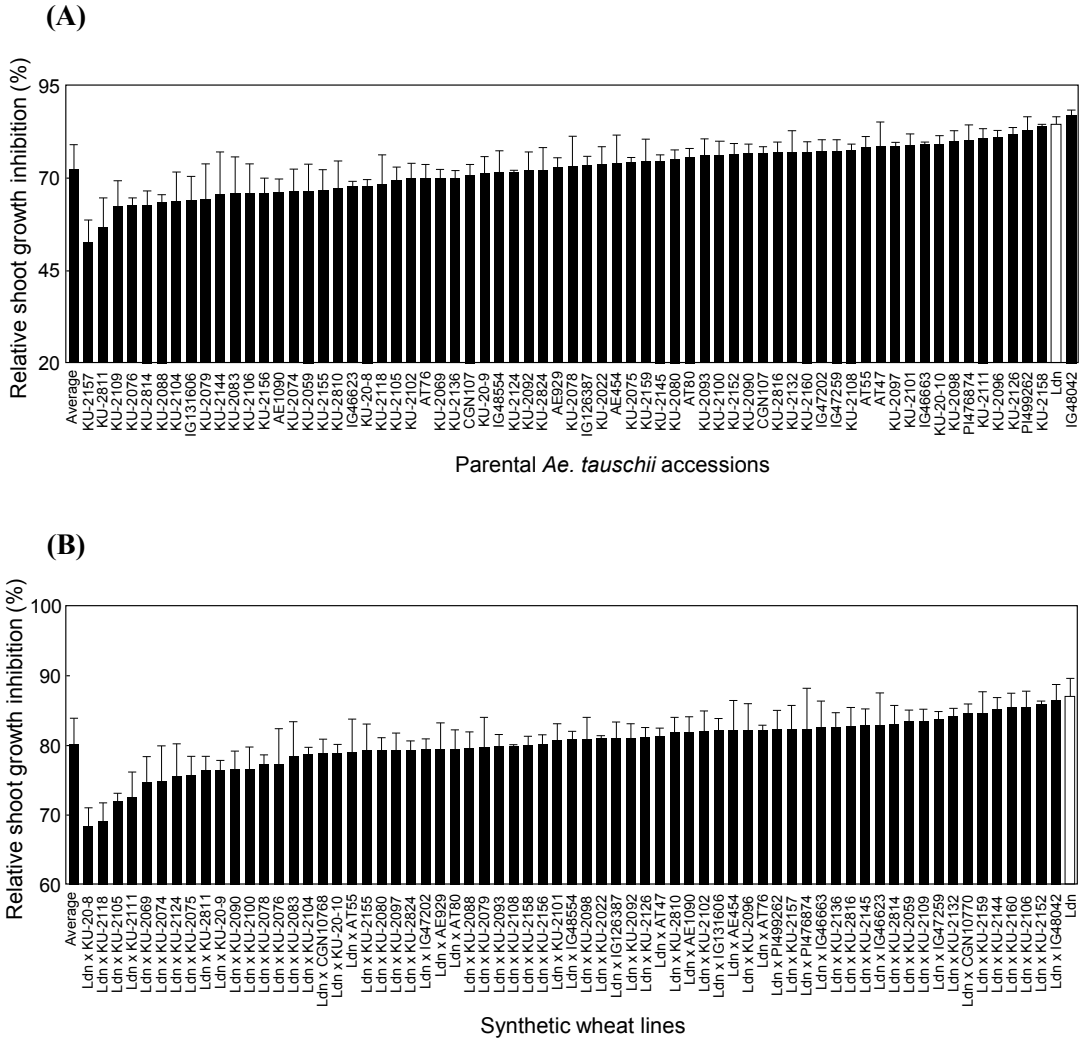

Figure VI-2. Variation in shoot growth inhibition by 20 μM ABA. (A) Relative shoot growth inhibition of 67 *Ae. tauschii* accessions. Data are means ± SD calculated from three or four independent experiments. Open bars: *T. turgidum* cv. Langdon. (B) Relative shoot growth inhibition of the synthetic wheat lines.

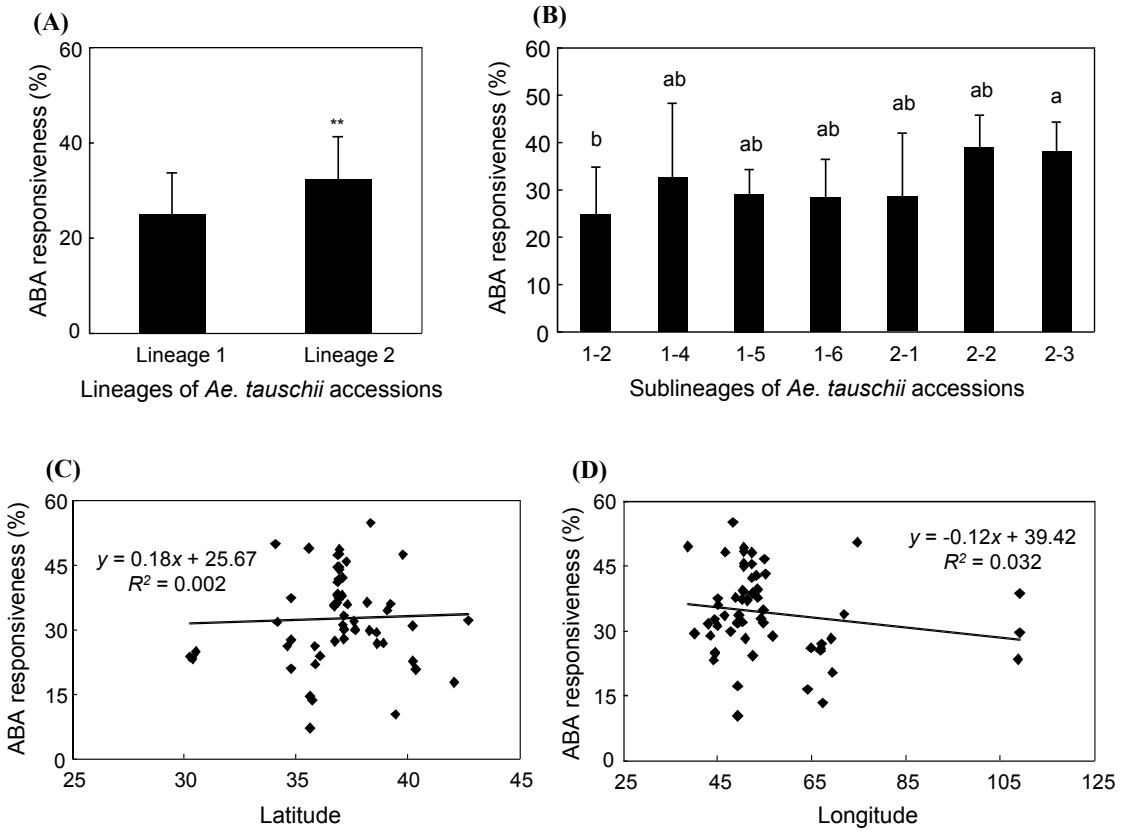

Figure VI-3. ABA responsiveness of *Ae. tauschii* accessions in the different lineages. (A) Mean ABA responsiveness in lineage 1 and 2 of *Ae. tauschii*. Student's *t*-test was used for statistical significance. $^{**}P < 0.01$. (B) ABA responsiveness of *Ae. tauschii* in the different sublineages. Tukey-Krammer's HSD test was performed. The number of accessions in each sublineage is: ten in 1-2, three in 1-4, eight in 1-5, three in 1-6, eleven in 2-1, six in 2-2, and 19 in 2-3. No accessions of sublineage 1-3 were found. (C, D) Relationship between ABA responsiveness and latitude (C) and longitude (D) of the original habitat.

responsiveness as the response variable. However, no significant effect of latitude and longitude on ABA responsiveness was observed (Figures VI-3C and VI-3D; Table VI-2).

4-2. Comparison of ABA responsiveness among synthetic wheat lines

To study whether the natural variation in ABA responsiveness of *Ae. tauschii* is expressed in the hexaploid genetic background, the ABA responsiveness of the synthetic wheat lines was evaluated. The relative rates of root growth inhibition varied widely, as observed in *Ae. tauschii*, and ranged from 44.59 ± 0.36% (Ldn x KU-2118) to 80.61 ± 6.40% (Ldn x KU-2160), with a mean value of 63.52 ± 7.92% (Figure VI-1B). The relative rates of shoot growth inhibition varied from 68.44 ± 2.60% (Ldn x KU-20-8) to 86.52 ± 2.27% (Ldn x IG48042), with a mean of 80.21 ± 3.69% (Figure VI-2B). Since root growth inhibition showed broader variation than shoot growth inhibition, and a positive correlation was found between them in synthetic hexaploids (r = 0.507, P < 0.01) as well as parental *Ae. tauschii*, root growth inhibition is equated to ABA responsiveness hereafter.

Next, the correlation between ABA responsiveness of synthetic hexaploids and their parental accessions was studied. The correlation was positive, with r = 0.248 and P < 0.05 (Figure VI-4A). Despite this positive correlation, in some cases the ABA responsiveness of *Ae. tauschii* is not reflected in synthetic wheat. For example, the *Ae. tauschii* accession KU-2111 is the highest responsive line but its derived synthetic showed a very low responsiveness. In contrast, IG48554 exhibited very low responsiveness but was relatively higher in hexaploid background. Synthetic wheat lines were classified into three groups according to their ABA responsiveness, and the mean value of their parental accessions was calculated for each group. This grouping was based on the total average and standard deviation (SD) of ABA responsiveness (63.52 ± 7.92%) in synthetic wheat. That is, low responsive group is composed by synthetics with ABA responsiveness less than mean - SD (55.60%), synthetics with responsiveness higher than mean + SD (71.44%) in the highly responsive group, and the intermediate group ranging from 55.60% to 71.44%. Eleven synthetic lines were classified into a less responsive group, 45 lines into an intermediate group, and 11 lines into a highly responsive group. The mean ABA responsiveness of the parental *Ae. tauschii* accessions in the less responsive group was 26.61 ± 12.87%, 32.92 ± 9.52% in the intermediate group and 36.50 ± 9.21% in the highly responsive group (Figure VI-4B). The ABA responsiveness of parental *Ae. tauschii* accessions was significantly higher in the intermediate (P < 0.05) and highly responsive group (P < 0.05) than in the less

Table VI-2. Multiple regression analysis of the effects of geographic clines on the ABA responsiveness

Source	Parameter estimate	Standard error	*t* statistic	*P* value
Latitude	-0.11	0.62	-0.18	0.86
Longitude	-0.13	0.10	-1.35	0.18

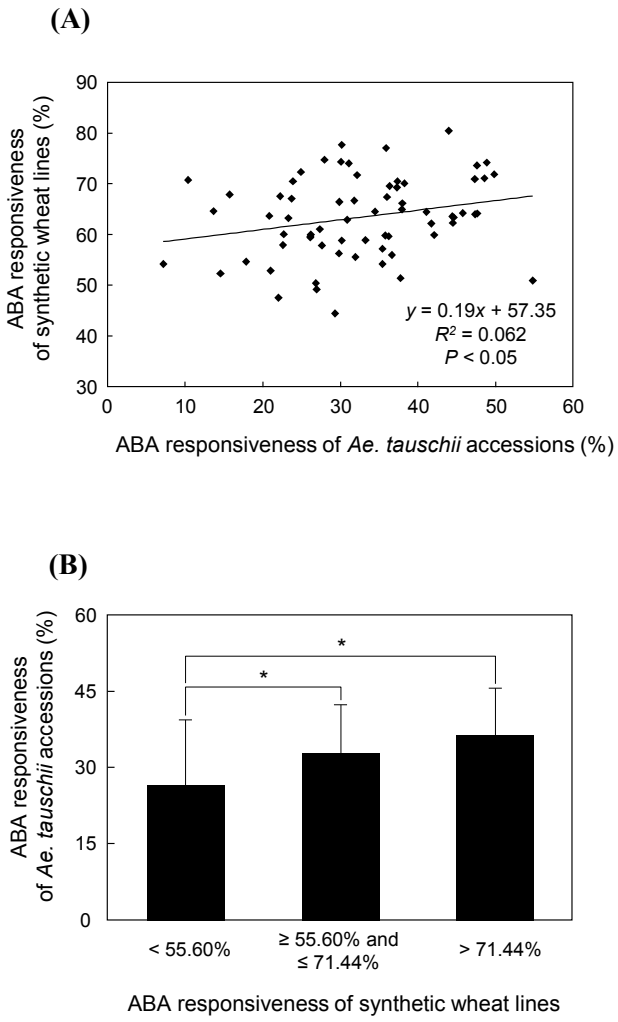

Figure VI-4. Correlation of ABA responsiveness between wheat synthetics and their parental accessions. (A) Scatter plot of ABA responsiveness in the synthetics wheat lines and their parental *Ae. tauschii* accessions. (B) Comparison of ABA responsiveness of parental *Ae. tauschii* accessions among synthetic lines classified according to their growth inhibition rates. Data are mean ± SD. Student's *t*-test was used for statistical significance (*$P < 0.05$).

responsive group. Of the 67 synthetic hexaploids, six lines that had parental *Ae. tauschii* accessions of KU-2159, KU-2078, KU-2152, KU-2102, KU-2101 and KU-2160 showed significantly higher ABA responsiveness than Ldn, 47 lines showed similar responsiveness, and 13 showed lower responsiveness. None of the synthetic wheat lines exhibited lower ABA responsiveness than their parental *Ae. tauschii* accessions.

4-3. Expression analysis of ABA-inducible genes

To compare gene expression patterns between less ABA-responsive and highly responsive lines, two pairs of synthetic hexaploids were randomly selected from the synthetic lines. Pair 1 included a low ABA-responsive line from Ldn x KU-2124 and a highly responsive line from Ldn x IG47259, and pair 2 included a low responsive line from Ldn x IG126387 and a highly responsive line from Ldn x KU-2159. These lines were selected because their parental *Ae. tauschii* accessions also exhibited similar response to ABA. In pair 2, ABA responsiveness of the parental *Ae. tauschii* accessions correlated with that of the synthetics, being high for KU-2159 and low for IG126387. The correspondence was similar in pair 1 except for the low ABA-responsive synthetic, for which its parental *Ae. tauschii* accession exhibited intermediate responsiveness.

In pair 1, the gene expression patterns of the *WABI5* transcription factor and three *Cor/Lea* genes, *Wrab17*, *Wrab18* and *Wdhn13*, were examined by real-time RT-PCR analysis. These genes were selected because they are known to express under low temperature, dehydration and ABA treatment (Tsuda et al. 2000; Ohno et al. 2003; Kobayashi et al 2004, 2008c). Transcript accumulation of *WABI5* in the highly ABA-responsive synthetic wheat (Ldn x IG47259) reached a maximum at 5 h after ABA treatment (Figure VI-5A). The expression of *WABI5* in the low responsive line (Ldn x KU-2124) was similar to that of the maximum value for the highly responsive line, but was reached more slowly (12 h after treatment). Both pair 1 synthetics showed higher *WABI5* expression than the tetraploid wheat Ldn. In the diploid *Ae. tauschii*, *WABI5* expression was lower in the low ABA-responsive accession (KU-2124) (Figure VI-6A). The highly responsive accession (IG47259) showed similar expression to that of Ldn, except 12 h after treatment. The expression of *Wrab17* was higher in the highly responsive synthetic and its parental *Ae. tauschii* line (Figures VI-5B and VI-6B). Transcript accumulation of *Wrab18* was similar for the synthetics in pair 1 (Figure VI-5C), but was higher in the low responsive *Ae. tauschii* than in the highly responsive accession (Figure VI-6C). The *Wdhn13* expression level was higher in the low responsive synthetic line of pair 1 (Figure VI-5D).

In pair 2, expression of *WABI5* was slightly higher in the intermediate responsive

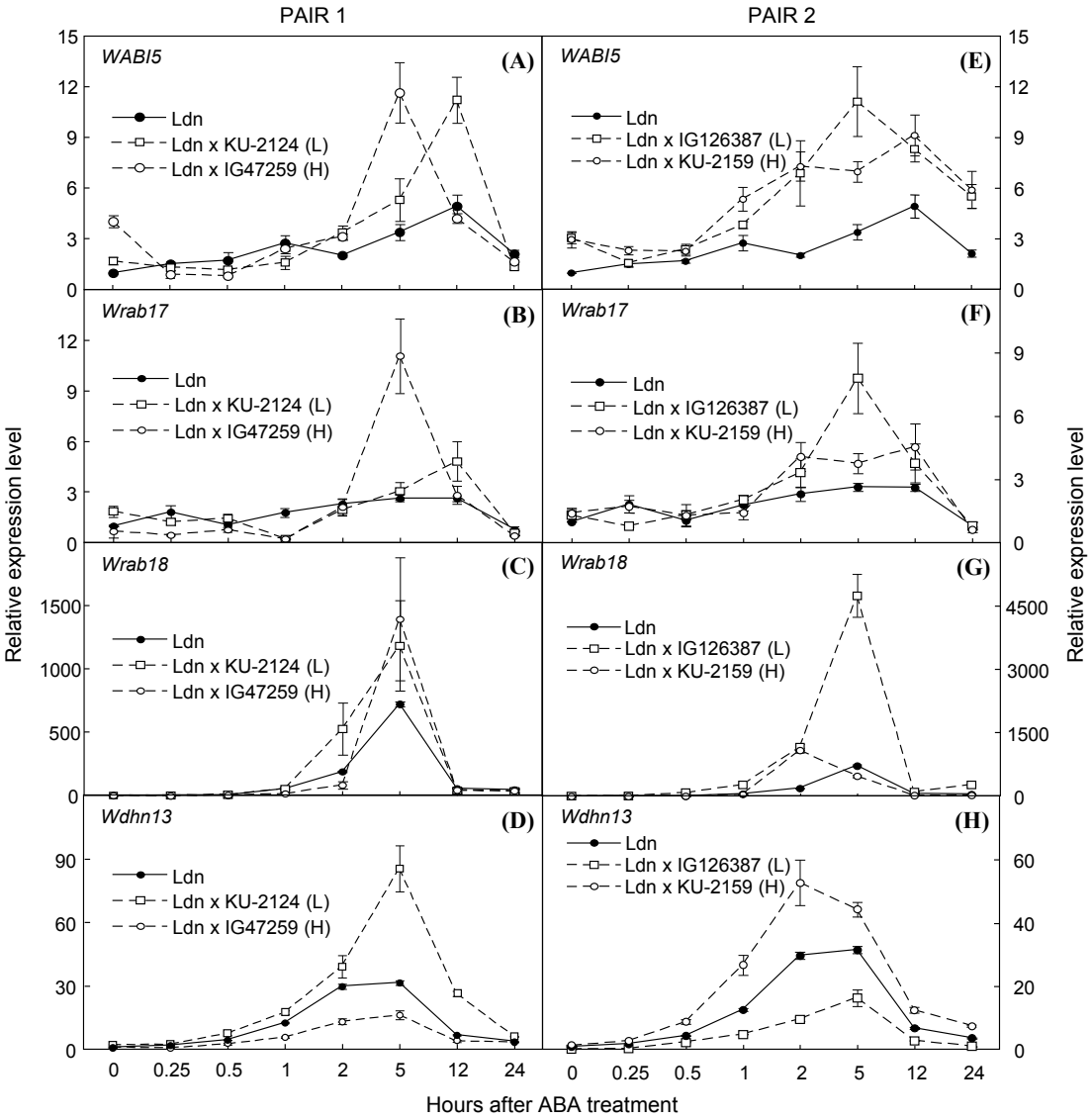

Figure VI-5. Expression patterns of ABA-inducible genes in the synthetic lines of pairs 1 (A-D) and 2 (E-H). Transcripts of *WABI5* (A, E), *Wrab17* (B, F), *Wrab18* (C, G), and *Wdhn13* (D, H) were examined by real-time RT-PCR analysis, and represented as mean ± SD. Synthetic wheat lines are indicated by dashed lines, and Ldn by continuous lines. Highly and low ABA-responsive synthetic lines are indicated as (H) and (L), respectively. The *Actin* gene was used as an internal control. Each transcript level is represented as the value relative to that of Ldn at 0 h.

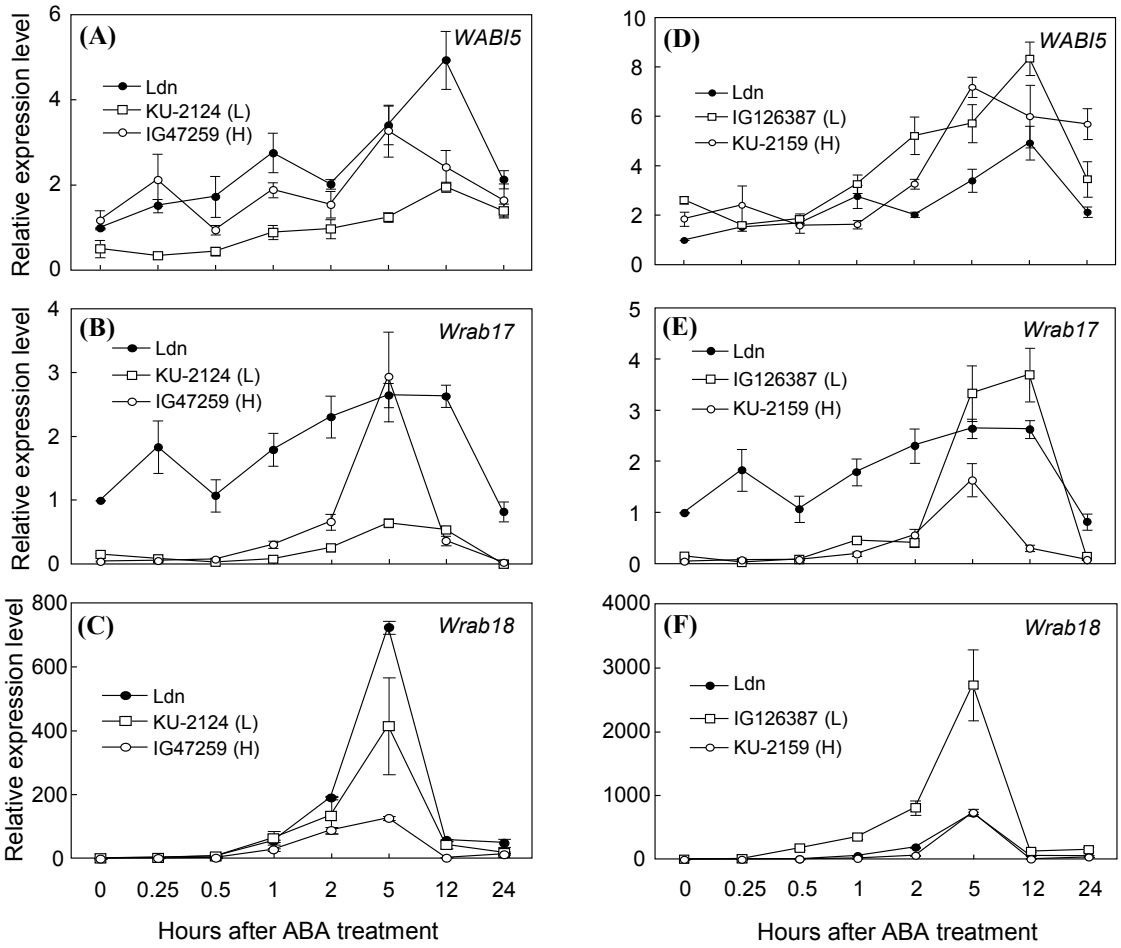

Figure VI-6. Expression patterns of ABA-inducible genes in the parental *Ae. tauschii* accessions of pairs 1 (A-C) and 2 (D-F). Transcripts of *WABI5* (A, D), *Wrab17* (B, E), and *Wrab18* (C, F) were examined by real-time RT-PCR analysis, and represented as mean ± SD. Highly and low ABA-responsive accessions are indicated as (H) and (L), respectively. The *Actin* gene was used as an internal control. Each transcript level is represented as the value relative to that of Ldn at 0 h.

Ae. tauschii (IG126387) and its derived (low responsive) synthetic wheat; Ldn showed the lowest expression (Figures VI-5E and VI-6D). The transcript accumulation of *Wrab17* was higher in IG126387 and its synthetic (Figures VI-5F and VI-6E). As in pair 1, *Ae. tauschii* accessions showed *Wrab17* expression that was lower than or similar to Ldn, but their derived hexaploids showed higher expression. *Wrab18* was also expressed at a higher level in IG126387 and its derived synthetic line (Figures VI-5G and VI-6F). In contrast, expression of *Wdhn13* was higher in the highly ABA-responsive synthetic line (Figure VI-5H).

To test whether these ABA-inducible genes are expressed additively in the pair 1 synthetics, a mid-parent value (MPV) was calculated as a 2:1 mixture of Ldn and the parental *Ae. tauschii* accession based on an AB-to-D genome ratio of 2:1 (Pumphrey et al. 2009). For example, the highest expression level of *WABI5* in Ldn x KU-2124 was reached at 12 h after ABA treatment. The MPV was calculated as the sum of Ldn expression level multiplied by two (4.95 x 2) and the parental *Ae. tauschii* expression (1.96), both at 12 h after treatment, and then dividing by three (11.86/3 = 3.95). The estimated MPVs for *WABI5* and *Wrab17* at their maximal expression were lower than the observed expression level in both IG47259- and KU-2124-derived synthetic hexaploids (Table VI-3). For *Wrab18*, the observed expression level was significantly higher than the MPV in the IG47259-derived synthetic line, but no difference was observed in the low responsive synthetic line. For *Wdhn13*, both synthetic lines exhibited non-additive expression, however, the observed expression level of the highly responsive line was lower than the MPV. These results indicate that these ABA-inducible genes are non-additively expressed in the pair 1 synthetic lines, except for *Wrab18* in the KU-2124-derived synthetic line. These three genes also showed non-additive expression in both pair 2 synthetic lines, similarly to the pair 1 synthetics.

4-4. Water loss and salinity tolerance in pairs 1 and 2

Since the expression analyses of ABA inducible genes showed no clear difference between higher and lower responsive lines, the relationship between ABA responsiveness and abiotic stress tolerance in wheat synthetics was studied. Dehydration and salt stress tolerance was compared in the two selected pairs of synthetic wheat lines. Dehydration tolerance was evaluated as water loss rate, whereas salt stress tolerance was estimated based on growth rate under hydroponic conditions.

In pair 1, the water loss rate was similar between the highly (Ldn x IG47259) and low (Ldn x KU-2124) ABA-responsive lines (Figure VI-7A). However, in pair 2, the KU-2159-derived synthetic (a higher ABA-responsive line) lost water more slowly than

Table VI-3. Altered expression levels of three ABA-inducible genes in synthetic wheat lines

Gene	Synthetic wheat	MPV (2:1)[a]	Observed value[b]
Pair 1			
WABI5	Ldn x KU-2124 (L)	3.95 ± 0.43	11.24 ± 1.37***
	Ldn x IG47259 (H)	3.37 ± 0.31	11.67 ± 1.79**
Wrab17	Ldn x KU-2124 (L)	1.94 ± 0.12	4.86 ± 1.18*
	Ldn x IG47259 (H)	2.75 ± 0.35	11.10 ± 2.21**
Wrab18	Ldn x KU-2124 (L)	621.20 ± 47.70	1523.67 ± 816.51ns
	Ldn x IG47259 (H)	525.05 ± 14.14	1424.92 ± 895.57*
Wdhn13	Ldn x KU-2124 (L)	24.61 ± 0.87	85.39 ± 10.90***
	Ldn x IG47259 (H)	21.28 ± 0.74	16.17 ± 2.09*
Pair 2			
WABI5	Ldn x IG126387 (L)	4.19 ± 0.56	11.15 ± 2.07**
	Ldn x KU-2159 (H)	5.30 ± 0.22	9.14 ± 1.21**
Wrab17	Ldn x IG126387 (L)	4.37 ± 0.67	7.82 ± 1.67*
	Ldn x KU-2159 (H)	1.86 ± 0.13	4.56 ± 1.09*
Wrab18	Ldn x IG126387 (L)	147.28 ± 2.91	4755.83 ± 500.00***
	Ldn x KU-2159 (H)	1393.32 ± 185.29	1085.93 ± 57.80***
Wdhn13	Ldn x IG126387 (L)	21.11 ± 0.73	16.37 ± 2.57*
	Ldn x KU-2159 (H)	23.07 ± 0.77	52.75 ± 7.13**

[a]MPVs (mean ± SD) were calculated using the expression level of parental Ldn and *Ae. tauschii* accessions at the time point at which each synthetic wheat line reached a maximum.

[b]Student's *t*-test was used for statistical significance (ns; not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(L); low responsive line, (H); highly responsive line.

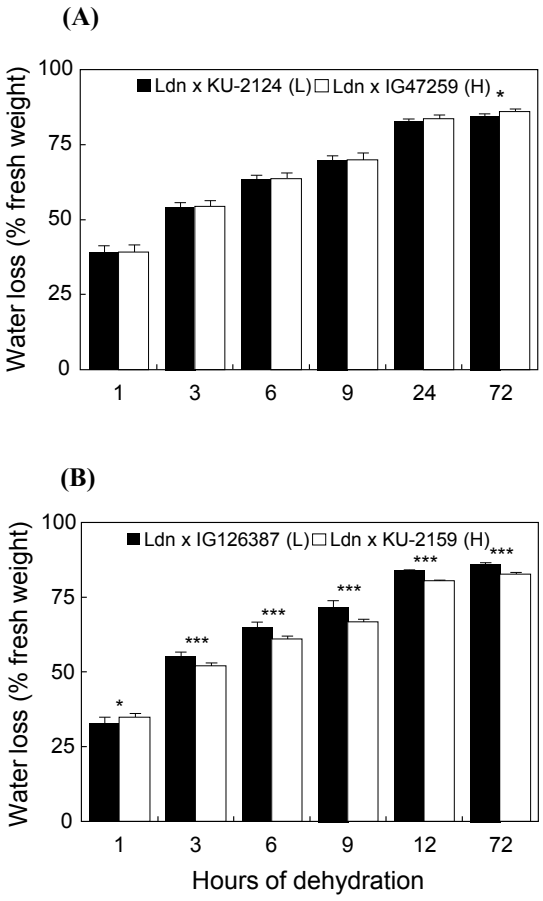

Figure VI-7. Comparison of stomatal response under dehydration in synthetic wheat lines. Water loss from whole plants was determined in pair 1 (A) and pair 2 (B), and represented as mean ± SD. Black and open bars represent the percentage of water loss in the low (L) and highly (H) ABA-responsive lines, respectively. Student's *t*-test was used for statistical significance (*$P < 0.05$, ***$P < 0.001$).

the low ABA-responsive IG126387-derived line (Figure VI-7B).

Growth rate under the hydroponic condition, implying salt tolerance, was slightly different among the synthetic wheat lines. In pair 1, no significant difference in growth rate was observed between the synthetic wheat lines (Figure VI-8A). In pair 2, both synthetic lines showed a similar weight gain between the first and second day of treatment. From the third day, the low responsive line (Ldn x IG126387) lost weight more rapidly than the highly responsive line (KU-2159-derived synthetic) (Figure VI-8B).

5. Discussion

Analysis of natural variation has contributed to understanding of many aspects of plant biology (Tonsor et al. 2005; Alonso-Blanco et al. 2009). *Ae. tauschii*, the D-genome progenitor of common wheat, is widely distributed in central Eurasia, ranging from northern Syria and Turkey to western China, and shows abundant natural variation in various traits including flowering time, morphological traits and fertile triploid F_1 formation (Matsuoka et al. 2007, 2008, 2009; Takumi et al. 2009b). Similarly, this study showed that the *Ae. tauschii* population provides wide natural variation in ABA responsiveness, based on rates of shoot and root growth inhibition by exogenous ABA treatment.

The 67 synthetic wheat lines used in the present study were generated through crossing Ldn with 67 different *Ae. tauschii* accessions. These diploid accessions exhibited wide natural variation in ABA responsiveness, and it was higher in the accessions belonging to L2 than in L1. A wide variation in ABA responsiveness was also observed in the 67 synthetic hexaploid lines. Although the contribution of AB-genomes on ABA responsiveness was relatively high, the natural variation of *Ae. tauschii* in ABA responsiveness is expressed well in the hexaploid genetic background.

In our previous study, a positive correlation between ABA responsiveness of 17 synthetics and their parental accessions was also found, but was not statistically significant (Kurahashi et al. 2009), likely due to the small population size. In the present study using a larger population size of both *Ae. tauschii* and synthetic wheat, the correlation was significant. In our previous studies, a significant correlation of heading time was observed between 82 wheat synthetics and their parental *Ae. tauschii* accessions (Kajimura et al. 2011), but not using 27 hexaploid synthetics (Takumi et al. 2009a). In spikelet morphology-related traits, on the other hand, positive correlations were observed using 27 hexaploid synthetics and their parental *Ae. tauschii* accessions (Takumi et al. 2009a). The set of 67 synthetic wheat lines is thus a valuable resource to

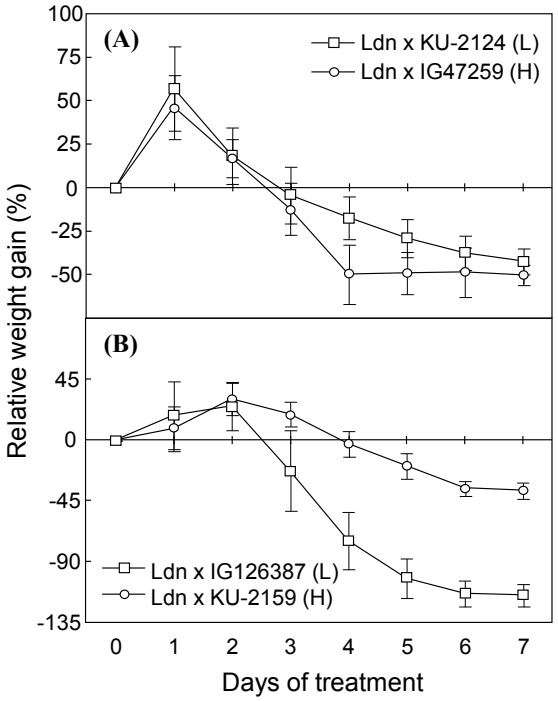

Figure VI-8. Comparison of salt stress tolerance in the synthetic wheat lines. Weight gain was determined in 7-d-old seedlings grown in 0.2% Hyponex solution containing 0.15 M NaCl. Percentage of weight gain was determined in pair 1 (A) and pair 2 (B). Low (L) and highly (H) ABA-responsive lines are indicated by open squares and circles, respectively. Data are mean ± SD.

study expression of D-genome variation in various traits in a hexaploid genetic background.

Correlation between ABA responsiveness and expression of abiotic stress-inducible genes has been reported in common wheat cultivars (Kobayashi et al. 2006, 2010). In this study, two pairs of synthetic wheat lines were selected based on differences in ABA responsiveness, and gene expression patterns and abiotic stress tolerance were compared using the highly and low ABA-responsive synthetic lines of each pair. In the highly ABA-responsive lines, not all the *Cor/Lea* genes were necessarily highly expressed, suggesting that a complex ABA signalling network regulates the expression of *Cor/Lea* genes and growth inhibition by ABA. In pair 2, only *Wdhn13* expression was correlated with ABA responsiveness and abiotic stress tolerance, suggesting that ABA-dependent development of abiotic stress tolerance might be tightly associated with *Wdhn13* expression in the synthetic wheat lines. On the other hand, the difference in ABA responsiveness was not reflected in the tolerance to dehydration and salinity in pair 1. One explanation could be due to the higher expression of *Wdhn13* in the low responsive line of pair 1, and the high *Wdhn13* expression led in a dehydration and high salinity tolerance similar to the highly responsive line, supporting the correlation between this *Cor/Lea* gene and abiotic stress tolerance. Another explanation is the difference among synthetic wheat lines in the endogenous ABA concentration, since this phytohormone is known to increase under dehydration and high salinity conditions. In *Arabidopsis thaliana*, a quantitative trait locus (QTL) for salt tolerance and ABA sensitivity has been reported (Ren et al. 2010). Kobayashi et al. (2006, 2008d) found a significant association between ABA responsiveness and freezing tolerance in common wheat. However, the association between QTL for ABA responsiveness and other abiotic stress tolerance have not been reported to our knowledge. Since higher accumulation of ABA in spikes causes pollen sterility and grain loss (Ji et al. 2011), it is important to select QTLs that do not decrease grain yield under abiotic stress. Therefore, identification of the QTLs that determine the difference in ABA responsiveness between the synthetic wheat lines is needed to assess the use of highly ABA-responsive *Ae. tauschii* accessions as genetic resources to improve the abiotic stress tolerance of common wheat.

Hybrid vigor or heterosis is the phenotypic superiority of hybrid offspring over their parents, generally referring to a superior level of biomass, growth rate, fertility and yield. In allopolyploids, permanent fixation of heterozygosity and hybrid vigor occur due to the whole genome duplication of interspecific hybrids (Chen 2010a). Here, permanent fixation of heterosis in ABA responsiveness was observed in some synthetic hexaploid

wheat lines. ABA responsiveness in most of the synthetic wheat lines surely ranged between the ABA responsiveness of their parents, which were Ldn and the respective *Ae. tauschii* accessions. However, in the six synthetic wheat lines, ABA responsiveness was significantly higher than in their parental *Ae. tauschii* accessions or Ldn, showing phenotypic superiority (heterosis) for ABA responsiveness over their parents. It was brought about through the allopolyploidization between Ldn and the respective *Ae. tauschii* accessions. Epistatic gene interactions between the AB and D genomes could result in heterosis. However in some cases like KU-2111, the AB and D interaction could also lead in a decrease of ABA responsiveness.

At the gene expression level, most of the ABA-inducible genes examined in this study showed higher expression than the estimated MPV in the synthetic wheat lines. Allopolyploids are well known to undergo changes at the genetic, epigenetic and gene expression levels (Chen 2010; Jackson and Chen 2010). Many of the non-additively expressed genes were reported to be underexpressed in synthetic hexaploid wheat (Chague et al. 2010). In particular, phytohormone- and stress-related genes tend to be underexpressed in *Arabidopsis* allopolyploids grown under normal conditions (Jackson and Chen 2010; Kim and Chen 2011). However, these underexpressed phytohormone- and stress-related genes are highly expressed under stress conditions (Kim and Chen 2011). In this study, many ABA-inducible genes were overexpressed (expression higher than MPV), and reaching expression level higher or similar to the sum of Ldn and parental *Ae. tauschii* level (AABB + DD, not the average). Kurahashi et al. (2009) reported that drought tolerance levels of synthetic wheat lines are generally higher than their D-genome parental accessions. ABA-inducible genes might be overexpressed under drought conditions, leading to higher tolerance in synthetics. Although non-additive expression was observed in all the synthetics analyzed in this study, heterosis for ABA responsiveness was commonly observed in only six lines. This indicates that not all epistatic interactions between the D- and AB-genomes necessarily lead to heterosis appearing in phenotypes, despite the non-additive expression of ABA-inducible genes.

Chapter VII

Identification of quantitative trait loci for abscisic acid responsiveness in the D genome of hexaploid wheat

1. Abstract

In crop species such as wheat, abiotic stresses and pre-harvest sprouting reduce grain yield and quality. Because the plant hormone abscisic acid (ABA) plays an important role in abiotic stress tolerance and seed dormancy, the study of ABA responsiveness in hexaploid wheat is important for the improvement of these traits. In our previous study, ABA responsiveness of 67 *Ae. tauschii* accessions and their synthetic hexaploid wheat lines has been evaluated, finding a wide variations due to D-genome. In this study, quantitative trait locus (QTL) analysis was performed using F_2 population derived from the highly ABA-responsive synthetic wheat Ldn/KU-2159 and the low responsive Ldn/IG126387. A significant QTL was detected on chromosome 6D, in a similar location to that reported for ABA responsiveness using recombinant inbred lines (RILs) derived from common wheat cultivars Chinese Spring (CS) and Mironovskaya 808 (M808). Comparative map and functional analyses of the 6D QTL of synthetic wheat and common wheat suggested that both are different locus. The common wheat 6D QTL was found to affect seed dormancy and the regulation of *cold-responsive* (*Cor*)/*late embryogenesis abundant* (*Lea*) genes during dehydration treatment. However in synthetic wheat, we failed in the detection of association of ABA responsiveness with abiotic stress tolerance and seed dormancy, in the experimented conditions. This QTL might have small effect on abiotic stress tolerance and/or seed dormancy. Development of near isogenic lines (NILs) is important for functional analyses of the synthetic wheat 6D QTL.

2. Introduction

Common wheat (*Triticum aestivum* L., AABBDD genome) is an allohexaploid species originated by natural hybridization between tetraploid *Triticum turgidum* L. (AABB genome) and the wild diploid relative *Aegilops tauschii* Coss. (DD genome) (Kihara 1944; McFadden and Sears 1944). *Ae. tauschii* is widely distributed in Eurasia and shows abundant genetic variation (Dvorak et al. 1998; Dudnikov and Kawahara 2006; Matsuoka et al. 2007, 2008, 2009; Takumi et al. 2009a). Based on population structure analyses, *Ae. tauschii* has been divided into two major evolutionary lineages: lineage 1 (L1) and L2 (Mizuno et al. 2010; Wang et al. 2013). It is supposed that the *Ae. tauschii*

populations involved in the origin of common wheat are limited to a narrow distribution range and restricted to L2, which has given rise to a founder effect in hexaploid wheat (Feldman 2001; Mizuno et al. 2010; Wang et al. 2013). Synthetic hexaploid wheat can be obtained by artificial hybridization of tetraploid wheat with *Ae. tauschii* (Kihara and Lilienfeld 1949; Matsuoka and Nasuda 2004), and can be used as intermediates to exploit the natural variation of *Ae. tauschii* in hexaploid wheat improvement (Trethowan and Mujeeb-Kazi 2008).

The phytohormone ABA serves as an endogenous messenger to mediate responses to environmental stress as well as in plant growth and development including seed maturation and dormancy (Finkelstein et al. 2002). During abiotic stress, ABA induces expression of a variety of genes that function in the regulation of gene expression, signal transduction and stress tolerance (Yamaguchi-Shinozaki and Shinozaki 2006). One family of genes encodes the COR/LEA proteins, which promote stress tolerance by protecting cellular components from environmental stress (Thomashow 1999). In common wheat, C-repeat (CRT)/dehydration-responsive element (DRE), ABA-responsive element (ABRE) and other cold-responsive motifs have been identified in the promoter regions of four *Cor/Lea* genes, *Wdhn13*, *Wrab17*, *Wrab18* and *Wrab19* (Kobayashi et al. 2008a). These *Cor/Lea* genes are responsive to low temperature, drought and ABA (Ohno et al. 2003; Kobayashi et al. 2004, 2006; Egawa et al. 2006). Some of the transcription factors acting in ABA signaling are bZIP-type transcription factors WLIP19 (Kobayashi et al. 2008b) and WABI5 (Kobayashi et al. 2008c), and ethylene-responsive factor (ERF)/APETALA2 (AP2) domain-containing transcription factors WDREB2 (Egawa et al. 2006) and TaDREB1 (Shen et al. 2003). *Trans*-activation analysis of the *Cor/Lea* gene promoters revealed that *Wdhn13*, *Wrab18* and *Wrab19* expression is induced by WABI5, WLIP19 and WDREB2, whereas *Wrab17* expression is activated by WLIP19 and WDREB2 but not by WABI5 (Kobayashi et al. 2008a, 2008b, 2008c).

In crop species, such as wheat, an adequate level of seed dormancy is required. It must be high enough to prevent pre-harvest sprouting or vivipary and low enough to promote uniform germination. The catabolism of ABA is a crucial step in dormancy release in several plant species (Gubler et al. 2005). The hydroxylation of ABA at the 8'-position, catalyzed by cytochromome P450 with ABA 8'-hydroxylase activity (ABA8'OH), is the key step of ABA inactivation. In wheat and barley, two genes encoding ABA8'OH have been reported (Millar et al. 2006; Nakamura et al. 2010; Chono et al. 2013). *TaABA8'OH1* has been located on the long arm of homoeologous group-6 chromosomes and a role in seed dormancy was suggested (Chono et al. 2013).

On the other hand, *TmABA8'OH2* has been mapped on the centromeric region of chromosome $5A^m$. Using a diploid wheat mapping population of RILs derived from a cross between accessions of *T. monococcum* L. and *T. boeoticum* L., *TmABA8'OH2* was found to be linked to QTL for seed dormancy (Nakamura et al. 2010).

QTL analysis for ABA responsiveness at the seedling stage has been performed using a mapping population of RILs derived from a cross between two common wheat cultivars, CS (a low ABA-responsive variety) and M808 (a highly ABA-responsive variety). Five significant QTLs were found, with 2A, 3A, 6D and 7B QTLs regulating both root growth arrest and *Cor/Lea* gene expression in ABA-treated seedlings (Kobayashi et al. 2010). However, the effect of QTL for ABA responsiveness on abiotic stress tolerance and seed dormancy still remains unclear. In our previous study, ABA responsiveness of 67 *Ae. tauschii* accessions and their derived synthetic wheats was evaluated. The highly ABA-responsive line Ldn/KU-2159 has been show to express higher levels of *Wdhn13* and higher tolerance to dehydration and salinity stress compared to Ldn/IG126387, a low ABA-responsive line (Chapter VI). The objective of this study is the identification of QTLs that determine the difference in ABA responsiveness at the seedling stage between these two synthetics using a F_2 mapping population. The effects of the identified QTL on abiotic stress tolerance and seed dormancy are discussed.

3. Materials and methods

3-1. Plant materials

The mapping population of 100 F_2 individuals derived from a cross between Ldn/KU-2159 and Ldn/IG126387 was used in this study. For the functional analyses of the identified QTL, five RILs derived from the cross between common wheat cultivars CS and M808 were also included. These RILs were selected by Kobayashi et al. (2010) based on the genotypes of the SSR markers at the 6D QTL region. Two of them (RIL199 and RIL200) carries low ABA-responsive CS allele and three (RIL53, RIL136 and RIL190) the highly-responsive M808 allele at this region. The 1B, 2A, 3A and 7B QTL regions are homozygous for CS allele in these five RILs (Kobayashi et al. 2010).

3-2. Bioassay for ABA responsiveness

ABA responsiveness was evaluated as in our previous study (Chapter VI) with some modifications. Briefly, after imbibition of seeds in tap water for 5 h, they were incubated overnight at 4°C. Seeds were pre-germinated for 24 h at 24°C in darkness. Five to six synchronously germinated seeds were placed in a plastic Petri dish

containing two overlapping sheets of filter paper wetted with 6 mL of distilled water (control) or 20 μM (±)-ABA (Wako Pure Chemical Industries, Osaka, Japan), then incubated at 24°C in the dark. After 48 h of treatment, the length of shoots and primary roots were recorded in four to five independent experiments. At least 20 seeds per each of control and ABA treated groups were used. Bioassay was performed using the F_3 generation, and the average of F_3 individuals was used as the value of their respective F_2 individuals in QTL analysis. Relative growth inhibition was calculated as the difference between growth of the control group and the ABA-treated group, relative to the control. The data were statistically analyzed using JMP software ver. 5.1.2 (SAS Institute, Cary, NC, USA). The correlations among the examined traits were estimated based on Pearson's correlation coefficient values.

3-3. Genetic map construction and QTL analysis

A previously established genetic map of Ldn/KU-2159//Ldn/IG126387 population was used (Nguyen et al. 2013). To supplement the chromosome 6D regions with scarce simple sequence repeat (SSR) markers, eight SSR markers and four single nucleotide polymorphisms (SNPs) derived from leaf and spike transcripts of *Ae. tauschii* (Table VII-1) (Chapter IV and V) were used. The additional markers were selected from a high confidence SNP dataset constructed by comparing the next generation sequencing of leaf and spike transcripts between two genetically distinct accessions of *Ae. tauschii* in our previous study (Chapter IV and V). Information on the SSR markers and their annealing temperatures were obtained from the National BioResource Project (NBRP) KOMUGI web site (http://www.shigen.nig.ac.jp/wheat/komugi/strains/aboutNbrpMarker.jsp) and the GrainGenes web site (http://wheat.pw.usda.gov/GG2/maps.shtml). The PCR conditions for cleaved amplified polymorphic sequence (CAPS) markers were 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 30 s. After amplification, PCR products were digested with a restriction enzyme, and the digested fragments were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Genetic mapping was performed using MAPMAKER/EXP version 3.0b (Lander et al. 1987). The threshold for log-likelihood scores was set at 3.0, and genetic distances were calculated with the Kosambi function (Kosambi 1944).

QTL analysis was carried out by the composite interval mapping with Windows QTL Cartographer ver. 2.5 software (Wang et al. 2011) using the forward and backward method. A log-likelihood (LOD) score threshold for each trait was determined by

computing a 1000 permutation test. The percentage of phenotypic variation explained by a QTL for a trait and any additive effects were also estimated using this software.

3-4. Mapping of *TaABA8'OH1-D*

The location of *ABA8'OH1* on *Ae. tauschii* genetic map was checked based on BlastN search of *TaABA8'OH1-D* (accession number AB714579, Chono et al. 2013) against the draft genome of *Ae. tauschii* (Jia et al. 2013). The hit (scaffold13064, position 85390 to 83229 bp) with *E*-value = 0 was found to be located around the centromeric region of chromosome 6D on Y2280/AL8/78 linkage map (position: 53.69 cM, Jia et al. 2013).

To map *TaABA8'OH1-D*, first, BlastN search was conducted against polymorphic contigs derived from leaf and spike transcripts (Chapter IV and V), but no hits were found. Next, SSR motifs were searched on the genomic scaffold containing *TaABA8'OH1-D* (scaffold13064) using SciRoKo version 3.4 (Kofler et al. 2007) (SSR search mode: mismatched, fixed penalty). One SSR motif was found at 3' untranslated region (UTR) of *TaABA8'OH1-D* (1-bp apart from the stop codon, position 83551 bp on scaffold13064). The primer set amplifying this SSR motif (*TaABA8'OH1*, Table VII-1) detected polymorphism between the parental lines of F_2 individuals.

3-5. Cloning and sequencing of *TaABA8'OH1* in CS and M808

Total DNA extracted from leaves of CS and M808 (Kobayashi et al. 2010) were used for cloning of *TaABA8'OH1*. Genomic sequence was amplified using the following primer set: 5'-CAGGCCATCTTCTTCCAGCA-3' and 5'-GGATGGATGGATGGCTCAGG-3', with PrimeSTAR HS DNA Polymerase (Takara-Bio, Shiga, Japan) according to the manufacturer's protocol. This primer set amplifies from the middle of the first exon to 3' untranslated region (UTR). The amplified PCR product was cloned into a pMD20-T vector using Mighty TA-cloning Reagent Set for PrimeSTAR (Takara-Bio). Sequencing was performed using Big Dye terminator cycle sequencing kit (Applied Biosystems, CA, USA) and an Applied Biosystems 3730*xl* DNA Analyzer (Applied Biosystems). D-genome sequence was selected by blastn search against *Aegilops tauschii* genomic sequence (Jia et al. 2013) and *TaABA8'OH1-D* mRNA sequence (accession number: AB714579, Chono et al. 2013).

Table VII-1. List of markers used in this study to supplement the chromosome 6D

Marker locus	Primer sequence	Marker type	Restriction enzyme
Xctg02103	GGTGTATTTCGGCACGACTT TTGCCACCATCCATTACAAA	CAPS	*Msp*I
Xctg05183	TCGCTTGATAGTGCATGTGA ACTAGCTGCACCCTTTGCAT	CAPS	*Hind*III
Xctg05512	TCGTCGCTGGTGAAGATGTA ATGACGACGACGGAGAAGAT	CAPS	*Mbo*I
Xctg05586	AGCTTTTCTTTAACAGACTATTTTGCT TGATGCCGAGAAACAAGAGCT	CAPS	*Xba*I
TaABA8'OH1	CTATCGCGCTGTTGATTTCC CAGATGGTCCACCTCCAAGT	SSR	-

3-6. Expression analysis of genes involved in the regulation of cell-cycle and ABA-inducible genes

For expression analysis at the seedling stage, seeds (F_3 generation) were pre-germinated and treated with ABA as in bioassay (section 3-2). After 48 h of ABA treatment, total RNA was extracted from root and shoot.

For expression analysis during dehydration, F_3 seedlings were grown in soil at 24°C under constant light condition. Seven-day-old seedlings were removed from soil and after washing of roots, they were kept on dry filter paper under standard conditions. Total RNA was extracted from leaves at various times after dehydration treatment using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan).

The transcript accumulation of each gene was detected by quantitative RT-PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics) with the gene-specific primer sets given in Table VII-2. These primers amplify genes expressed from all A-, B- and D-genomes. The *Actin* gene was used as an internal control. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. The relative expression level was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Actin*.

3-7. Determination of ABA content in seedlings

Seeds of Ldn/KU-2159, Ldn/IG126387, CS and M808 were pre-germinated as in section 3-2. Extraction, purification and quantification of ABA from 1-day-old seedlings, including the endosperm, were performed as described by Tokuda et al. (2013). Quantification was performed using four seedlings in three independent experiments.

3-8. Bioassay for salt stress tolerance

Six-day-old F_3 seedlings grown under standard conditions were removed from soil, roots were washed with tap water and then transferred to 0.2% Hyponex (Hyponex Japan, Osaka, Japan) solution. After 24 h, the solution was changed to that containing or not (control group) NaCl. In the salt-treated group, the concentration of NaCl was increased 25 mM every day until reach 125 mM. Fresh weight of plants was measured every two days, and weight gain was calculated as the percentage of weight gain (weight at the indicated time - initial fresh weight) of the salt-treated group versus weight gain of the control group. Experiments were performed using 11-12 individuals in each group.

Table VII-2. Primer sets used for real time RT-PCR analysis in this study

Gene	Forward primer	Reverse primer
CycA3	AGGAGTACAAGCTCGTTGCC	CCAAGGAGCTGCAGCCTATT
CycB1	TGCTCGTGTACTCGCCATCC	CCTTCTGCTTGCTGCTCCC
CycB2	CGCAAGTACAGCACCTTCAA	AACATCGAACGTTGGTCTCC
CDKA1	TCCTTCTTGGAGCAAGGCAG	AGTCAGGCAAGGAACTCACG
CDKB1	GGAATTGCACACTGCCATGG	CGTCACGATCTCATGGGTGT
CDKD1	CAGAGTGTCTGCCCCTGATG	AATGTTGGTCGAGGAGCTGG
KRP1	CAGCGCCATTGCACTATGAC	CGTCGAAGTTGTACTTGGCG
KRP5	CAGCCTGATCTACAGCTCGG	GGCGAAGAACTCGTCCATCT
Histone H2B	GAGAAGAAGGGCAAGAAGAAGT	TGGGCTTCTTGTTGTACCTG
Histone H4	CACCACCAGCAACAAAGCCA	TGGTGATGCCCTGGATGTTG
TaABA8'OH1	ACAGATGGTCCACCTCCAAG	CCTCTATCGTGCCGTTGATT
TaABA8'OH2	GGTGATTTTGGAGAGCCTGA	AAGTAGTCCGGGCTGTGATG
Wdhn13	GGCGAGAAGAAGGGCGTCAT	GTGGTGGCTGTGGTGGCAT
Wrab18	TCGATTCATCCAAGCCAGAG	ACCAAACGAGTAAAGGAAGCAA
Wrab15	GGGATTCTTTCTTCGCGTCT	AGCCTCGGCCTTGAGTATGT
WABI5	CACCTCAGCGCCAAGAC	CTCCCATACCAACTGCCCTC
WDREB2	CTCTCTACCTGGATGGCTTG	CCGACCAAACACCATAGACA
Actin	GCCGTGCTTTCCCTCTATG	GCTTCTCCTTGATGTCCCTTA

3-9. Determination of water loss

F_3 plants were grown under standard conditions as for expression analysis, and the first leaves of 7-day-old seedlings (n = 10) were detached. The leaves were kept at 24ºC (60-70% relative humidity) and their fresh weight was measured. Water loss was calculated as the weight difference between initial fresh weight and weight at the indicated time, divided by initial fresh weight. Two independent experiments were performed.

3-10. Bioassay for drought tolerance

F_3 plants were grown under constant light condition at 24 ºC in soil. The soil of five-day-old seedlings was saturated with water and after that, the control group was daily irrigated but it was stopped in the drought-treated group. Soil moisture was controlled using soil moisture tester (model DM-18, Takemura Electric Works Ltd., Tokyo, Japan) every day and final moisture of ~25% was kept in the drought-treated group. Each group was composed by eleven F_3 plants and plant height was recorded every three days.

3-11. Evaluation of seed dormancy

The degree of seed dormancy for the selected F_2 individuals was evaluated using 15 freshly harvested seeds (collected from 1st and 2nd florets of the center part of spike) per F_2 plant in three independent experiments. The seeds were sown on filter paper (8-cm diameter) wetted with 4 mL of distilled water in plastic petri dishes (9-cm diameter). The number of germinated seeds was counted daily during 5 days and expressed as the percentage of germination and weighted germination index (Walker-Simmons 1988). This index gives maximum weight to seeds that germinate rapidly and is calculated from the following formula:

$$\text{Germination index (GI)} = \frac{(5 \times N_1 + 4 \times N_2 + 3 \times N_3 + 2 \times N_4 + 1 \times N_5)}{(total\ days\ of\ test \times total\ seeds)}$$

where N_1, N_2, N_3, N_4 and N_5 are the number of seeds that had germinated in day 1 to day 5, respectively. The maximum index is 1.0 if all seeds germinate by day 1 whilst lower indices are indicative of increasing level of seed dormancy. The results are presented as the mean GI of three replicates.

4. Results

4-1. ABA responsiveness in the F_2 population

ABA responsiveness was evaluated based on inhibition of root and shoot growth by exogenous ABA. In the highly ABA-responsive synthetic, Ldn/KU-2159, root and shoot length were significantly short in the presence of ABA (RL-ABA and SL-ABA) and the relative root and shoot growth inhibition (RRGI and RSGI) were significantly higher (Table VII-3) as in our previous study (Chapter VI). Root and shoot length in control group (RL and SL) also differed significantly between the parents. The values of these traits in most of F_2 plants ranged within that of parents, indicating the involvement of multiple loci.

Although RL differed significantly between the parental lines, low correlation was observed between RL and RL-ABA in F_2 population (Table VII-4) suggests that the response of root to ABA was independent of root growth in the absence of ABA. Similar observation was found between SL and SL-ABA. The variation in RRGI was better explained by RL-ABA (R^2 = 0.517) than RL (R^2 = 0.266). In contrast, the variation in RSGI was better explained by SL (R^2 = 0.718) than SL-ABA (R^2 = 0.272). Although these observations, a positive and significant correlation was observed between RSGI and RRGI in F_2 population ($P < 0.001$, Table VII-4). Moreover, the similar coefficient of determination between RRGI and SL-ABA (R^2 = 0.293) and SL (R^2 = 0.316) suggest that the difference in post-germination growth of F_2 plants are also reflected in RRGI. These results indicate that RL-ABA is sufficient for evaluation of ABA responsiveness in this population.

4-2. QTL analysis for ABA responsiveness

The genetic map, established in previous study (Figure VII-1, Nguyen et al. 2013), with 110 markers were used for QTL analysis. QTLs were detected for RL-ABA, RRGI and SL-ABA (Table VII-5). One QTL for RL-ABA was detected on the short arm of chromosome 6D (*QRla.kpg-6D.1*) with LOD score of 4.77 and can explain 22.8% of the variation in RL-ABA. For RRGI, one QTL was detected on the long arm of chromosome 4D (*QRgi.kpg-4D.1*) with LOD score of 7.62 and another QTL on chromosome 6D (*QRgi.kpg-6D.1*, LOD score of 4.74) which overlapped with *QRla.kpg-6D.1*. On chromosome 6D, one QTL for SL-ABA (*QSla.kpg-6D.1*) was also detected with a LOD score of 3.30 and explained 15.3% of the variation in SL-ABA. This QTL was located at the distal region of long arm and did not overlap with *QRla.kpg-6D.1* and *QRgi.kpg-6D.1* (Figure VII-2).

Table VII-3. Parental and F_2 population means for ABA responsiveness

	Ldn/KU-2159	Ldn/IG126387	F_2 population
RL-ABA (mm)	11.85 ± 2.16***	18.81 ± 5.02	14.42 ± 2.39
RL (mm)	62.30 ± 8.36***	76.74 ± 12.64	61.82 ± 7.63
RRGI (%)	80.98 ± 3.47***	75.48 ± 6.54	76.38 ± 4.60
SL-ABA (mm)	4.44 ± 0.49***	6.30 ± 1.54	4.98 ± 0.45
SL (mm)	32.14 ± 4.31**	36.93 ± 7.12	32.62 ± 4.13
RSGI (%)	86.17 ± 1.52***	82.95 ± 4.17	84.44 ± 2.72

Data are expressed as mean ± standard deviation

Table VII-4. Correlation coefficient (r) matrices for ABA responsiveness in F_2 population

	RL	RRGI	SL-ABA	SL	RSGI
RL-ABA	0.2084	-0.7192***	0.4419***	-0.0430	-0.2421*
RL		0.5153***	-0.2071	0.7645***	0.7486***
RRGI			-0.5409***	0.5624***	0.7414***
SL-ABA				-0.0220	-0.5215***
SL					0.8473***

Levels of significance are indicated by asterisks, * $P < 0.05$, *** $P < 0.001$

Table VII-5. Summary of identified QTLs for ABA responsiveness

Traits	Locus	Map location	LOD score	LOD threshold	Contribution (%)	Additive effect
RL-ABA	*QRla.kpg-6D.1*	*Xcfd42-Xcfd80*	4.77	3.4	22.8	1.08
RRGI	*QRgi.kpg-6D.1*	*Xcfd42-Xpgw312*	4.74	3.2	18.3	-2.22
RRGI	*QRgi.kpg-4D.1*	*Xwmc399-Xcfd84*	7.62	3.2	30.2	2.88
SL-ABA	*QSla.kpg-6D.1*	*Xgdm98-Xcfd5*	3.30	3.3	15.3	0.23

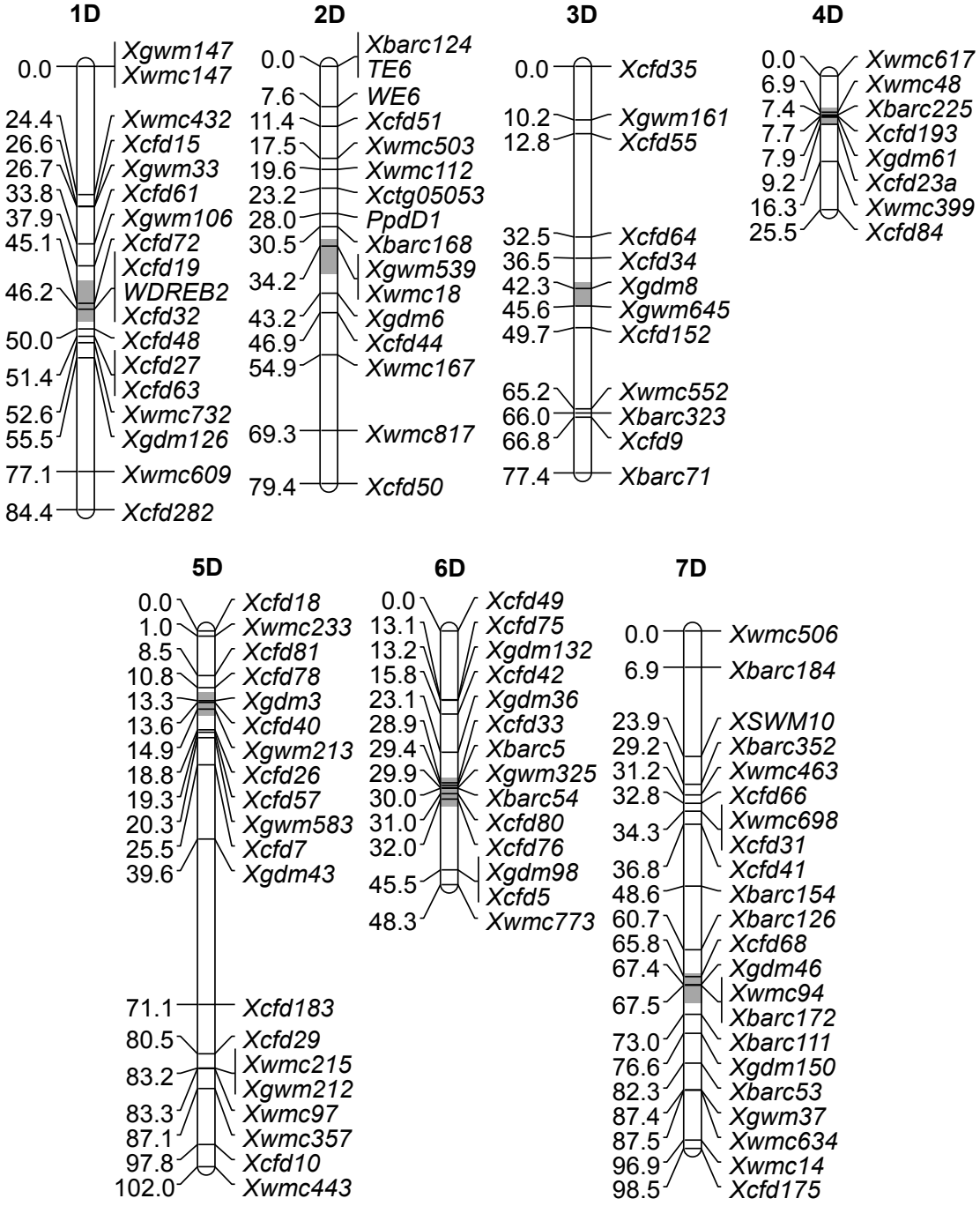

Figure VII-1. Linkage map established in previous study. This map has been constructed using publicly available 105 SSR markers and five EST markers. Genetic distances are given in centiMorgans (cM). Gray bars indicate the putative positions of centromeres.

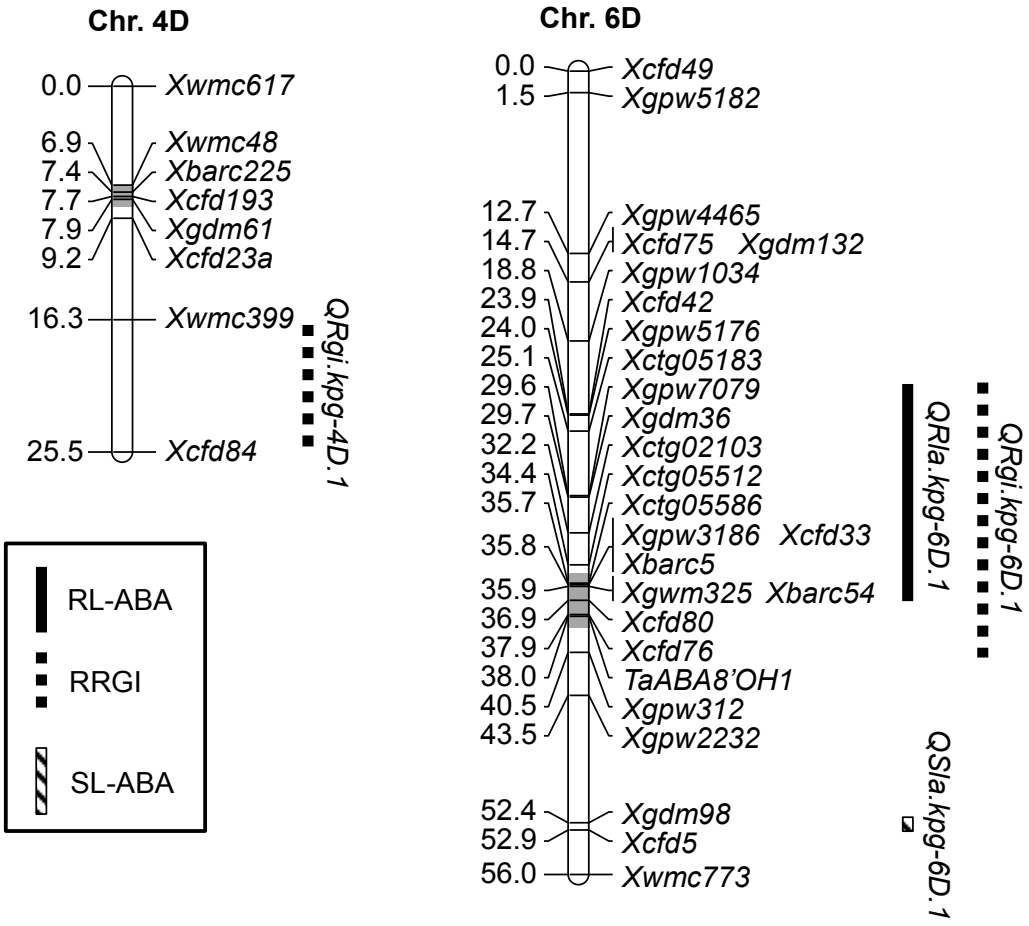

Figure VII-2. Linkage maps and positions of identified QTLs for ABA responsiveness. The linkage map of chromosome 6D was supplemented with 13 markers. QTLs with LOD score above the threshold are indicated, and genetic distances are given in cM. Gray bars indicate the putative positions of centromeres.

In QTLs identified for RL-ABA and SL-ABA, allele from the lower ABA-responsive parent showed positive values of additive affect (Table VII-5), indicating that the allele derived from the lower responsive line produced low ABA-responsive phenotype (longer root and shoot length in the presence of exogenous ABA). There were significant differences ($P < 0.05$) between F_2 individuals carrying alleles from the highly responsive and low responsive parents at both QTLs (Figure VII-3). *QRgi.kpg-6D.1* showed a negative additive effect, indicating that the allele for high ABA responsiveness was derived from the highly responsive synthetic wheat line. Conversely, the allele derived from the lower ABA-responsive line produced high ABA-responsive phenotype in *QRgi.kpg-4D.1*, indicated by its positive value of additive effect. The genotype effect at these two QTLs also differed significantly ($P < 0.05$) in F_2 individuals (Figure VII-3).

Because RL-ABA was suggested to be suitable for the evaluation of ABA responsiveness (Table VII-4), QTL for ABA responsiveness was equated to the 6D QTL region of *QRla.kpg-6D.1*, where *QRgi.kpg-6D.1* also overlapped. Moreover, at this region the allele with highly ABA-responsive effect was derived from the highly responsive parental line, in contrast to *QRgi.kpg-4D.1*. Therefore, we attempted to study in more detail the 6D QTL for ABA responsiveness. Since the number of markers mapped on this region is scarce, polymorphism of 13 publicly available SSR markers and seven CAPS markers were tested between parents. CAPS markers were developed using the information of SNPs derived from leaf and spike transcripts of *Ae. tauschii* (Chapter IV and V). In total, eight (61.5%) SSR markers and four (57.1%) CAPS markers were available for genotyping of F_2 plants. All these markers were assigned on chromosome 6D, of which seven markers were placed in the QTL region (Figure VII-2).

4-3. Mapping of *TaABA8'OH1* on chromosome 6D

In previous study, the gene encoding an ABA catabolic enzyme *TaABA8'OH1* has been found to locate on homoeologous group-6 chromosomes in wheat (Chono et al. 2013). In order to check the location on chromosome 6D, BlastN search was performed using as query *TaABA8'OH1-D*, derived from D-genome (accession number AB714579, Chono et al. 2013), against the draft genome of *Ae. tauschii* (Jia et al. 2013). A hit with *E*-value = 0 was obtained (scaffold13064) and this scaffold has been located around the centromeric region of the diploid genetic map (53.69 cM, Jia et al. 2013). Because the QTL regions of *QRla.kpg-6D.1* and *QRgi.kpg-6D.1* also overlap with the centromeric region, we attempted to map *TaABA8'OH1-D*. No hits were found in the blast search of *TaABA8'OH1-D* against the leaf and spike transcripts (Chapter IV and V). One SSR

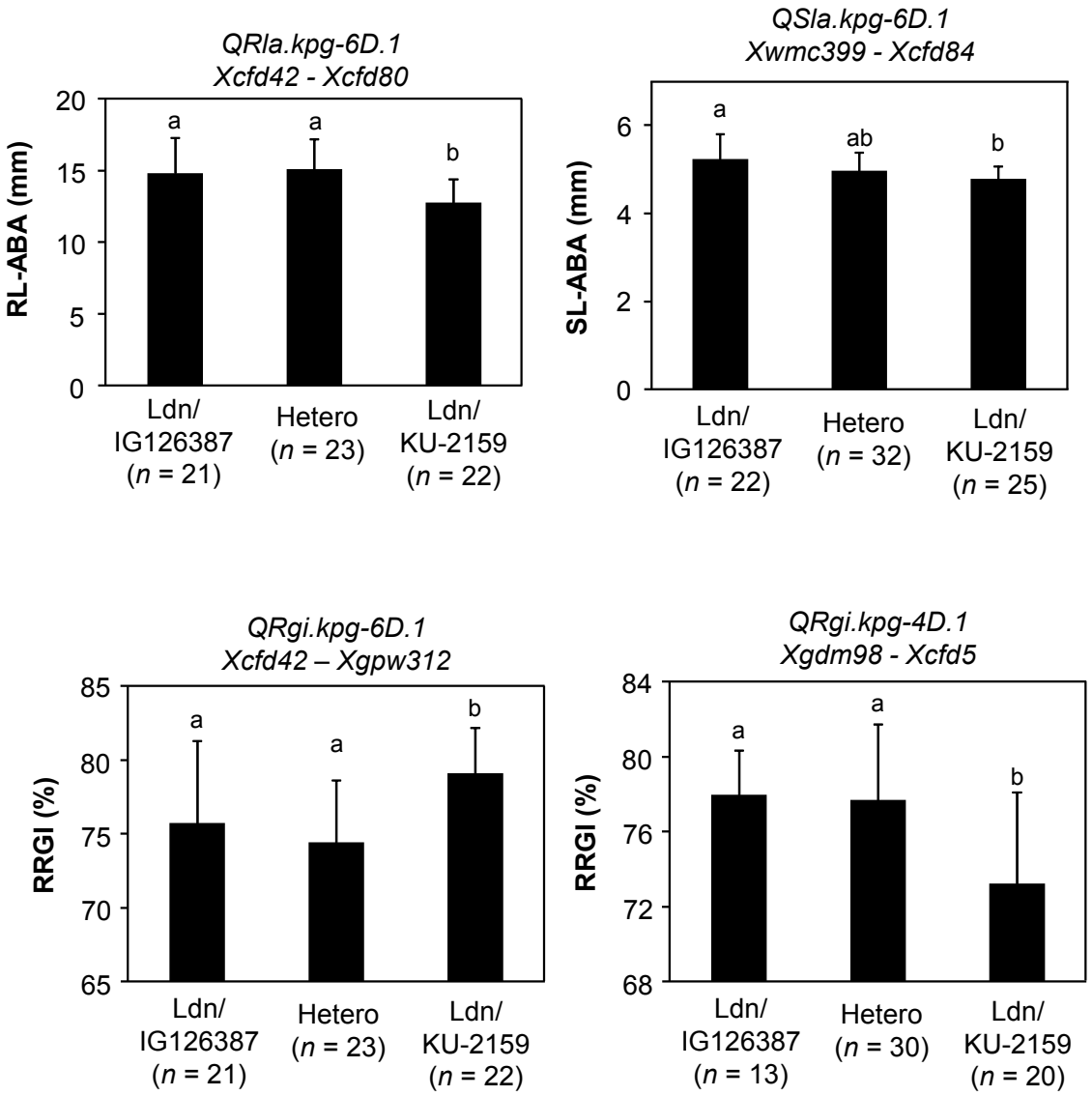

Figure VII-3. The genotype effect at identified QTLs for ABA responsiveness. Lower values of RL-ABA and SL-ABA, and higher values of RRGI indicates higher ABA responsiveness. The genotypes at the marker interval listed above each graph were used for analysis. Mean ± standard deviation with the same letter were not significantly different ($P > 0.05$, Tukey-Krammer HSD test).

motif was found at the 3' UTR of *TaABA8'OH1-D* and was polymorphic between parents of the F_2 population. This SSR marker (*TaABA8'OH1*) was tightly linked to *Xcfd76* and was included in the QTL region of *QRgi.kpg-6D.1* but not in that of RL-ABA (Figure VII-2).

In a previous study, Kobayashi et al. (2010) reported a QTL for ABA responsiveness (based on RRGI) on chromosome 6D. Comparison of the QTL regions showed that *QRgi.kpg-6D.1*, but not *QRla.kpg-6D.1*, overlaps with the QTL region for ABA responsiveness detected in CS/M808 mapping population (Figure VII-4). To test whether the 6D QTL for ABA responsiveness identified in this study and that reported by Kobayashi et al. (2010) are different loci, five RILs (two carrying CS-type 6D QTL and three carrying M808-type QTL) were included for further studies.

4-4. Expression analysis during post-germination growth

For functional analysis of the QTLs, seven F_2 individuals were selected based on the genotypes at SSR markers flanking the 6D QTL for ABA responsiveness, and F_3 plants derived from each F_2 individuals were bulked for further analysis. Because ABA regulates genes involved in cell-cycle control (Wang et al. 1998; Gendreau et al. 2012; Chapter III), we performed gene expression analysis of three *Cyclins* (*CYCA3*, *CYCB1* and *CYCB2*), three *CDKs* (*CDKA1*, *CDKB1* and *CDKD1*), two *KRPs* (*KRP1* and *KRP5*) and two *Histones* (*Histone H2B* and *Histone H4*) during post-germination growth. As in ABA bioassay, pre-germinated seeds were grown in the presence or absence of 20 μM ABA and total RNA was extracted after 48 h of incubation.

Of the analyzed genes in root, allelic difference at the 6D QTL affected the transcript accumulation of *CYCB2* (Figure VII-5C) and *CDKA1* (Figure VII-5D). Expression level of *CYCB2* was significantly higher ($P < 0.05$) in individuals with Ldn/IG126387-type 6D QTL (low responsive lines, average of 0.62 ± 0.06) than in those with Ldn/KU-2159 allele (highly responsive lines, average of 0.42 ± 0.05). Similarly, transcript accumulation of *CDKA1* was significantly higher ($P < 0.05$) in individuals carrying the low responsive parental allele (1.44 ± 0.25) than in those with the highly responsive parental allele (1.07 ± 0.08). *KRP1* was up-regulated by > 2-fold as in our previous study (Chapter III) but no significant difference was observed between the two alleles (Figure VII-5G). Although the expression level of *Histone H2B* tended to be lower in plants carrying highly responsive allele, the effect of allelic difference was not significant (Figure VII-5I). In shoot, the expression patterns of the cell-cycle controlling genes were similar to those in root except for *KRP1*, which was

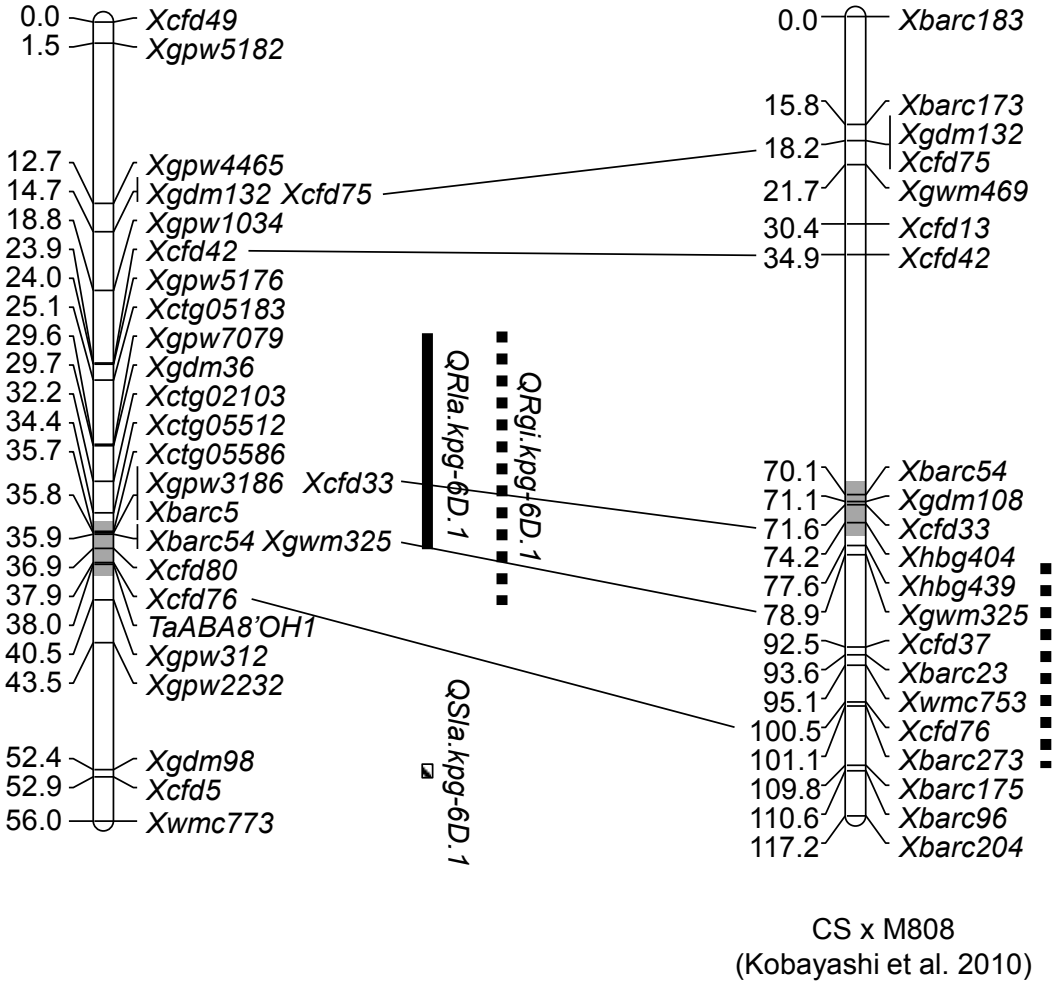

Figure VII-4. Comparative map of QTL regions for ABA responsiveness on chromosome 6D. The QTL region for RRGI (broken bar) detected in the present study (left linkage map) partially overlapped with the QTL region for ABA responsiveness (broken bar) reported by Kobayashi et al. (2010) (right map). Genetic distances are given in cM.

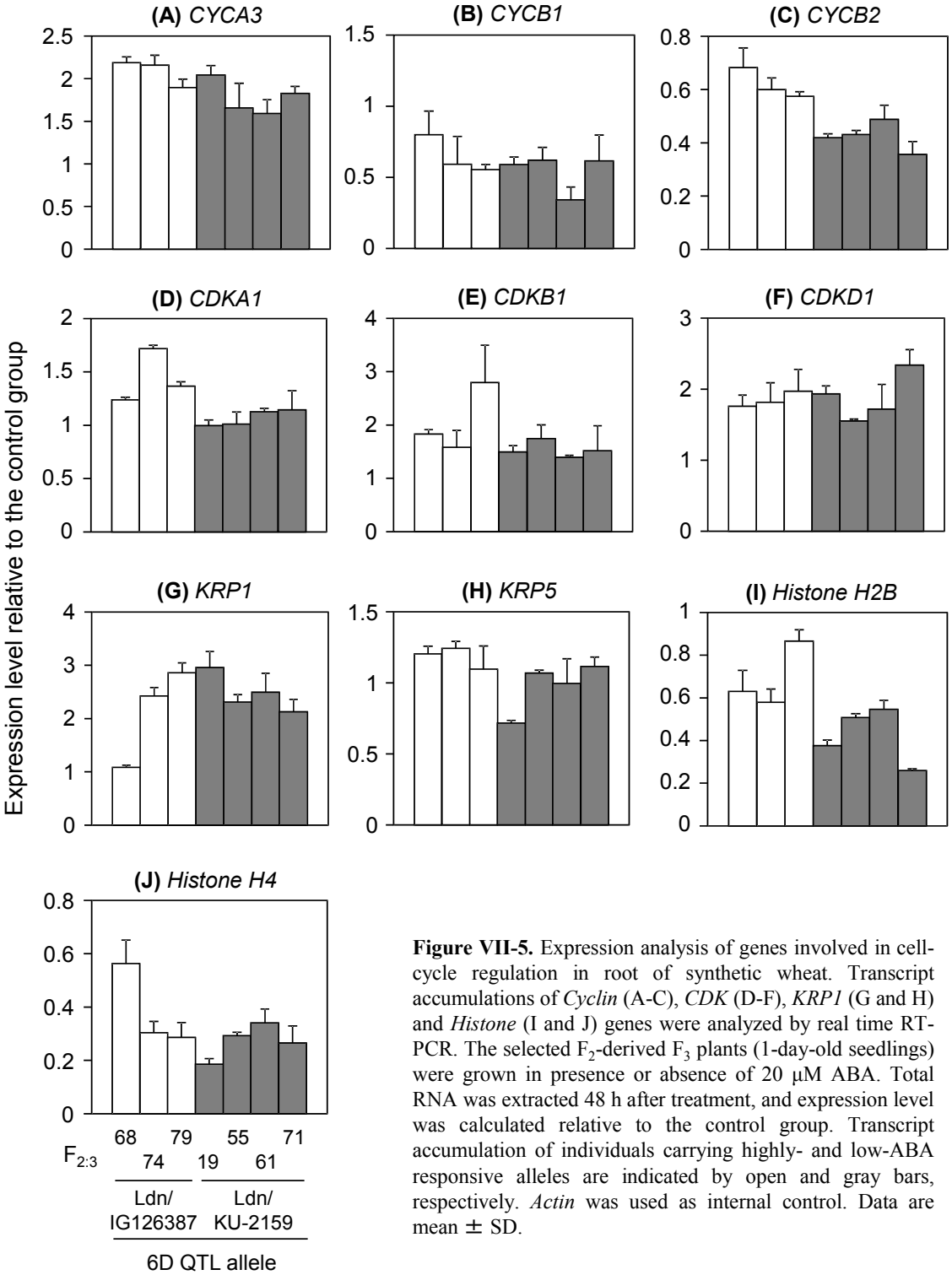

Figure VII-5. Expression analysis of genes involved in cell-cycle regulation in root of synthetic wheat. Transcript accumulations of *Cyclin* (A-C), *CDK* (D-F), *KRP1* (G and H) and *Histone* (I and J) genes were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 µM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying highly- and low-ABA responsive alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

not up-regulated (Figure VII-6). However, significant difference in expression levels was not observed between the two alleles.

On the other hand, expression levels of ABA 8'-hydroxylase genes *TaABA8'OH1* (located on homoeologous group-6 chromosomes) and *TaABA8'OH2* (located on homoeologous group-5 chromosomes) tended to be higher in individuals with low responsive allele in root (Figures VII-7A and VII-7C), but this difference was not significant. Conversely, in shoot expression levels of *TaABA8'OH1* were similar among the F_3 plants (Figure VII-7B) and *TaABA8'OH2* tended to be higher in individuals carrying the highly responsive allele (Figure VII-7D).

We performed the same analysis using the selected RILs derived from CS and M808. Unlike in root of synthetic wheat lines, *CYCB2* and *CDKA1* expression did not differ between RILs with CS-type (low responsiveness) and M808-type (high responsiveness) 6D QTL (Figures VII-8B and VII-8C), transcript accumulation of *KRP1* tended to be higher ($P = 0.08$) in RILs carrying highly responsive parental allele (Figure VII-8D), and that of *Histone H2B* differed significantly ($P < 0.05$, 0.75 ± 0.14 [CS allele] vs. 0.33 ± 0.05 [M808 allele]) (Figure VII-8D). Expression levels of the ABA catabolic gene *TaABA8'OH1* were similar among RILs (Figure VII-8F), and transcript accumulation of *TaABA8'OH2* tended to be higher in lines with M808 allele as observed in in F_3 lines of synthetic wheat mapping population (Figure VII-8G). In shoot, as in synthetic wheat, no significant differences were observed between the two alleles, and *KRP1* was not induced by ABA (Figure VII-9). These results suggest that 6D QTL for ABA responsiveness identified in synthetic wheat and common wheat are different loci that regulate cell-cycle through partially distinct pathway. However, *TaABA8'OH2* seems to be regulated in a similar manner by these two QTLs.

To test whether the difference in endogenous ABA content between parental lines caused the differential ABA responsiveness, ABA content of the 1-day-old seedling, just before ABA treatment, was measured in parents. Although the difference in endogenous ABA was not significant between Ldn/IG126387 and Ldn/KU-2159 and between CS and M808, it was slightly higher in parental lines with higher ABA responsiveness (Figure VII-10). Because *TaABA8'OH1-D* was located in the 6D QTL region for ABA responsiveness in common wheat RIL population, we cloned and sequenced from genomic DNA of CS and M808. No difference in *TaABA8'OH1-D* genomic sequences were observed (Figure VII-11).

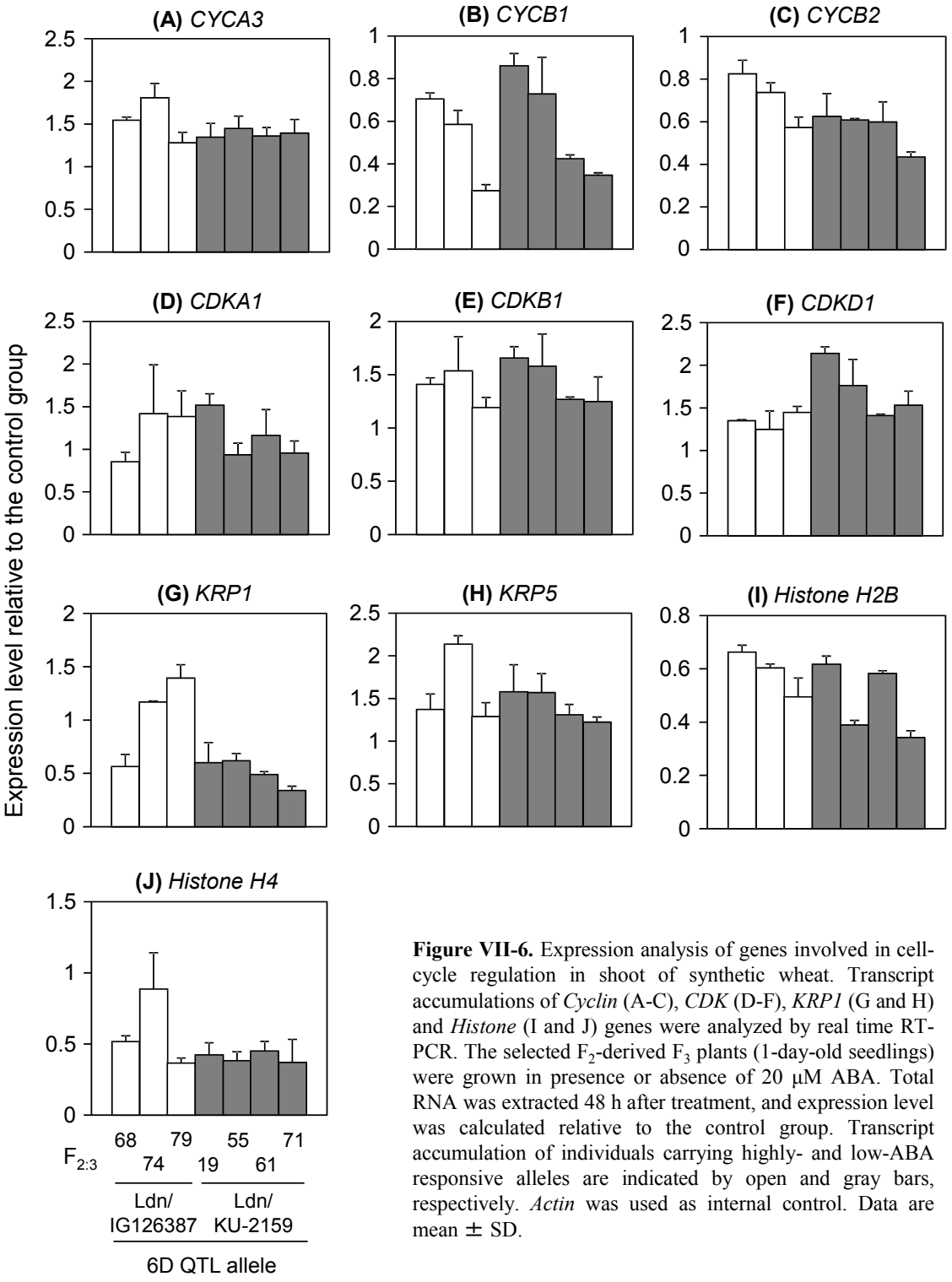

Figure VII-6. Expression analysis of genes involved in cell-cycle regulation in shoot of synthetic wheat. Transcript accumulations of *Cyclin* (A-C), *CDK* (D-F), *KRP1* (G and H) and *Histone* (I and J) genes were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 µM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying highly- and low-ABA responsive alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

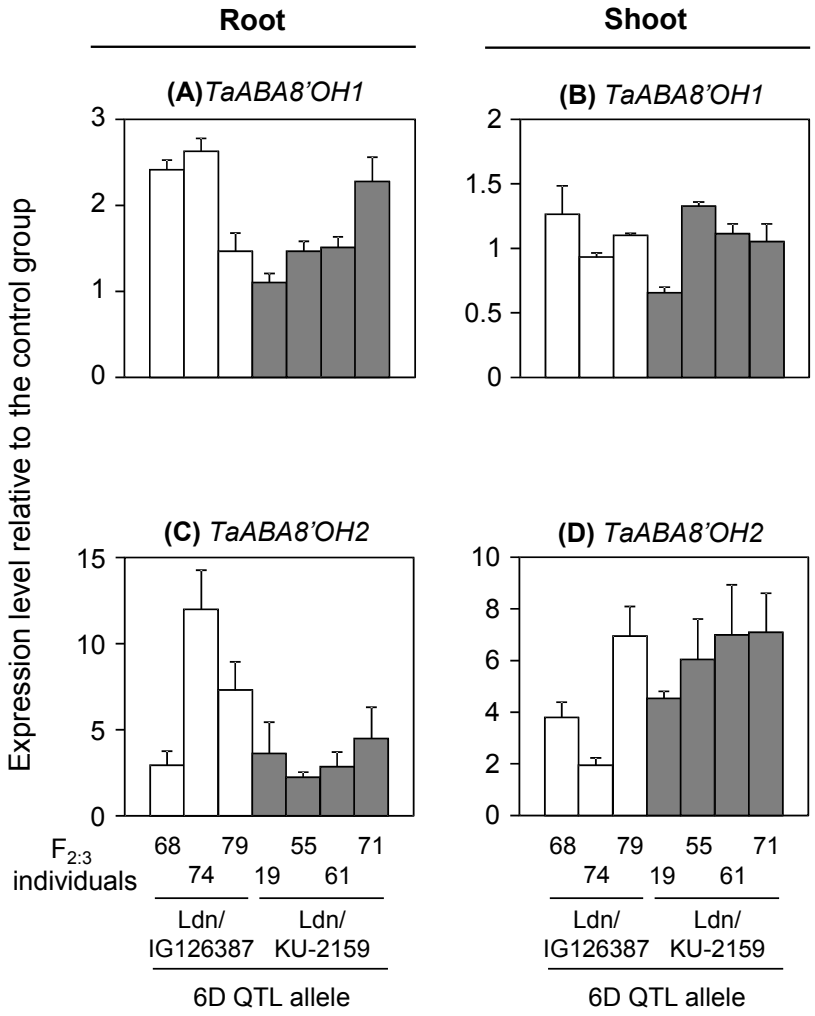

Figure VII-7. Expression analysis of genes involved in the catabolism of ABA in root and shoot of synthetic wheat. Transcript accumulations of *TaABA8'OH1* (A and B) and *TaABA8'OH2* (C and D) were analyzed by real time RT-PCR. The selected F_2-derived F_3 plants (1-day-old seedlings) were grown in presence or absence of 20 µM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. Transcript accumulation of individuals carrying highly- and low-ABA responsive alleles are indicated by open and gray bars, respectively. *Actin* was used as internal control. Data are mean ± SD.

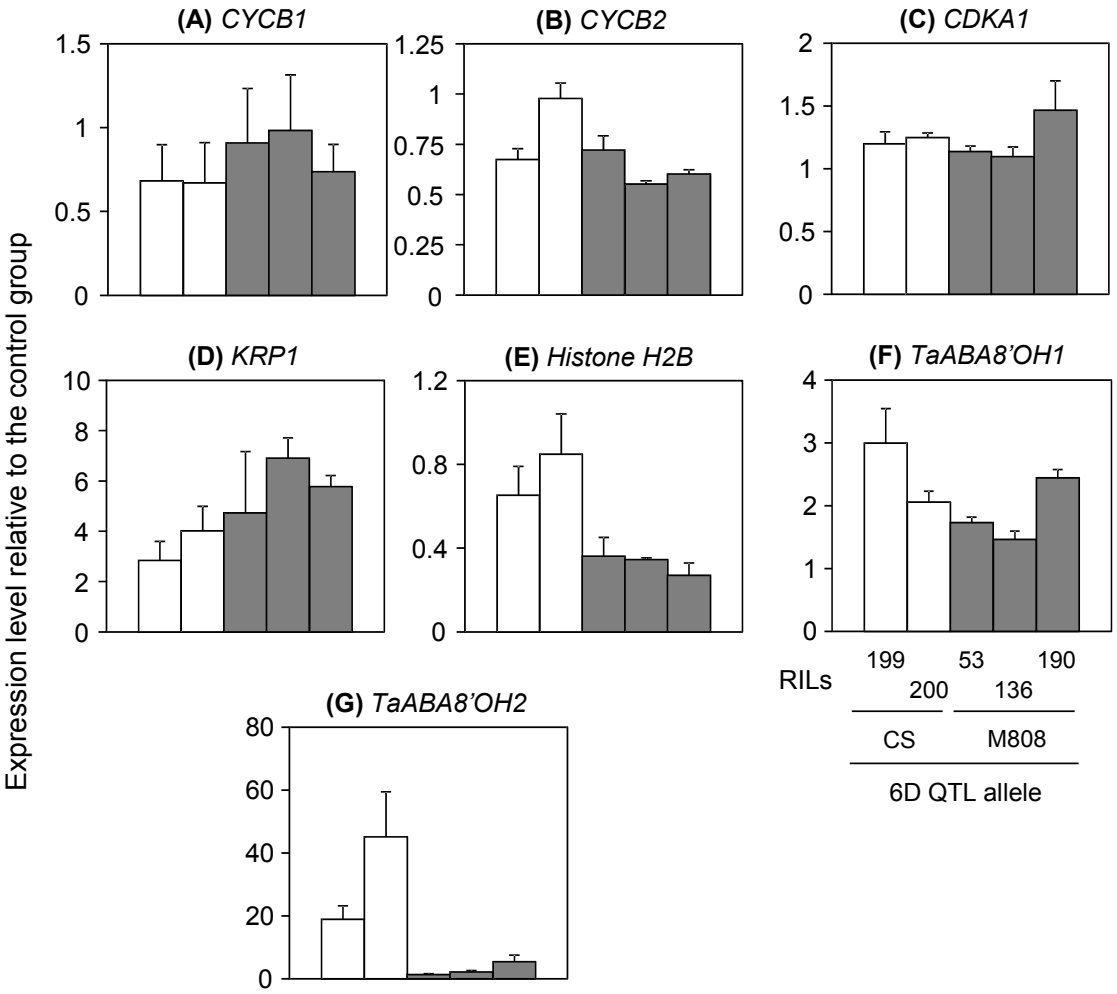

Figure VII-8. Expression analysis of genes involved in cell-cycle regulation and ABA catabolism in root of common wheat. Transcript accumulations of B-type cyclins (A and B), *CDKA1* (C), *KRP1* (D), *Histone H2B* (E) and *ABA 8'-hydroxylases* (F and G) were analyzed by real time RT-PCR. RILs used by Kobayashi et al. (2010), carrying CS-type 6D QTL (low responsiveness, open bars) or M808 allele (highly responsive, gray bars), were grown in presence or absence of 20 μM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. *Actin* was used as internal control. Data are mean ± SD.

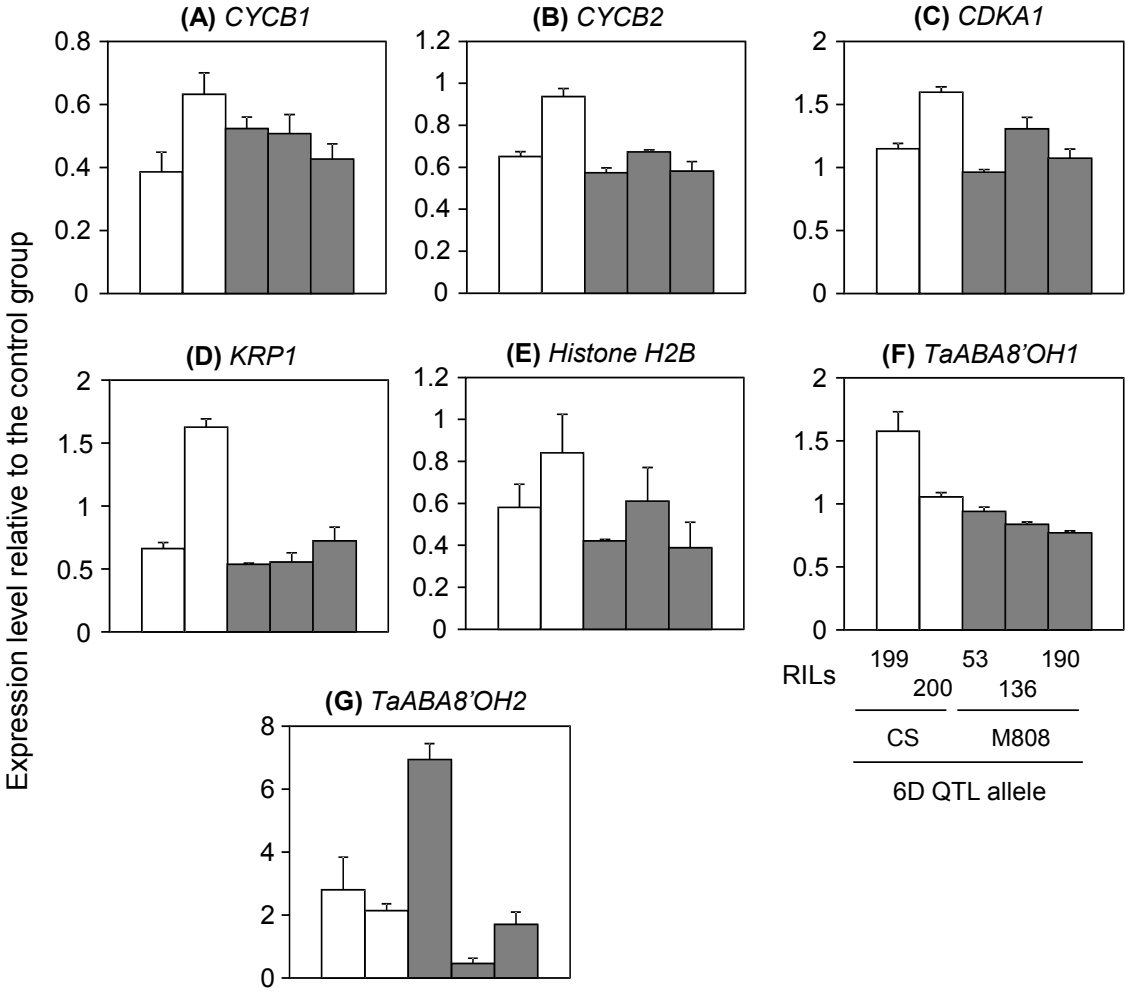

Figure VII-9. Expression analysis of genes involved in cell-cycle regulation and ABA catabolism in shoot of common wheat. Transcript accumulations of B-type cyclins (A and B), *CDKA1* (C), *KRP1* (D), *Histone H2B* (E) and *ABA 8'-hydroxylases* (F and G) were analyzed by real time RT-PCR. RILs used by Kobayashi et al. (2010), carrying CS-type 6D QTL (low responsiveness, open bars) or M808 allele (highly responsive, gray bars), were grown in presence or absence of 20 μM ABA. Total RNA was extracted 48 h after treatment, and expression level was calculated relative to the control group. *Actin* was used as internal control. Data are mean ± SD.

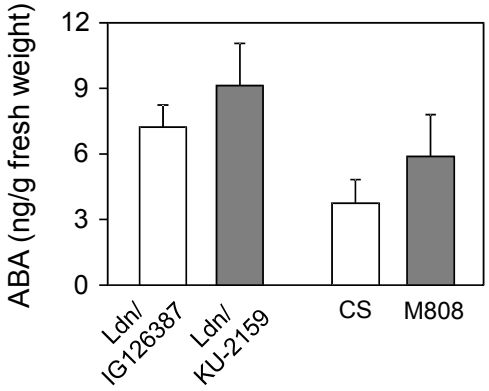

Figure VII-10. ABA content in whole seedlings of synthetic and common wheat lines. ABA was quantified using 1-day-old seedlings (n = 4), including endosperm. Low and highly responsive parental lines are indicated by open and gray bars, respectively. Data are mean ± SD of three independent measurements.

```
CS        1 CAGGCCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGCCGTCTCGTCTCACGC 60
M808      1 CAGGCCATCTTCTTCCAGCAGGGGGACTACCATGCCCACCTCCGCCGTCTCGTCTCACGC 60
            ************************************************************

CS       61 GCCTTCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAGGCTATCGCCCTCCGC 120
M808     61 GCCTTCTCTCCCGAGGCCATCCGCGGTTCCGTCCCTGCCATCGAGGCTATCGCCCTCCGC 120
            ************************************************************

CS      121 TCCCTCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGT 180
M808    121 TCCCTCGGCTCCTGGGAAGACCTGCAAGTCAACACCTTCCAAGAGATGAAGACTGTGAGT 180
            ************************************************************

CS      181 GCTTCTTCTTCTTCCTCTTCCATTCCCGCTTGCTCTGCTTTCCTCTGCTCTGCTCTACTG 240
M808    181 GCTTCTTCTTCTTCCTCTTCCATTCCCGCTTGCTCTGCTTTCCTCTGCTCTGCTCTACTG 240
            ************************************************************

CS      241 CTAAATGATTGGAGCTCGAGGCTGATCCTTCTCTTGGTGTCGTGGCGCAGTACGCTCTGA 300
M808    241 CTAAATGATTGGAGCTCGAGGCTGATCCTTCTCTTGGTGTCGTGGCGCAGTACGCTCTGA 300
            ************************************************************

CS      301 ATGTGGCATTGCTGTCCATCTTCGGCGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGC 360
M808    301 ATGTGGCATTGCTGTCCATCTTCGGCGAGGAGGAGATGCAGTACATCGAGGAGCTGAAGC 360
            ************************************************************

CS      361 AGTGCTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGC 420
M808    361 AGTGCTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCTGCCGGGCACGC 420
            ************************************************************

CS      421 TGTTCCACAAGGCCATGAAGGCCCGAAAGCGGCTGGGCGCCATTGTGGCCCACATCATCT 480
M808    421 TGTTCCACAAGGCCATGAAGGCCCGAAAGCGGCTGGGCGCCATTGTGGCCCACATCATCT 480
            ************************************************************

CS      481 CGGCCCGGCGCGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCG 540
M808    481 CGGCCCGGCGCGAGCGCGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCCGCG 540
            ************************************************************

CS      541 AGGCGCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCG 600
M808    541 AGGCGCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGCG 600
            ************************************************************

CS      601 ACACCACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCC 660
M808    601 ACACCACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCC 660
            ************************************************************

CS      661 TCAAAGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAA 720
M808    661 TCAAAGCCGTCACCGTAAGTCGCCATCAACCAGCTGACCCGCTTGGTACCCGATCGAAAA 720
            ************************************************************

CS      721 GCAGTGGCTGACCCGTGCGTCGTACAATTAACAGGAAGAGCACGCTGAGATCGCGAGGGA 780
M808    721 GCAGTGGCTGACCCGTGCGTCGTACAATTAACAGGAAGAGCACGCTGAGATCGCGAGGGA 780
            ************************************************************
```

Figure VII-11. Alignment of CS and M808 *TaABA8'OH1-D* genomic sequence. From the middle of first exon of *TaABA8'OH1-D* to 3'-UTR was sequenced. D-genome derived clone was selected based on blastn against *Ae. tauschii* genome and *TaABA8'OH1-D* mRNA sequences. Asterisks indicate the identical nucleotides between CS and M808.

```
CS    781 GAAGGCGTTGTCCGGCGAGCCACTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGG 840
M808  781 GAAGGCGTTGTCCGGCGAGCCACTGTCGTGGGCCGACACGCGGCGGATGCGGATGACGGG 840
          ************************************************************

CS    841 CCGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGC 900
M808  841 CCGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGGGAGGC 900
          ************************************************************

CS    901 CGTGGAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAATCGCTTTGGTCGTTT 960
M808  901 CGTGGAGGACGTGGAGTACCAAGGTGAGCAGAGCAGAGACATCAATCGCTTTGGTCGTTT 960
          ************************************************************

CS    961 GTGGCAGCGCAGTGCTGTACTCCGCTGTCCCTCTCGGAGTACAGCAGTGAGCTGCCTGCC 1020
M808  961 GTGGCAGCGCAGTGCTGTACTCCGCTGTCCCTCTCGGAGTACAGCAGTGAGCTGCCTGCC 1020
          ************************************************************

CS   1021 TGCCTGCGCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAACGAACGAAATAGACC 1080
M808 1021 TGCCTGCGCATGAACTGGCTCGGAAAGGACGCGCTCCTAACCGAACGAACGAAATAGACC 1080
          ************************************************************

CS   1081 AACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTACCTGATTC 1140
M808 1081 AACTCAAACTCGCAACTCACCTCGACTTGCTCTCCTCTGTGCGTGCAGGGTACCTGATTC 1140
          ************************************************************

CS   1141 CCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGACCACTTCC 1200
M808 1141 CCAAGGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGACCACTTCC 1200
          ************************************************************

CS   1201 CCTCCCCTGAAAAGTTCGATCCTTCACGATTCGAGGTCAGCATCATCACAGCCCTCTGTT 1260
M808 1201 CCTCCCCTGAAAAGTTCGATCCTTCACGATTCGAGGTCAGCATCATCACAGCCCTCTGTT 1260
          ************************************************************

CS   1261 TGACGAGTCTGCTTCGATTCGATTGATCATTATCTGATTATACGTTTTGGTTGCTGACTG 1320
M808 1261 TGACGAGTCTGCTTCGATTCGATTGATCATTATCTGATTATACGTTTTGGTTGCTGACTG 1320
          ************************************************************

CS   1321 CAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACGGGACCCACTCGTGCCCC 1380
M808 1321 CAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTTCGGGAACGGGACCCACTCGTGCCCC 1380
          ************************************************************

CS   1381 GGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCACCAAGTAC 1440
M808 1381 GGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCACCAAGTAC 1440
          ************************************************************

CS   1441 AGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCCTCCCCATC 1500
M808 1441 AGATGGTCCACCTCCAAGTCCGAGAGCGGCGTCCAGTTCGGCCCCTTCGCCCTCCCCATC 1500
          ************************************************************

CS   1501 AACGGCCTCCCCATGACCTTCACCCGCAAGGACGACAAGAACAAAGCCTGAGCCATCCAT 1560
M808 1501 AACGGCCTCCCCATGACCTTCACCCGCAAGGACGACAAGAACAAAGCCTGAGCCATCCAT 1560
          ************************************************************

CS   1561 CCATCC 1566
M808 1561 CCATCC 1566
          ******
```

Figure VII-11. Continued.

4-5. Effect of the QTL on abiotic stress tolerance

To study the effect of QTL for ABA responsiveness, salinity and dehydration tolerance were evaluated. Salinity tolerance was evaluated by weight gain of the selected F_3 synthetic wheat seedlings grown under stress conditions (0.2% Hyponex solution [Hyponex Japan] containing NaCl). The concentration of NaCl was increased 25 mM per day to finally keep 125 mM. Salinity tolerance did not differ between individuals carrying the low and highly responsive alleles at the 6D QTL in this experimental condition (Figure VII-12).

Because ABA induces stomatal closure during drought stress to limit water loss, the stomatal response during leaf dehydration was evaluated based on the rate of water loss from detached leaves. Water loss rate was similar among the tested F_3 individuals, except the individual number 74 (carrying low responsive parental allele), which showed lower stomatal response to leaf dehydration (Figure VII-13A). The long-term dehydration tolerance was also evaluated. Five day after sowing, irrigation was withheld to finally keep soil moisture of ~25%. The growth rate, evaluated based on the plant height relative to control group, did no differ among these selected synthetic wheats (Figure VII-13B). Similarly, the effect of 6D QTL on stomatal response to leaf dehydration was not detected among the selected RILs (Figure VII-13C).

To test whether the 6D QTL for ABA responsiveness regulate stress inducible genes, expression analysis during dehydration was performed. Transcript accumulations of three *Cor/Lea* genes (*Wrab18*, *Wrab15* and *Wdhn13*), two transcription factors (*WDREB2* and *WABI5*) and ABA 8'-hydroxylases (*TaABA8'OH1* and *TaABA8'OH2*) were analyzed by real time RT-PCR. In synthetic wheat F_3 individuals, all these genes were induced by dehydration treatment, but no differences between the two alleles were observed (Figure VII-14). Conversely, in the RILs derived from CS and M808, transcript accumulations of *Cor/Lea* genes and transcription factors were higher in two of the three RILs (RIL53 and RIL190) carrying the highly-responsive M808 allele at 6D QTL (Figure VII-15A–E). The expression levels of the two ABA 8'-hydroxylase genes were also higher in RIL53 and RIL190, indicating that *TaABA8'OH1-D* might not be causal gene (Figure VII-15F and VII-15G).

4-6. Effect of the 6D QTL on seed dormancy

ABA is also involved in the regulation of seed dormancy (Finkelstein et al. 2002). The seeds of each selected F_2-derived F_3 individuals and RILs, were imbibed at 20°C for 5 days. In synthetic wheat F_3 lines, germination rate was around 20% after five days of imbibition at 40 DPA (Figure VII-16A), around 50% at 50 DPA (Figure VII-16B) and

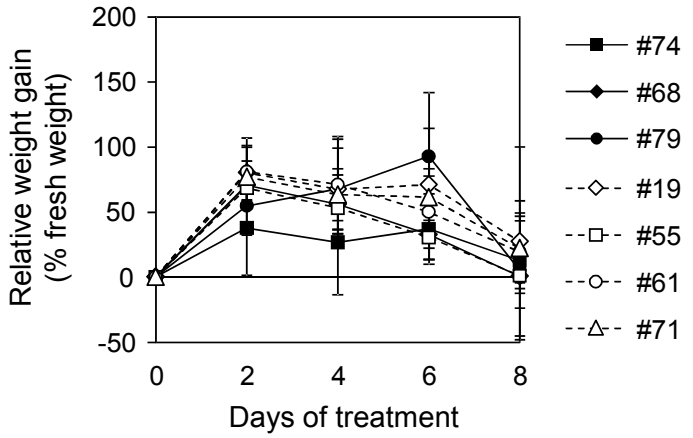

Figure VII-12. Salinity tolerance of the selected F_2-derived F_3 plants. Weight gain was determined in 7-day-old seedlings grown in 0.2% Hyponex solution containing NaCl. The concentration of NaCl was increased 25 mM per day to finally keep 125 mM. The percentage of weight gain of individuals carrying low ABA-responsive allele is indicated by filled symbols and solid lines, and those carrying highly responsive allele by open symbols and broken lines. Data are means ± SD from 10–12 individuals.

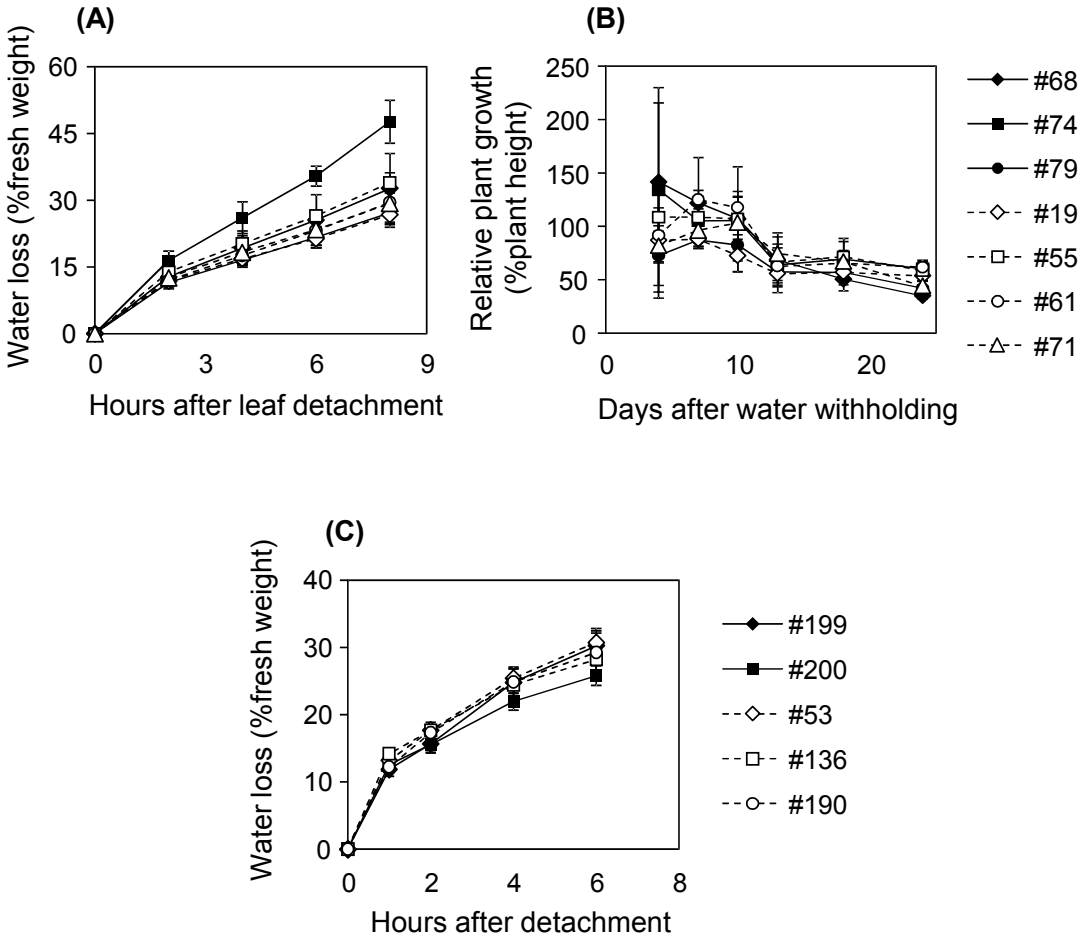

Figure VII-13. Dehydration tolerance of the selected F_2-derived F_3 plants and RILs. Water loss from detached leaves in synthetic wheat lines (A) and RILs derived from CS and M808 (C). The first leaf of 7-day-old seedlings (n = 10-12) was detached and its fresh weight was measured at the indicated times. (B) Growth rate of drought stressed plants. Irrigation of 5-day-old seedlings (n = 10) was withheld to finally keep soil moisture of ~25%. The growth rate was evaluated based on the plant height relative to control group. Mean ± SD of individuals carrying low ABA-responsive allele is indicated by filled symbols and solid line, and those carrying highly responsive allele by open symbols and broken lines.

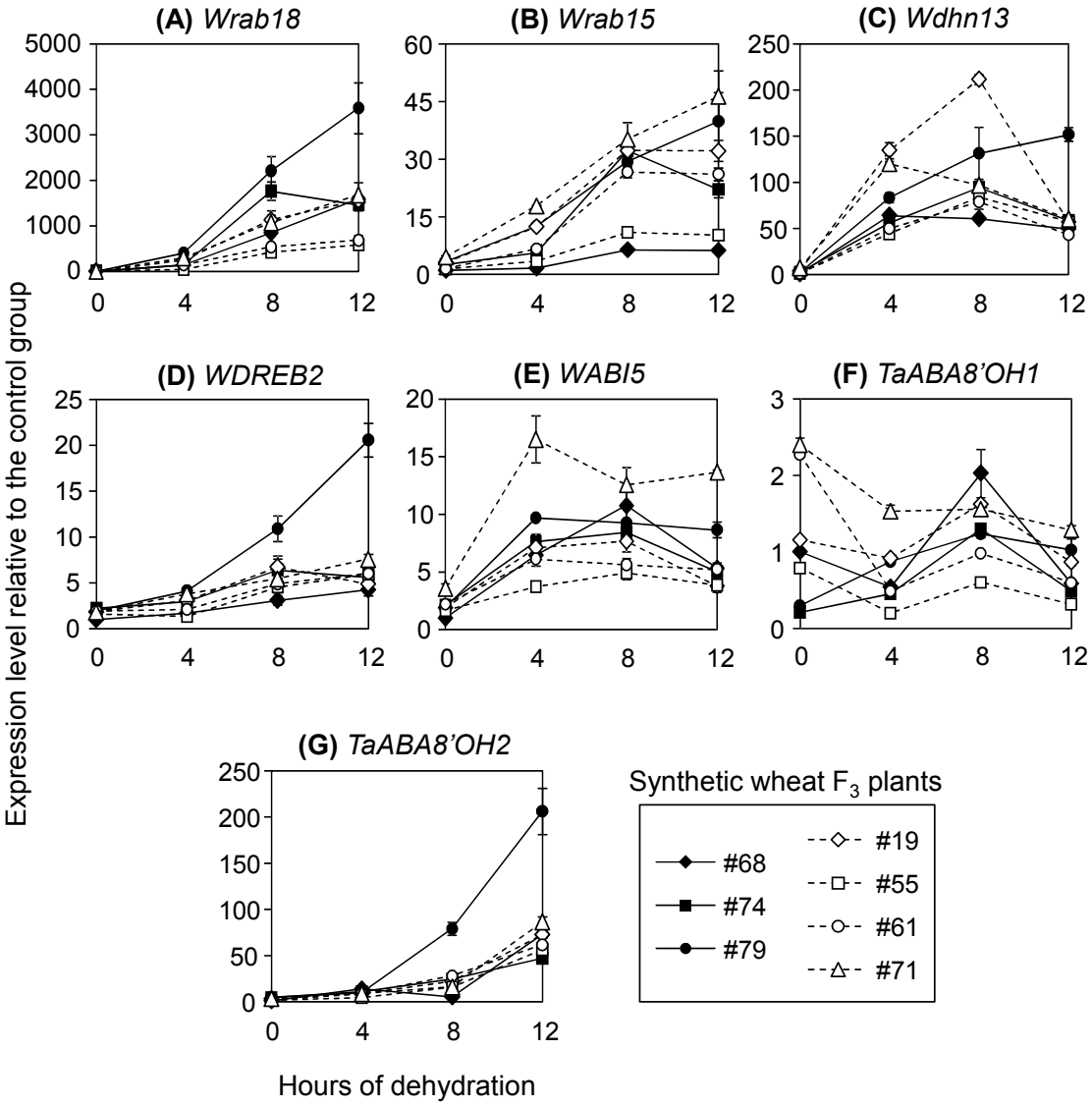

Figure VII-14. Gene expression analysis of ABA-inducible genes and ABA catabolic genes during dehydration in synthetic wheat lines. Transcript accumulations of *Cor/Lea* (A–C), transcription factor (D and E) and ABA 8'-hydroxylase (F and G) genes were analyzed by real time RT-PCR. Total RNA was extracted at indicated times after dehydration treatment from leaves. Transcript accumulation of individuals carrying low and highly ABA-responsive alleles are indicated by solid and broken lines, respectively. *Actin* was used as internal control. Data are mean ± SD relative to individual #68 at 0 h.

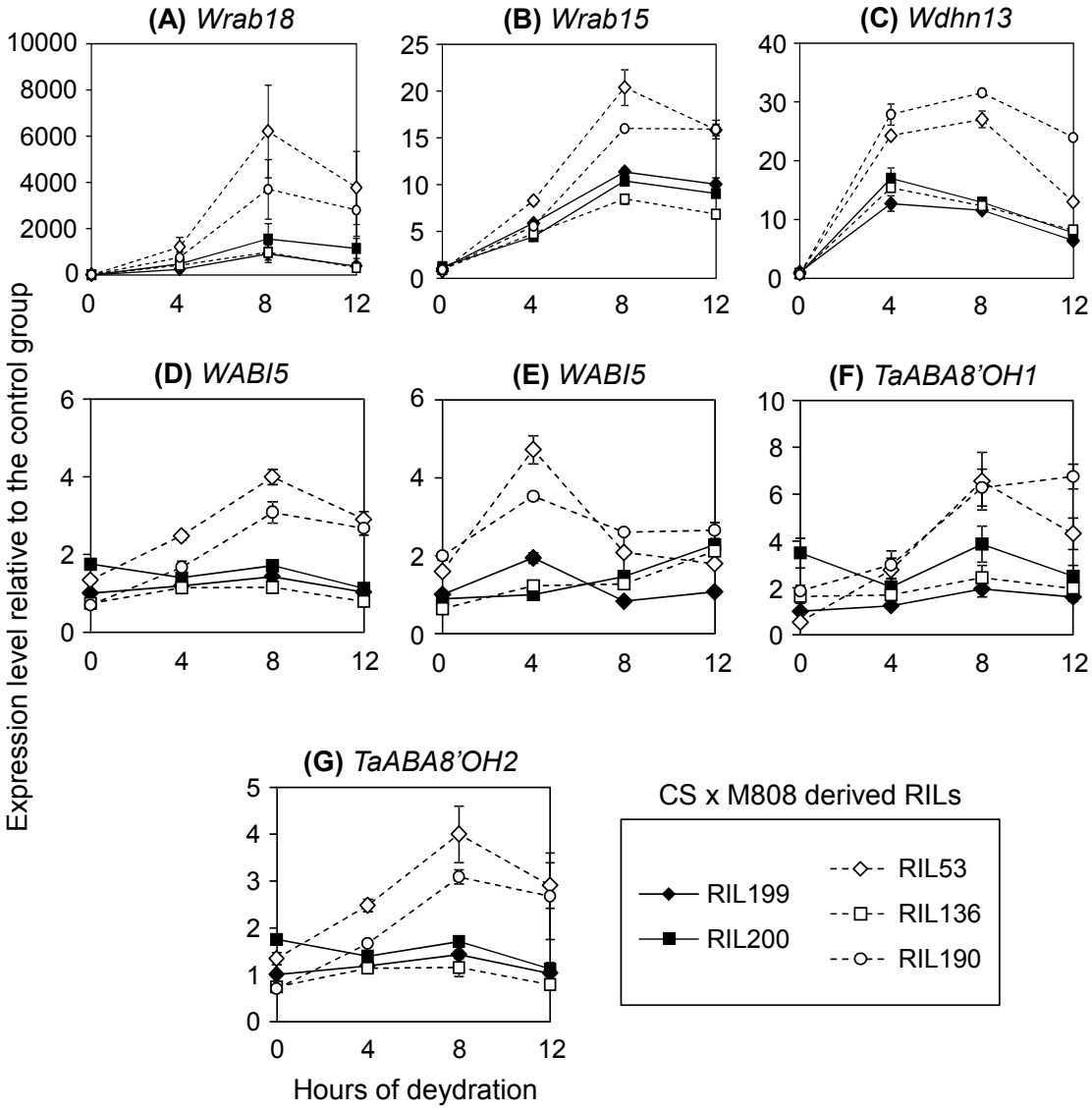

Figure VII-15. Gene expression analysis of ABA-inducible genes and ABA catabolic genes during dehydration in CS x M808 derived RILs. Transcript accumulations of *Cor/Lea* (A–C), transcription factor (D and E) and ABA 8'-hydroxylase (F and G) genes were analyzed by real time RT-PCR. Total RNA was extracted at indicated times after dehydration treatment from leaves. Transcript accumulation of individuals carrying low and highly ABA-responsive alleles are indicated by solid and broken lines, respectively. *Actin* was used as internal control. Data are mean ± SD relative to RIL199 at 0 h.

most of the seeds were germinated at 60 and 70 DPA (Figure VII-16C and VII-16D). There was no significant difference in GI between the two alleles (Figure VII-16E), suggesting that the 6D QTL of synthetic wheat has little or no effect on seed dormancy.

Seed dormancy of the highly ABA-responsive RILs (RIL136 and RIL190) was compared with that of CS. Low germination rate was observed in seeds harvested at 35 and 45 DPA (Figure VII-17A and VII-7B). At 55 DPA, seeds of CS started to germinate 2 days after imbibition and completely germinated after 5 days of imbibition (Figure VII-17C). In contrast, RILs with M808-type 6D QTL showed low germination rates. GI of the RILs with highly ABA-responsive allele was significantly lower ($P < 0.001$) than that of CS at 55 and 65 DPA (Figure VII-17E), indicating that this QTL has an effect on seed dormancy.

5. Discussion

In our previous study, a wide variation in ABA responsiveness was found in the diploid *Ae. tauschii* accessions and their derived synthetic hexaploids (Chapter VI). To identify the genetic factor affecting the difference in ABA responsiveness among synthetic wheat lines, QTL analysis was performed using F_2 plants derived from Ldn/IG126387 (a low ABA-responsive line) and Ldn/KU-2159 (a highly responsive line). ABA responsiveness at the seedling stage has been evaluated based on RRGI because the cultivar difference is clearer in this parameter than RSGI (Kobayashi et al. 2010; Chapter II; Chapter VI). In the present study, correlations among the six evaluated traits showed that RL-ABA is sufficient for evaluating the ABA responsiveness (Table VII-4). Although root growth in the absence of ABA differed significantly between the parental lines (Table VII-3), root growth inhibition by ABA was not affected by RL. However, RL was reflected on RRGI. QTL analysis of the six traits identified two QTLs for RRGI, on chromosomes 4D and 6D, but only one for RL-ABA on chromosome 6D (Table VII-5). The 4D QTL, in which highly responsive allele was derived from the low responsive parent, might be detected due to the difference in RL. Therefore, QTL for ABA responsiveness was defined as the chromosomal region where QTL for RL-ABA and RRGI overlapped (Figure VII-2).

The 6D QTL identified in synthetic wheat was located in a chromosomal region similar to that reported in common wheat (Kobayashi et al. 2010). Comparative map suggested that these two QTLs are different loci (Figure VII-4). Moreover, during post-germination root growth arrest by ABA, expression pattern of genes involved in cell-cycle regulation was slightly different between synthetic wheat and common wheat. However, *TaABA8'OH2* expression seems to be regulated by a common signaling

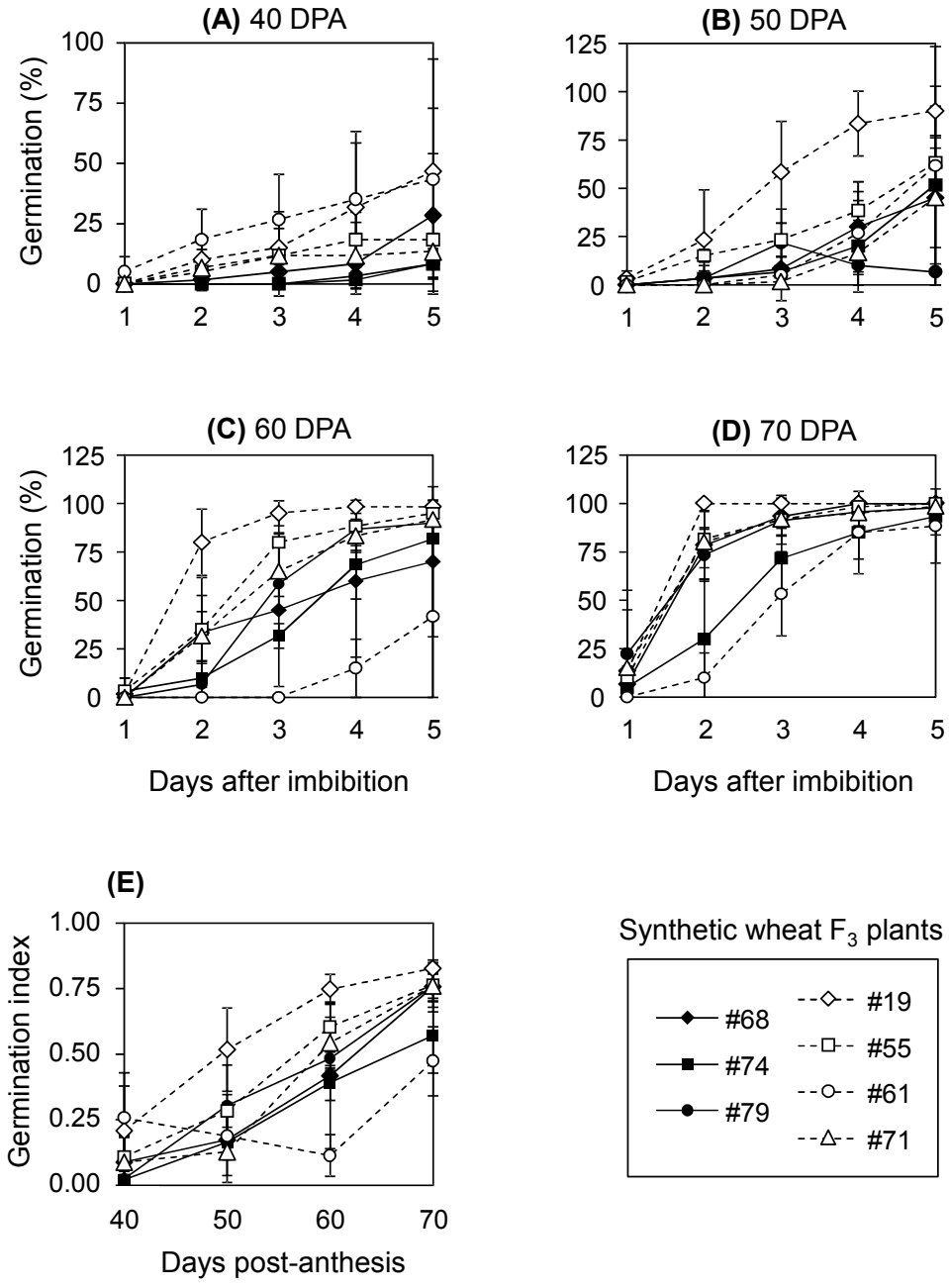

Figure VII-16. Analysis of seed dormancy using the selected F_2-derived F_3 synthetic wheats. Germination rate at 40 days post-anthesis (40 DPA, A), 50 DPA (B), 60 DPA (C) and 70 DPA (D). Based on these data, GI was also calculated (E). Data are mean ± SD of four independent experiments using fifteen freshly harvested seeds. Individuals carrying low and highly ABA-responsive alleles are indicated by solid and broken lines, respectively.

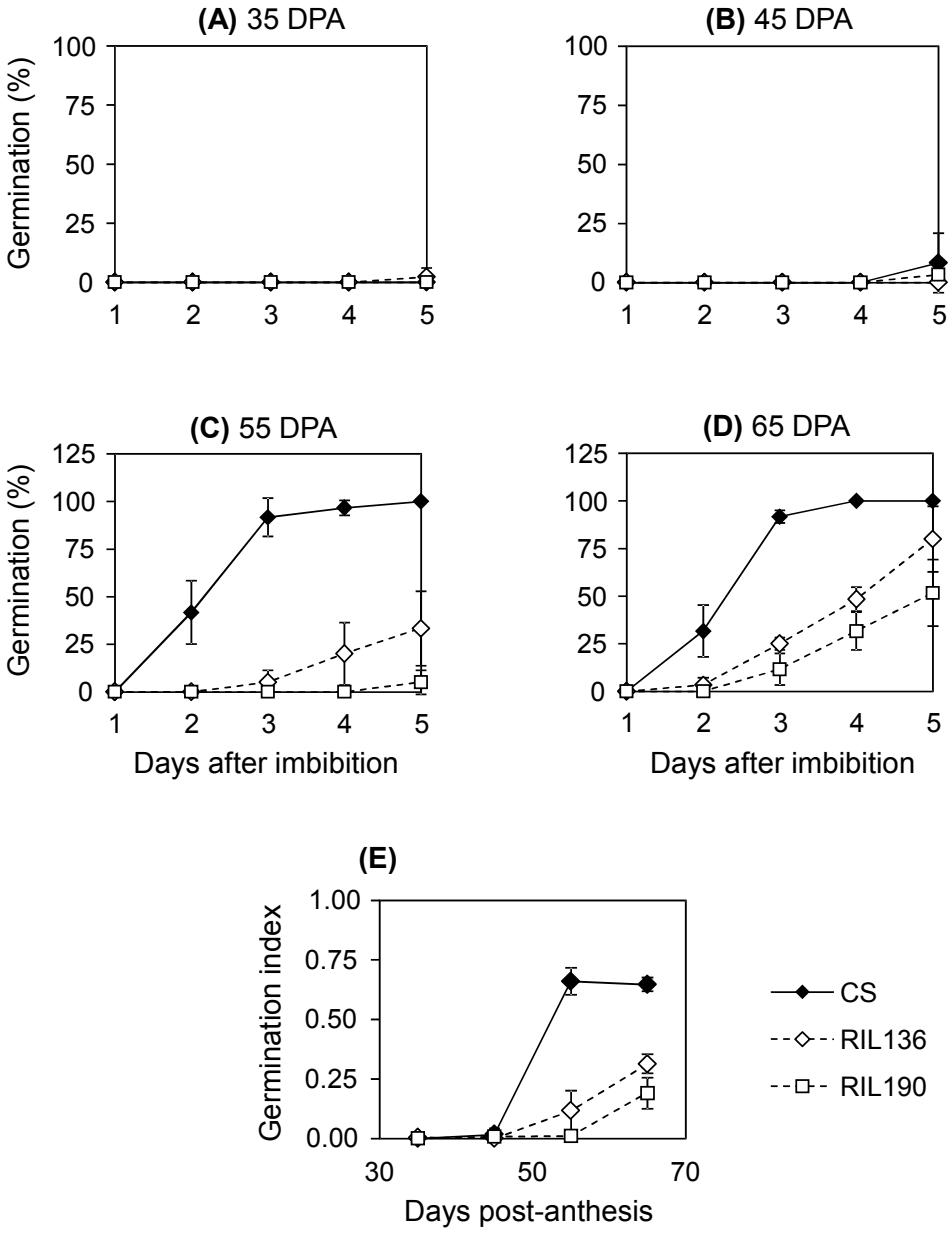

Figure VII-17. Analysis of seed dormancy using the selected RILs. Germination rate at 35 days post-anthesis (35 DPA, A), 45 DPA (B), 55 DPA (C) and 65 DPA (D). Based on these data, GI was also calculated (E). Data are mean ± SD of four independent experiments using fifteen freshly harvested seeds. Germination rate and GI of CS and RILs carrying M808-type 6D QTL are indicated by solid and broken lines, respectively.

pathway to both 6D QTLs (Figure VII-7). ABA content of 1-day-old seedlings, before ABA treatment, might also contribute in part in differential growth arrest (Figure VII-10).

In our previous report, the highly ABA-responsive synthetic Ldn/KU-2159 has been show to express higher levels of *Wdhn13* by ABA treatment and higher tolerance to dehydration and salinity stress compared to the low responsive Ldn/IG126387 (Chapter VI). However, functional analyses of the 6D QTL failed in the detection of association of ABA responsiveness with abiotic stress tolerance and seed dormancy, in the experimented conditions. One possibility is that this QTL has small effect on abiotic stress tolerance and/or seed dormancy. In addition, the use of F_3 individuals that have heterogeneous genetic background may difficult the analysis of the effects of allelic difference at specific QTL. Development of near isogenic lines (NILs) may resolve this problem, including the analysis of QTLs with small effects.

The 6D QTL for ABA responsiveness found in RIL population of CS and M808 has been located in a similar region to the QTL for pre-harvest sprouting and cold tolerance (Kobayashi et al. 2010). Functional analyses revealed an association with seed dormancy (Figure VII-17), indicating that this QTL for ABA responsiveness may correspond to that for pre-harvest sprouting. In this 6D chromosomal region, *TaABA8'OH1-D* was located (Figure VII-4) which has been reported to plays a role in seed germination (Chono et al. 2013). However, no difference in the expression levels (Figure VII-8F) and genomic sequence (Figure VII-11) was found suggesting that *TaABA8'OH1* is not the causal gene.

Although this QTL showed no effects on stomatal response under leaf dehydration (Figure VII-13C), transcript accumulations of ABA-inducible genes tended to be higher in individuals with highly responsive allele (Figure VII-15). Cultivar difference in the expression levels of *Cor/Lea* genes and transcription factors were associated with the difference in freezing tolerance (Tsuda et al. 2000; Egawa et al. 2006; Kobayashi et al. 2004, 2008c). Because COR/LEA proteins protect cellular components from abiotic stresses to tolerate them (Thomashow 1999), higher *Cor/Lea* expression in individuals with M808-type 6D QTL might contribute in dehydration tolerance. Considering that expression QTL (eQTL) analysis of *Cor/Lea* genes in the RIL population of CS and M808 did not detect any eQTLs on chromosome 6D (Motomura et al. 2013), the QTL for ABA responsiveness and that for cold tolerance (Båga et al. 2007) might be different loci. These results indicate that the 6D QTL for ABA responsiveness of common wheat affects seed dormancy and may contribute in dehydration tolerance. However, we cannot discard the possibility that multiple loci are present in the 6D QTL region

because of the detection of multiple LOD peaks (Kobayashi et al. 2010). Since drought tolerance has been found to be a complex quantitative trait controlled by a large number of genes or QTLs with small effects (reviewed in Mir et al. 2012), this QTL might improve the drought tolerance of wheat by pyramiding with other related genes/QTLs.

Chapter VIII

General discussion

Improvement of abiotic stress tolerance and seed dormancy in common wheat may be conducted studying the genetic factors that affect ABA responsiveness. Two different genetic resources were used: common wheat cultivars and synthetic hexaploid wheat lines.

In common wheat, the cultivar difference (between Cnn, Hope and CS) in ABA responsiveness was found to be commonly determined by specific chromosomes (3A, 5A and 6D). In contrast, the difference in ABA responsiveness between synthetic wheat lines might be determined by different set of genes present on D-genome. In both plant materials, higher ABA responsiveness was associated with higher dehydration tolerance and higher induction of *Cor/Lea* genes by ABA treatment. Therefore, highly ABA-responsive common wheat cultivars and synthetic hexaploid wheats might be used as genetic resources to improve the abiotic stress tolerance of common wheat.

ABA responsiveness at the seedling stage has been evaluated based on RRGI because the cultivar difference is clearer in this trait than RSGI. In the present study, correlations among the six evaluated traits in common wheat and synthetic wheat F_2 populations showed that root growth in absence of ABA is reflected on RRGI but not in RL-ABA. Therefore, RL-ABA is sufficient for evaluating the ABA responsiveness in these mapping populations.

For functional analyses of QTLs, F_2-derived F_3 individuals were selected based on the genotypes of SSR markers at the 5A (for common wheat mapping population) and 6D (for synthetic wheat) QTL region. RILs carrying CS-type and M808-type 6D QTL were also included. These three QTLs seem to regulate the transcript accumulation of the genes involved in cell-cycle control through different ABA signaling pathways. However, the observation that the expression level of *TaABA8'OH2* is higher in roots of individuals with highly-responsive allele seems to be common effect of all the QTLs for ABA responsiveness.

The 5A QTL for ABA responsiveness affected the stomatal response to leaf dehydration and transcript accumulation of *Cor/Lea* genes during dehydration, but not the salinity tolerance and seed dormancy. On the other hand, the previously identified 6D QTL of common wheat (Kobayashi et al. 2010) was found to be associated with seed dormancy and regulation of *Cor/Lea* expression during dehydration treatment. Because COR/LEA proteins protect cellular components from abiotic stresses, the

higher accumulation of their transcripts might contribute in drought tolerance. Drought tolerance has been found to be a complex quantitative trait controlled by a large number of genes or QTLs with small effects (Mir et al. 2012). These results suggest that the QTLs for ABA responsiveness of common wheat have a small effect on dehydration tolerance, and might contribute in the improvement of wheat drought tolerance by pyramiding with other genes/QTLs. Pyramiding of multiple QTLs by the conventional marker assisted selection is time-consuming. Thus, efficient modern breeding approaches such as marker-assisted recurrent selection or genomic selection should be used (Mir et al. 2012).

For 6D QTL of synthetic wheat, we failed in the detection of association of ABA responsiveness with abiotic stress tolerance and seed dormancy, in the experimented conditions. One possibility is that this QTL has small effect on abiotic stress tolerance and/or seed dormancy. In addition, the use of F_3 individuals that have heterogeneous genetic background may difficult the analysis of the effects of allelic difference at specific QTL. In fact, functional analyses were successfully performed in RILs and F_3 individuals derived from CS and its chromosome substitution line. In this latter, genetic background is more homogeneous, similar to NILs. Therefore, development of NILs is important for characterization of the 6D QTL for ABA responsiveness of synthetic wheat.

High-resolution genetic map construction is important for genetic and genomic research as well as for molecular breeding (Yano 2001). In organisms with large and complex genomes, such as wheat, RNA-seq analysis is cost effective for discovery of genome-wide SNPs. Sequencing of the leaf and spike transcriptome from two *Ae. tauschii* accessions representing two major lineages led to discovery of 732 HC polymorphic contigs on each D-genome chromosome. SNP markers developed from polymorphisms between the two distinct lineages of *Ae. tauschii* were found to be useful for analysis not only of the diploid but also of the hexaploid wheat genome, including common wheat. Thus, these SNPs might help to exploit the wide natural variation found in *Ae. tauschii*, especially in the L1 accessions.

Chapter IX

Summary

The phytohormone abscisic acid (ABA) plays an important role in responses to environmental stress and dormancy. Abiotic stresses seriously affect plant growth and reduce yield in crop species. Therefore, the study of ABA responsiveness in common wheat might lead in an improvement of abiotic stress tolerance and/or seed dormancy. Common wheat is an allohexaploid species originated by natural hybridization between tetraploid wheat and the diploid *Aegilops tauschii*. It is supposed that only a small population of *Ae. tauschii* was involved in the origin of common wheat. Because tetraploid wheat can be crossed with *Ae. tauschii* to obtain synthetic hexaploid wheat, it is possible to exploit the wide natural variation of *Ae. tauschii* in common wheat improvement. In this study, two different plant material sets, common wheat cultivars and synthetic hexaploid wheat lines, were used to identify the genetic factors determining the difference in ABA responsiveness among hexaploid wheat accessions.

Comparison of responsiveness to exogenous ABA, based on root growth inhibition by ABA, revealed that common wheat cultivars Cheyenne (Cnn) and Hope are highly responsive than seedlings of Chinese Spring (CS). The chromosomes involved in the regulation of ABA responsiveness and *Cor* (*cold-responsive*)*/Lea* (*late embryogenesis abundant*) expression were identified using chromosome substitution lines, in which a chromosome pair of CS was substituted for the corresponding homologous pair of Cnn or Hope. In both CS-Cnn and CS-Hope substitution lines, chromosomes 3A and 5A were involved in the regulation of ABA responsiveness, *Cor/Lea* gene expression and stomatal response during leaf dehydration.

In common wheat, quantitative trait locus (QTL) for ABA responsiveness at the seedling stage has been reported on chromosomes 1B, 2A, 3A, 6D and 7B. Using a F_2 population derived from a cross between common wheat cultivar CS and CS-Hope5A, a novel QTL for ABA responsiveness on chromosome 5A was identified. Functional analyses of the identified QTL found that stomatal response during leaf dehydration and transcript accumulation levels of the *Cor/Lea* genes *Wrab18* and *Wdhn13* tended to be higher in individuals carrying Hope allele, which showed higher ABA responsiveness. These results suggest that the 5A QTL for ABA responsiveness has a small effect on dehydration tolerance.

ABA responsiveness of 67 *Ae. tauschii* accessions and their synthetic hexaploid wheat lines was evaluated. The wide variations in ABA responsiveness due to

D-genome found at the diploid level were also expressed in a hexaploid genetic background. Two pairs of synthetic wheat lines differing in ABA responsiveness were selected for gene expression analysis and to test abiotic stress tolerance. In one pair, the highly responsive line exhibited higher induction of *Wrab17* by ABA treatment, but no significant difference in dehydration or salinity tolerance was observed. In contrast, in the second pair, the highly ABA-responsive line showed higher levels of *Wdhn13* expression and dehydration and salinity tolerance. Therefore, highly ABA-responsive common wheat cultivars and synthetic hexaploid wheats might be used as genetic resources to improve the abiotic stress tolerance of common wheat.

QTL analysis for ABA responsiveness was performed using F_2 population derived from the highly ABA-responsive synthetic wheat Ldn/KU-2159 and the low responsive Ldn/IG126387. Significant QTL was detected on chromosome 6D, located in a similar region to the previously reported common wheat 6D QTL for ABA responsiveness. Functional analyses of the synthetic wheat and common wheat 6D QTL were performed. In common wheat, individuals carrying highly-responsive parental allele exhibited higher seed dormancy and transcript accumulation of ABA-inducible genes during dehydration. However, in synthetic wheat, association of ABA responsiveness with abiotic stress tolerance and seed dormancy could not be detected in the experimented conditions, possibly due to the heterogeneous genetic background of F_2 individuals.

Drought tolerance has been found to be a complex trait controlled by a large number of genes/QTLs with small effects. These results suggest that the identified QTLs for ABA responsiveness have a small effect on dehydration tolerance, and might contribute in the improvement of wheat drought tolerance by pyramiding with other genes/QTLs. These results also demonstrated that the study of ABA responsiveness in wheat is useful for discovery of genes or QTLs involved in abiotic stress tolerance and/or seed dormancy.

Development of molecular markers are crucial for molecular breeding. In plant species having large and complex genomes and insufficient reference information, RNA-seq is cost effective for single nucleotide polymorphism (SNP) discovery. Deep sequencing of the leaf and spike transcriptomes from two *Ae. tauschii* accessions representing two major lineages led to discovery of 16,148 high-confidence (HC) SNPs in 5,808 contigs. On average, 732 HC polymorphic contigs were mapped *in silico* to each *Ae. tauschii* chromosome. The results indicated that molecular markers developed from polymorphisms between two distinct lineages of *Ae. tauschii* are useful for analysis not only of the diploid but also of the hexaploid wheat genome.

Acknowledgement

I would like to express my deepest gratitude to Associate Professor Dr. S. Takumi for his continuous guidance and encouragement throughout this work.

I wish to express my acknowledge to Associate Professor Dr. C. Miyake, Professor Dr. T. Sugimoto and Professor Dr. Y. Tosa for their kind review of this manuscript and for valuable comments and suggestions.

I am also very grateful to Dr. K. Sato, Dr. A. Shimizu and Dr. S. Nasuda for sequencing and analysis of leaf and spike transcriptome, and their kind supports and suggestions.

I want to thank Dr. T. Matsuura and Dr. I. C. Mori for the measurement of phytohormones in wheat grains.

I thank to Dr. N. Mizuno, Dr. R. Ohno, Mr. Y. Kurahashi and Mr. Y. Kuki for their kind technical supports and suggestions. I am also thankful to Mr. R. Matsuda, Mr. R. Nishijima and Mr. K. Sakaguchi for supports in genetic map construction and to Mr. H. Yokota for technical supports.

I am also very thankful for Dr. F. Kobayashi for his constructive comments and advices.

I wish to express my gratitude to Professor Dr. C. Nakamura for give me the opportunity to study at Kobe University.

I would like to thank to Ministry of Education, Culture, Sports, Science and Technology of Japan for a scholarship that made it possible to study in Japan.

References

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J Biol Chem* 281: 37636-37645

Akhunov E, Nicolet C, Dvorak J (2009) Single nucleotide polymorphism genotyping in polyploid wheat with the Illumina GoldenGate assay. *Theor Appl Genet* 119: 507-517

Allen AM, Barker GLA, Berry ST, Coghill JA, Gwilliam R, Kirby S, Robinson P, Brenchley RC, D'Amore R, McKenzie N, Waite D, Hall A, Bevan M, Hall N, Edwards KJ (2011) Transcript-specific, single-nucleotide polymorphism discovery and linkage analysis in hexaploid bread wheat (*Triticum aestivum* L.). *Plant Biotechnol J* 9: 1086-1099

Allen AM, Barker GLA, Wilkinson P, Burridge A, Winfield M, Coghill J, Uauy C, Griffiths S, Jack P, Berry S, Werner P, Melichar JPE, McDougall J, Gwilliam R, Robinson P, Edwards KJ (2013) Discovery and development of exome-based, co-dominant single nucleotide polymorphism markers in hexaploid wheat (*Triticum aestivum* L.). *Plant Biotechnol J* 11: 279-295

Alonso-Blanco C, Aarts MGM, Bentsink L, Keurentjes JJB, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? *Plant Cell* 21: 1877-1896

Badawi M, Danyluk J, Boucho B, Houde M, Sarhan F (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBFs*. *Mol Genet Genomics* 277: 533-554

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat *ICE* (Inducer of CBF Expression) genes. *Plant Cell Physiol* 49: 1237-1249

Båga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB, Chibbar RN (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. *Funct Integr Genomics* 7: 53-68

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5'-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol Biol* 24: 701-713

Barker GL, Edwards KJ (2009) A genome-wide analysis of single nucleotide polymorphism diversity in the world's major cereal crops. *Plant Biotechnol J* 7: 318-325

Benech-Arnold RL, Gualano N, Leymarie J, Côme D, Corbineau F (2006) Hypoxia interferes with ABA metabolism and increases ABA sensitivity in embryos of dormant barley grains. *J Exp Bot* 57: 1423-1430

Berkman PJ, Skarshewski A, Lorenc MT, Lai K, Duran C, Ling EYS, Stiller J, Smits L, Imelfort M,

Manoli S, McKenzie M, Kubalakova M, Simkova H, Batley J, Fleury D, Dolezel J, Edwards D (2011) Sequencing and assembly of low copy and genic regions of isolated *Triticum aestivum* chromosome arm 7DS. *Plant Biotechnol J* 9: 768-775

Berkman PJ, Skarshewski A, Manoli S, Lorenc MT, Stiller J, Smits L, Lai K, Campbell E, Kubalakova M, Simkova H, Batley J, Dolezel J, Hernandez P, Edwards D (2012) Sequencing wheat chromosome arm 7BS delimits the 7BS/4AL translocation and reveals homoeologous gene conservation. *Theor Appl Genet* 124: 423-432

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proc Natl Acad Sci USA* 103: 17042-17047

Caldwell KS, Dvorak J, Lagudah ES, Akhunov E, Luo MC, Wolters P, Powell W (2004) Sequence polymorphism in polyploid wheat and their D-genome diploid ancestor. *Genetics* 167: 941-947

Carollo V, Matthews DE, Lazo GR, Blake TK, Hummel DD, Lui N, Hane DL, Anderson OD (2005) GrainGenes 2.0. An improved resource for the small-grains community. *Plant Physiol* 139: 643-651

Casao MC, Igartua E, Karsai I, Bhat PR, Cuadrado N, Gracia MP, Lasa JM, Casas AM (2011) Introgression of an intermediate *VRNH1* allele in barley (*Hordeum vulgare* L.) leads to reduced vernalization requirement without affecting freezing tolerance. *Mol Breed* 28: 475-484

Cattivelli L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM, Pecchiolini N, Stanca AM (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. *Plant Mol Biol* 48: 649-665

Cavanagh CR, Chao S, Wang S, Huang BE, Stephen S, Kiani S, Forrest K, Saintenacc C, Brown-Guedira GL, Akhunova A, See D, Bai G, Pumphrey M, Tomar L, Wong D, Kong S, Reynolds M, Lopez da Silva M, Bockelman H, Talbert L, Anderson JA, Dreisigacker S, Baenziger S, Carter A, Korzun V, Morrell PL, Dubcovsky J, Morell MK, Sorrells ME, Hayden MJ, Akhunov E (2013) Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars. *Proc Natl Acad Sci USA* 110: 8057-8062

Chague V, Just J, Mestiri I, Balzergue S, Tanguy AM, Huneau C, Huteau V, Belcram H, Coriton O, Jahier J, Chalhoub B (2010) Genome-wide gene expression changes in genetically stable synthetic and natural wheat allohexaploids. *New Phytol* 187: 1181-1194

Chao S, Zhang W, Akhunov E, Sherman J, Ma Y, Luo MC, Dubcovsky J (2009) Analysis of gene-derived SNP marker polymorphism in US wheat (*Triticum aestivum* L.) cultivars. *Mol Breed* 23: 23-33

Chapman V, Miller TE, Riley R (1976) Equivalence of the A genome of bread wheat with that of *Triticum urartu*. *Genet Res* 27: 69-76

Chen ZJ (2010a) Molecular mechanisms of polyploidy and hybrid vigor. *Trends Plant Sci* 15: 57-71

Chen X, Hackett CA, Niks RE, Hedley PE, Booth C, Druka A, Marcel TC, Vels A, Bayer M, Milne I et al. (2010b) An eQTL analysis of partial resistance to *Puccinia hordei* in barley. *PLoS ONE* 5: e8598

Chen X, Niks RE, Hedley PE, Morris J, Druka A, Marcel TC, Vels A, Waugh R (2010c) Differential gene

expression in nearly isogenic lines with QTL for partial resistance to *Puccinia hordei* in barley. *BMC Genomics* 11: 629

Chinnusamy V, Ohta M, Kanrar S, Lee B, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 17: 1043-1054

Chono M, Matsunaka H, Seki M, Fujita M, Kiribuchi-Otobe C, Oda S, Kojima H, Kobayashi D, Kawakami N (2013) Isolation of a wheat (*Triticum aestivum* L.) mutant in ABA 8'-hydroxylase gene: effect of reduced ABA metabolism on germination inhibition under field condition. *Breed Sci* 63: 104-115

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. *BMC Genomics* 10: 582

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21: 3674-3676

Del Pozo JC, Lopez-Matas MA, Ramirez-Parra E, Gutierrez C (2005) Hormonal control of the plant cell cycle. *Physiol Plant* 123: 173-183

Delker C, Quint M (2011) Expression level polymorphisms: heritable traits shaping natural variation. *Trend Plant Sci* 16: 481-488

Deschamps S, Campbell MA (2010) Utilization of next-generation sequencing platforms in plant genomics and genetic variation discovery. *Mol Breed* 25: 553-570

Deschamps S, la Rota M, Ratashak JP, Biddle P, Thureen D, Farmer A, Luck S, Beatty M, Nagasawa N, Michael L, Llaca V, Sakai H, May G, Lightner J, Campbell MA (2010) Rapid genome-wide single nucleotide polymorphism discovery in soybean and rice via deep resequencing of reduced representation libraries with the Illumina Genome Analyzer. *Plant Genome* 3: 53-68

Dhillon T, Pearce SP, Stockinger EJ, Distelfeld A, Li C, Knox AK, Vashegyi I, Vágújfalvi A, Galiba G, Dubcovsky J (2010) Regulation of freezing tolerance and flowering in temperate cereals: the *VRN-1* connection. *Plant Physiol* 153: 1846-1858

Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* 21: 972-984

Dubcovsky J, Luo MC, Dvorak J (1995) Linkage relationships among stress-induced genes in wheat. *Theor Appl Genet* 91:795-801

Dudnikov AJ, Goncharov NP (1993) Allozyme variation in *Aegilops squarrosa*. *Hereditas* 119: 117-122

Dudnikov AJ, Kawahara T (2006) *Aegilops tauschii*: genetic variation in Iran. *Genet Resour Crop Evol* 53: 579-586

Dvorak J, Zhang HB (1990) Variation in repeated nucleotide sequences sheds light on the phylogeny of

the wheat B and G genomes. *Proc Natl Acad Sci USA* 87:9640-9644

Dvorak J, Terlizzi PD, Zhang HB, Resta P (1993) The evolution of polyploidy wheats: identification of the A genome donor species. *Genome* 36: 21-31

Dvorak J, Luo MC, Yang ZL, Zhang HB (1998) The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. *Theor Appl Genet* 97: 657-670

Egawa C, Kobayashi F, Ishibashi M, Nakamura T, Nakamura C, Takumi S (2006) Differential regulation of transcript accumulation and alternative splicing of a *DREB2* homolog under abiotic stress conditions in common wheat. *Genes Genet Syst* 81: 77-91

Eig A. Monographisch-kritische Übersicht der Gatteung *Aegilops*. *Repertorium Specierum Novarum Rgni Vegetabilis. Beihefte* 1929, *55*, 1-228

Ewen-Campen B, Shaner N, Panfilio KA, Suzuki Y, Roth S, Extavour CG (2011) The maternal and early embryonic transcriptome of the milkweed bug *Oncopeltus fasciatus*. BMC Genomics 12: 61

FAOstat (2008) Food and Agriculture Organization of the United Nations [WWW document]. URL http://faostat.fao.org (accessed on 12 March 2010)

Feldman M (2001) Origin of cultivated wheat. In:*The World Wheat Book: A History of Wheat Breeding* (eds Bonjean AP, Angus WJ). pp. 3-53, Lavoisier Publishing, rue Lavoisier, Paris

Filiault DL, Wessinger CA, Dinneny JR, Lutes J, Borevitz JO, Weigel D, Chory J, Maloof JN (2008) Amino acid polymorphisms in Arabidopsis phytochrome B cause differential responses to light. *Proc Natl Acad Sci USA* 105: 3157-3162

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell* 14 (Suppl): S15-S45

Fowler DB, Gusta LV (1979) Selection for winterhardiness in wheat. I. Identification of genotypic variability. *Crop Sci* 19: 769-772

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK (2009) *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature* 462: 660-664

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci USA* 103: 1988-1993

Fursova OV, Pogorelko GV, Tarasov VA (2009) Identification of *ICE2*, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. *Gene* 429: 98-103

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. *Theor Appl Genet* 90: 1174-1179

Galiba G, Vágújfalvi A, Li C, Soltész A, Dubcovsky J (2009) Regulatory genes involved in the determination of frost tolerance in temperate cereals. *Plant Sci* 176: 12-19

Gendreau E, Cayla T, Corbineau F (2012) S phase of the cell cycle: a key phase for the regulation of

thermodormancy in barley grain. *J Exp Bot* 63: 5535-5543

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *Plant J* 16: 433-442

Groos C, Gay G, Perretant MR, Gervais L, Bernard M, Dedryver F, Charmet G (2002) Study of the relationship between pre-harvest sprouting and grain color by quantitative trait loci analysis in a white×red grain bread-wheat cross. *Theor Appl Genet* 104: 39-47

Gubler F, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr Opin Plant Biol* 8: 183-187

Hammer K (1980) Vorarbeiten zur monographischen Darstellung von Wildpflanzensortimenten: *Aegilops* L. *Kulturpflanze* 28: 33-180

Hansey CN, Vaillancourt B, Sekhon RS, de Leon N, Kaeppler SM, Buell CR (2012) Maize (*Zea mays* L.) genome diversity as revealed by RNA-sequencing. *PLoS ONE* 7: e33071

Hatano H, Mizuno N, Matsuda R, Shitsukawa N, Park P, Takumi S (2012) Dysfunction of mitotic cell division at shoot apices triggered severe growth abortion in interspecific hybrids between tetraploid wheat and *Aegilops tauschii*. *New Phytol* 194: 1143-1154

Hernandez P, Martis M, Dorado G, Pfeifer M, Galvez S, Schaaf S, Jouve N, Simkova H, Valarik M, Dolezel J, Mayer KFX (2012) Next-generation sequencing and syntenic integration of flow-sorted arms of wheat chromosome 4A exposes the chromosome structure and gene content. *Plant J* 69: 377-386

Hobo T, Asada M, Kowyama Y, Hattori T (1999) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. *Plant J* 19: 676-689

Hori K, Kobayashi T, Shimizu A, Sato K, Takeda K, Kawasaki S (2003) Efficient construction of high-density linkage map and its application to QTL analysis in barley. *Theor Appl Genet* 107: 806-813

Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. *Genome Res* 9: 868-877

Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo ZF (2012) Signal transduction during cold, salt, and drought stresses in plants. *Mol Biol Rep* 39: 969-987

International Barley Genome Sequencing Consortium et al. (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491:711-716

Jackson S, Chen ZJ (2010) Genomic and expression plasticity of polyploidy. *Curr Opin Plant Biol* 13: 153-159

Ji X, Dong B, Shiran B, Talbot MJ, Edlington JE, Hughes T, White RG, Gubler F, Dolferus R (2011) Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant Physiol* 156: 647-662

Jia J, Shancen Zhao S, Kong X, Li Y, Zhao G, He W, Appels R, Pfeifer M, Tao Y, Zhang X, Jing R, Zhang

C, Ma Y, Gao L, Gao C, Spannagl M, Mayer KFX, Dong Li D, Pan S, Fengya Zheng F, Hu Q, Xia X, Li J, Liang Q, Chen J, Wicker T, Gou C, Kuang H, He G, Luo Y, Keller B, Xia Q, Lu P, Wang J, Zou H, Zhang R, Gao J, Middleton C, Quan Z, Liu G, Wang J, IWGSC, Yang H, Xu Liu X, He Z, Mao L, Wang J (2013) *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496:91-95

Jiang C, Iu B, Singh J (1996) Requirement of a CCGAC *cis*-acting element for cold induction of the *BN115* gene from winter *Brassica napus*. *Plant Mol Biol* 30: 679-684

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C (2000) Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. *Science* 290: 344-347

Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK (2002) The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant Physiol* 130: 837-846

Jones H, Gosman N, Horsnell R, Rose GA, Everest LA, Bentley AR, Tha S, Uauy C, Kowalski A, Novoselovic D, Simek R, Kobiljski B, Kondic-Spika A, Brbaklic K, Mitrofanova O, Chesnokov Y, Bonnett D, Greenland A (2013) Strategy for exploiting exotic germplasm using genetic, morphological, and environmental diversity: the *Aegilops tauschii* Coss. example. *Theor Appl Genet* 126: 1793-1808

Jordan MC, Somers DJ, Banks TW (2007) Identifying regions of the wheat genome controlling seed development by mapping expression quantitative trait loci. *Plant Biotech J* 5: 442-453

Kajimura T, Murai K, Takumi S (2011) Distinct genetic regulation of flowering time and grain-filling period based on empirical study of D-genome diversity in synthetic hexaploid wheat lines. *Breed Sci* 61: 130-141

Kaur S, Francki MG, Forster JW (2012) Identification, characterization and interpretation of single-nucleotide sequence variation in allopolyploid crop species. *Plant Biotechnol J* 10: 125-138

Kihara H (1944) Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. *Agriculture and Horticulture* 19: 889-890 (In Japanese)

Kihara H, Lilienfeld F (1949) A new-synthesized 6x-wheat. *Hereditas* 35 (Suppl): 307-319

Kim ED, Chen ZJ (2011) Unstable transcripts in Arabidopsis allotetraploids are associated with nonadditive gene expression in response to abiotic and biotic stresses. *PLoS ONE* 6: e24251

Klingler JP, Batelli G, Zhu JKK (2010) ABA receptors: the START of a new paradigm in phytohormone signalling. *J Exp Bot* 61: 3199-3210

Kobayashi F, Takumi S, Nakata M, Ohno R, Nakamura T, Nakamura C (2004) Comparative study of the expression profiles of the *Cor/Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. *Physiol Plantarum* 120: 585-594

Kobayashi F, Takumi S, Egawa C, Ishibashi M, Nakamura C (2006) Expression patterns of low temperature responsive genes in a dominant ABA-less-sensitive mutant line of common wheat.

Physiol Plantarum 127: 612-623

Kobayashi F, Ishibashi M, Takumi S (2008a) Transcriptional activation of *Cor/Lea* genes and increase in abiotic stress tolerance through expression of a wheat *DREB2* homolog in transgenic tobacco. *Transgenic Res* 17: 755-767

Kobayashi F, Maeta E, Terashima A, Kawaura K, Ogihara Y, Takumi S (2008b) Development of abiotic stress tolerance via bZIP-type transcription factor LIP19 in common wheat. *J Exp Bot* 59: 891-905

Kobayashi F, Maeta E, Terashima A, Takumi S (2008c) Positive role of a wheat *HvABI5* ortholog in abiotic stress response of seedlings. *Physiol Plantarum* 134: 74-86

Kobayashi F, Takumi S, Nakamura C (2008d) Increased freezing tolerance in an ABA-hypersensitive mutant of common wheat. *J Plant Physiol* 165: 224-232

Kobayashi F, Takumi S, Handa H (2010) Identification of quantitative trait loci for ABA responsiveness at the seedling stage associated with ABA-regulated gene expression in common wheat. *Theor Appl Genet* 121: 629-641

Kofler R, Schlötterer C, Lelley T (2007) SciRoKo: A new tool for whole genome microsatellite search and investigation. *Bioinformatics* 23: 1683-1685

Komori T, Nitta N (2005) Utilization of the CAPS/dCAPS method to convert rice SNPs into PCR-based markers. *Breed Sci* 55: 93-98

Kosambi DD (1944) The estimation of map distance from recombination values. *Ann Eugen* 12:172-175

Kosová K, Vitamvas P, Prasil IT (2011) Expression of dehydrins in wheat and barley under different temperatures. *Plant Sci* 180: 46-52

Koyama K, Hatano H, Nakamura J, Takumi S (2012) Characterization of three *VERNALIZATION INSENSITIVE3-like* (*VIL*) homologs in wild wheat, *Aegilops tauschii* Coss. *Hereditas* 149: 62-71

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA (2009) Circos: An information aesthetic for comparative genomics. *Genome Res* 19: 1639-1645

Kulwal PL, Kumar N, Gaur A, Khurana P, Khurana JP, Tyagi AK, Balyan HS, Gupta PK (2005) Mapping of a major QTL for pre-harvest sprouting tolerance on chromosome 3A in bread wheat. *Theor Appl Genet* 111: 1052-1059

Kume S, Kobayashi F, Ishibashi M, Ohno R, Nakamura C, Takumi S (2005) Differential and coordinated expression of *Cbf* and *Cor/Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. *Genes Genet Syst* 80: 185-197

Kurahashi Y, Terashima A, Takumi S (2009) Variation in dehydration tolerance, ABA sensitivity and related gene expression patterns in D-genome progenitor and synthetic hexaploid wheat lines. *Intl J Mol Sci* 10: 2733-2751

Lai J, Li R, Xu X, Jin W, Xu M, Zhao H, Xiang Z, Song W, Ying K, Zhang M, Jiao Y, Ni P, Zhang J, Li D, Guo X, Ye K, Jian M, Wang B, Zheng H, Liang H, Zhang X, Wang S, Chen S, Li J, Fu Y, Springer NM, Yang H, Wang J, Dai J, Schnable PS, Wang J (2010) Genome-wide patterns of genetic

variation among elite maize inbred lines. *Nature Genet* 42: 1027-1030

Lam HM, Xu X, Liu X, Chen W, Yang G, Wong FL, Li MW, He W, Qin N, Wang B, Li J, Jian M, Wang J, Shao G, Wang J, Sun SSM, Zhang G (2010) Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. *Nature Genet* 42: 1053-1059

Lander ES, Green P, Abrahamson J, Barlow A, Daly, MJ (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:178-181

Law CN, Worland AJ (1996) Inter-varietal chromosome substitution lines in wheat – revised. *Euphytica* 89: 1-10

Lelley T, Stachel M, Grausgruber H, Vollmann J (2000) Analysis of relationships between *Aegilops tauschii* and the D genome of wheat utilizing microsatellites. *Genome* 43: 661-668

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22: 1658-1659

Ling HQ, Zhao S, Liu D, Wang J, Sun H, Zhang C, Fan H, Li D, Dong L, Tao Y, Gao C, Wu H, Li Y, Cui Y, Guo X, Zheng S, Wang B, Yu K, Liang Q, Yang W, Lou X, Chen J, Feng M, Jian J, Zhang X, Luo G, Jiang Y, Liu J, Wang Z, Sha Y, Zhang B, Wu H, Tang D, Shen Q, Xue P, Zou S, Wang X, Liu X, Wang F, Yang Y, An X, Dong Z, Zhang K, Zhang X, Luo MC, Dvorak J, Tong Y, Wang J, Yang H, Li Z, Wang D, Zhang A, Wang J (2013) Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* 496: 87-90

Liu X, Yue Y, Li B, Nie Y, Li W, Wu WH, Ma L (2007) A G Protein–Coupled Receptor Is a Plasma Membrane Receptor for the Plant Hormone Abscisic Acid. *Science* 315: 1712-1716

Lopez-Molina L, Mongrand S, Chua NH (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proc Natl Acad Sci USA* 98: 4782-4787

Lubbers EL, Gill KS, Cox TS, Gill Bs (1991) Variation of molecular markers among geographically diverse accessions of *Triticum tauschii*. *Genome* 34: 354-361

Luo MC, Gu YQ, You FM, Deal KR, Ma Y, Hu Y, Huo N, Wang Y, Wang J, Chen S, Jorgensen CM, Zhang Y, McGuire PE, Pasternak S, Stein JC, Ware D, Kramer M, McCombie WR, Kianian SF, Martis MM, Mayer KFX, Sehgal SK, Li W, Gill BS, Bevan MW, Šimková H, Doležel J, Weining S, Lazo GR, Anderson OD, Dvorak J (2013) A 4-gigabase physical map unlocks the structure and evolution of the complex genome of *Aegilops tauschii*, the wheat D-genome progenitor. *Proc Natl Acad Sci USA* 110:7940-7945

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors. *Science* 324: 1064-1068

Maloof JN, Borevitz JO, Dabi T, Lutes J, Nehring RB, Redfern JL, Trainer GT, Wilson JM, Asami T, Berry CC, Weigel D, Chory J (2001) Natural variation in light sensitivity of Arabidopsis. *Nature*

Genet 29: 441-446

Mao X, Zhang H, Tian S, Chang X, Jing R (2010) TaSnRK2.4, an SNF1-type serine/threonine protein kinase of wheat (*Triticum aestivum* L.), confers enhanced multistress tolerance in *Arabidopsis*. *J Exp Bot* 61: 683-696

Marcotte WR Jr, Russell SH, Quatrano RS (1989) Abscisic acid-responsive sequences from the Em gene of wheat. *Plant Cell* 1: 969-976

Matsuda R, Iehisa JCM, Takumi S (2012) Application of real-time PCR-based SNP detection for mapping of *Net2*, a causal D-genome gene for hybrid necrosis in interspecific crosses between tetraploid wheat and *Aegilops tauschii*. *Genes Genet Syst* 87: 137-143

Matsumoto T, Tanaka T, Sakai H, Amano N, Kanamori H, Kurita K, Kikuta A, Kamiya K, Yamamoto M, Ikawa H, Fujii N, Hori K, Itoh T, Sato K (2011) Comprehensive sequence analysis of 24,783 barley full-length cDNAs derived from 12 clone libraries. *Plant Physiol* 156: 20-28

Matsuoka Y, Nasuda S (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F_1 hybrid with *Aegilops tauschii* Coss. *Theor Appl Genet* 109: 1710-1717

Matsuoka Y, Takumi S, Kawahara T (2007) Natural variation for fertile triploid F_1 formation in allohexaploid wheat speciation. *Theor Appl Genet* 115: 509-518

Matsuoka Y, Takumi S, Kawahara T (2008) Flowering time diversification and dispersal in central Eurasian wild wheat *Aegilops tauschii* Coss.: genealogical and ecological framework. *PLoS ONE* 3: e3138

Matsuoka Y, Nishioka E, Kawahara T, Takumi S (2009) Genealogical analysis of subspecies divergence and spikelet-shape diversification in central Eurasian wild wheat *Aegilops tauschii* Coss. *Plant Syst Evol* 279: 233-244

Mayer KFX, Martis M, Hedley PE, Simková H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubaláková M, Suchánková P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Dolezel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. *Plant Cell* 23:1249-1263

McCourt P, Creelman R (2008) The ABA receptors – we report you decide. *Curr Opin Plant Biol* 11: 474-478

McFadden ES, Sears ER (1994) The artificial synthesis of *Triticum spelta*. *Rec Genet Soc Am* 13: 26-27

McKibbin RS, Wilkinson MD, Bailey PC, Flintham JE, Andrew LM, Lazzeri PA, Gale MD, Lenton JR, Holdsworth MJ (2002) Transcripts of *Vp-1* homeologues are misspliced in modern wheat and ancestral species. *Proc Natl Acad Sci USA* 99: 10203-10208

Medina J, Bargues M, Terol J, Pérez-Alonso M, Salinas J (1999) The Arabidopsis *CBF* gene family is composed of three genes encoding AP2 domain-containing proteins Whose expression is regulated by

low temperature but not by abscisic acid or dehydration. *Plant Physiol* 119: 463-469

Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V, Kovach A, Li J, Wang Y, Li J, Peterson FC, Jensen DR, Yong EL, Volkman BF, Cutler SR, Zhu JK, Xu HE (2009) A gate–latch–lock mechanism for hormone signalling by abscisic acid receptors. *Nature* 462: 602-608

Menges M, de Jager SM, Gruissem W, Murray JAH (2005) Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. *Plant J* 41: 546-566

Michaels SD, Amasino RM (1998) A robust method for detecting single-nucleotide changes as polymorphic markers by PCR. *Plant J* 14: 381-385

Michaels SD, He Y, Scortecci KC, Amasino RM (2003) Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. *Proc Natl Acad Sci USA* 100: 10102-10107

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F (2006) Seed dormancy and ABA metabolism in *Arabidopsis* and barley: the role of ABA 8'-hydroxylase. *Plant J* 45: 942-954

Miller AK, Galiba G, Dubcovsky J (2006) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. *Mol Genet Genomics* 275: 193-203

Mir RR, Zaman-Allah M, Sreenivasulu N, Trethowan R, Varshney RV (2012) Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. *Theor Appl Genet* 125: 625-645

Miyazono K, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira K, Yamaguchi-Shinozaki K, Tanokura M (2009) Structural basis of abscisic acid signaling. *Nature* 462: 609-614

Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim Biophys Acta* 1819: 86-96

Mizuno N, Yamasaki M, Matsuoka Y, Kawahara T, Takumi S (2010a) Population structure of wild wheat D-genome progenitor *Aegilops tauschii* Coss.: implications for intraspecific lineage diversification and evolution of common wheat. *Mol Ecol* 19: 999-1013

Mizuno N, Hosogi N, Park P, Takumi S (2010b) Hypersensitive response-like reaction is associated with hybrid necrosis in interspecific crosses between tetraploid wheat and *Aegilops tauschii* Coss. *PLoS ONE* 5: e11326

Mizuno N, Shitsukawa N, Hosogi N, Park P, Takumi S (2011) Autoimmune response and repression of mitotic cell division occur in inter-specific crosses between tetraploid wheat and *Aegilops tauschii* Coss. that show low temperature-induced hybrid necrosis. *Plant J* 68:114-128

Motomura Y, Kobayashi F, Iehisa JCM, Takumi S (2013) A major quantitative trait locus for

cold-responsive gene expression is linked to frost-resistance gene *Fr-A2* in common wheat. *Breed Sci* 63: 58-67

Mouchel CLF, Briggs GC, Hardtke CS (2004) Natural genetic variation in Arabidopsis identifies BREVIS RADIX, a novel regulator of cell proliferation and elongation in the root. *Genes Dev* 18: 700-714

Nakamura S, Toyama T (2001) Isolation of a VP1 homologue from wheat and analysis of its expression in embryos of dormant and non-dormant cultivars. *J Exp Bot* 52: 875-876

Nakamura S, Komatsuda T, Miura H (2007) Mapping diploid wheat homologues of *Arabidopsis* seed ABA signaling genes and QTLs for seed dormancy. *Theor Appl Genet* 114: 1129-1139

Nakamura S, Chono M, Abe F, Miura H (2010) Mapping a diploid wheat abscisic acid 8'-hydroxylase homologue in the seed dormancy QTL region on chromosome $5A^m$. *Euphytica* 171: 111-120

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiol* 149: 88-95

Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J* 14: 387-392

Neff MM, Turk E, Kalishaman M (2002) Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet* 18: 613-615

Nguyen AT, Iehisa JCM, Kajimura T, Murai K, Takumi S. Identification of quantitative trait loci for flowering-related traits in the D-genome of synthetic hexaploid wheat lines. *Euphytica* 192: 401-412

Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED (2009) Structural Mechanism of Abscisic Acid Binding and Signaling by Dimeric PYR1. *Science* 326: 1373-1379

Niu X, Helentjaris T, Bate NJ (2002) Maize ABI4 binds Coupling Element 1 in Abscisic acid and sugar response genes. *Plant Cell* 14: 2565-2575

Ogbonnaya FC, Halloran GM, Lagudah ES (2005) D genome of wheat – 60 years on from Kihara, Sears and McFadden. *In*: Tsunewaki K (ed) Frontiers of Wheat Bioscience, the 100th Memorial Issue of Wheat Information Service, Kihara Memorial Foundation for the Advancement of Life Sciences, Yokohama, Japan, pp 205-220

Ohno R, Takumi S, Nakamura C (2003) Kinetics of transcript and protein accumulation of a low-molecular-weight wheat LEA D-11 dehydrin in response to low temperature. *J Plant Physiol* 160: 193-200

Okamoto Y, Kajimura T, Ikeda M, Takumi S (2012) Evidence from principal component analysis for improvement of grain shape- and spikelet morphology-related traits after hexaploid wheat speciation. *Genes Genet Syst* 87: 299-310

Osa M, Kato K, Mori M, Shindo C, Torada A, Miura H (2003) Mapping QTLs for seed dormancy and the

Vp1 homologue on chromosome 3A in wheat. *Theor Appl Genet* 106: 1491-1496

Ossowski S, Schneeberger K, Clark RM, Lanz C, Warthmann N, Weigel D (2008) Sequencing of natural strains of *Arabidopsis thaliana* with short reads. *Genome Res* 18: 2024-2033

Pandey S, Nelson DC and Assmann SM (2009) Two novel GPCR-type G proteins are Abscisic acid receptors in *Arabidopsis*. *Cell* 136: 136-148

Park SDE (2001) Trypanotolerance in west African cattle and the population genetic effects of selection [Ph.D. thesis] University of Dublin

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rogriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler S (2009) Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins. *Science* 324: 1068-1071

Paux E, Sourdille P, Salse J, Saintenac C, Choulet F, Leroy P, Korol A, Michalak M, Kianian S, Spielmeyer W, Lagudah E, Somers D, Kilian A, Alaux M, Vautrin S, Bergès H, Eversole K, Appels R, Safar J, Simkova H, Dolezel J, Bernard M, Feuillet C (2008) A physical map of the 1-gigabase bread wheat chromosome 3B. *Science* 322: 101-104

Pestsova E, Korzun V, Goncharov NP, Hammer K, Ganal MW, Röder MS (2000) Microsatellite analysis of *Aegilops tauschii* germplasm. *Theor Appl Genet* 101: 100-106

Pumphrey M, Bai J, Laudencia-Chingcuanco D, Anderson O, Gill BS (2009) Nonadditive expression of homoeologous genes is established upon polyploidization in hexaploid wheat. *Genetics* 181: 1147-1157

Quarrie SA, Gulli M, Calestani C, Steed A, Marmiroli N (1994) Location of a gene regulating drought-induced abscisic acid production on the long arm of chromosome 5A of wheat. *Theor Appl Genet* 89: 794-800

Qi LL, Echalier B, Chao S, Lazo GR, Butler GE, Anderson OD, Akhunov ED, Dvořák, Linkiewicz AM, Ratnasiri A, Dubcovsky J, Bermudez-Kandianis CE, Greene RA, Kantety R, La Rota CM, Munkvold JD, Sorrells SF, Sorrells ME, Dilbirligi M, Sidhu D, Erayman M, Randhawa HS, Sandhu D, Bondareva SN, Gill KS, Mahmoud AA, Ma XF, Miftahudin, Gustafson JP, Conley EJ, Nduati V, Gonzalez-Hernandez JL, Anderson JA, Peng JH, Lapitan NLV, Hossain KG, Kalavacharla V, Kianian SF, Pathan MS, Zhang DS, Nguyen HT, Choi DW, Fenton RD, Close TJ, McGuire PE, Qualset CO, Gill BS (2004) A chromosome bin map of 16,000 expressed sequence tag loci and distribution of genes among the three genomes of polyploid wheat. *Genetics* 168: 701-712

Raghavendra AS, Gonugunta VK, Christmann A and Grill E (2010) ABA perception and signalling. *Trends Plant Sci* 15: 395-401

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. *Nature* 439: 290-294

Ren Z, Zheng Z, Chinnusamy V, Zhu J, Cui X, Iida K and Zhu JK (2010) RAS1, a quantitative trait locus

for salt tolerance and ABA sensitivity in Arabidopsis. *Proc Natl Acad Sci USA* 107: 5669-5674

Rice P, Longden I, Bleasby A (2000) EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet* 16: 276-277

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier MH, Leroy P, Ganal MW (1998) A microsatellite map of wheat. *Genetics* 149: 2007-2023

Rushton DL, Tripathi P, Rabara RC, Lin J, Ringler P, Boken AK, Langum TJ, Smidt L, Boomsma DD, Emme NJ, Chen X, Finer JJ, Shen QJ, Rushton PJ (2012) WRKY transcription factors: key components in abscisic acid signalling. *Plant Biotechnol J* 10: 2-11

Rustenholz C, Choulet F, Laugier C, Safar J, Simkova H, Dolezel J, Magni F, Scalabrin S, Cattonaro F, Vautrin S, Bellec A, Berges H, Feuillet C, Paux E (2011) A 3,000-loci transcription map of chromosome 3B unravels the structural and functional features of gene islands in hexaploid wheat. *Plant Physiol* 157: 1596-1608

Saeidi H, Rahiminejad MR, Vallian S, Heslop-Harrison JS (2006) Biodiversity of diploid D-genome *Aegilops tauschii* Coss. in Iran measured using microsatellites. *Genet Resour Crop Evol* 53: 1477-1484

Sakai A, Larcher W (1987) Frost survival of plants: responses and adaptation to freezing stress. Springer-Verlag, Berlin, Heidelberg, New York

Salse J, Chague V, Bolot S, Magdelenat G, Huneau C, Pont C, Belcram H, Couloux A, Gardais S, Evrard A, Segurens B, Charles M, Ravel C, Samain S, Charmet G, Boudet N, Chalhoub B (2008) New insights into the origin of the B genome of hexaploid wheat: Evolutionary relationships at the *SPA* genomic region with the S genome of the diploid relative *Aegilops speltoides*. *BMC Genomics* 9: 555

Sato K, Shin-I T, Seki M, Shinozaki K, Yoshida H, Takeda K, Yamazaki Y, Conte M, Kohara Y (2009) Development of 5006 full-length cDNAs in barley: a tool for accessing cereal genomics resources. *DNA Res* 16: 81-89

Snape JW, Semikhodskii A, Fish L, Sarma RN, Quarrie SA, Galiba G, Sutka J (1997) Mapping frost resistance loci in wheat and comparative mapping with other cereals. *Acta Agron Hung* 45: 265-270

Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Márquez JA (2009) The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* 462: 665-668

Sasanuma T, Miyashita NT, Tsunewaki K (1995) Wheat phylogeny determined by RFLP analysis of nuclear DNA. 3. Intra and interspecific variation of five *Aegilops* Sitopsis species. *Theor Appl Genet* 92: 928-934

Shang Y, Yan L, Liu ZQ, Cao Z, Mei C, Xin Q, Wu FQ, Wang XF, Du SY, Jiang T, Zhang XF, Zhao R, Sun HL, Liu R, Yu YT, Zhang DP (2010) The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* 22: 1909-1935

Shen Q, Ho THD (1995) Functional dissection of an Abscisic acid (ABA)-inducible gene reveals two

independent ABA-responsive complexes each containing a G-box and novel *cis*-acting element. *Plant Cell* 7: 295-307

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

Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q, Chen SY (2003) An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. *Theor Appl Genet* 106: 923-930

Shen YY, Wang XF, Wu FG, Du SY, Cao Z, Shang Y, Wang XL, Peng CC, Yu XC, Zhu SY, Fan RC, Xu YH, Zhang DP (2006) The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* 443: 823-826

Sohail Q, Shehzad T, Kilian A, Eltayeb AE, Tanaka H, Tsujimoto H (2012) Development of diversity array technology (DArT) markers for assessment of population structure and diversity in *Aegilops tauschii*. *Breed Sci* 62: 38-45

Somers DJ, Isaac P, Edwards K (2004) A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). *Theor Appl Genet* 109: 1105-1114

Subbaiyan GK, Waters DLE, Katiyar SK, Sadananda AR, Vaddadi S, Henry RJ (2012) Genome-wide DNA polymorphisms in elite *indica* rice inbreds discovered by whole-genome sequencing. *Plant Biotechnol J* 10: 623-634

Sung S, Amasino RM (2005) Remembering winter: toward a molecular understanding of vernalization. *Annu Rev Plant Biol* 56: 491-508

Sutka J (1981) Genetic studies of frost resistance in wheat. *Theor Appl Genet* 59: 145-152

Sutka J, Galiba G, Vagujfalvi A, Gill BS, Snape JW (1999) Physical mapping of the *Vrn-A1* and *Fr1* genes on chromosome 5A of wheat using deletion lines. *Theor Appl Genet* 99: 199-202

Szalai G, Janda T, Páldi E, Dubacq JP (2001) Changes in the fatty acid unsaturation after hardening in wheat chromosome substitution lines with different cold tolerane. *J Plant Physiol* 158: 663-666

Takumi S, Nasuda S, Lin YG, Tsunewaki K (1993) Wheat phylogeny determined by RFLP analysis of nuclear DNA. 1. Erinkorn wheat. *Jpn J Genet* 68: 73-79

Takumi S, Shimamura C, Kobayashi F (2008) Increased freezing tolerance through up-regulation of downstream genes via the wheat *CBF* gene in transgenic tobacco. *Plant Physiol Biochem* 46: 205-211

Takumi S, Nishioka E, Morihiro H, Kawahara T, Matsuoka Y (2009a) Natural variation of morphological traits in wild wheat progenitor *Aegilops tauschii* Coss. *Breed Sci* 59: 579-588

Takumi S, Naka Y, Morihiro H, Matsuoka Y (2009b) Expression of morphological and flowering time variation through allopolyploidization: an empirical study with 27 wheat synthetics and their parental *Aegilops tauschii* accessions. *Plant Breed* 128: 585-590

Terashima A, Takumi S (2009) Allopolyploidization reduces alternative splicing efficiency for transcripts of the wheat *DREB2* homolog, *WDREB2*. *Genome* 52: 100-105

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Mol Biol* 50: 571-599

Thomashow MF, Gilmour SJ, Stockinger EJ, Jaglo-Ottosen KR, Zarka DG (2001) Role of the Arabidopsis CBF transcriptional activators in cold acclimation. *Physiol Plant* 112: 171-175

Tokuda M, Jikumaru Y, Matsukura K, Takebayashi Y, Kumashiro S, Matsumura M, Kamiya Y (2013) Phytohormones related to host plant manipulation by a gall-inducing leafhopper. *PLoS ONE* 8: e62350

Tonsor SJ, Alonso-Blanco C, Koornneef M (2005) Gene function beyond the single trait; natural variation, gene effects, and evolutionary ecology in *Arabidopsis thaliana*. *Plant Cell Environ* 28: 2-20

Tóth B, Galiba G, Fehér E, Sutka J, Snape W (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. *Theor Appl Genet* 107: 509-514

Trebbi D, Maccaferri M, Heer P, Sorensen A, Giuliani S, Salvi S, Sanguineti MC, Massi A, van der Vossen E, Tuberosa R (2011) High-throughput SNP discovery and genotyping in durum wheat (*Triticum durum* Desf.). *Theor Appl Genet* 123: 555-569

Trethowan RM, Mujeeb-Kazi A (2008) Novel germplasm resources for improving environmental stress tolerance of hexaploid wheat. *Crop Sci* 48: 1255-1265

Trick M, Adamski NM, Mugford SG, Jiang CC, Febrer M, Uauy C (2012) Combining SNP discovery from next-generation sequencing data with bulked segregant analysis (BSA) to fine-map genes in polyploid wheat. *BMC Plant Biol* 12: 14

Tsuda K, Tsvetanov S, Takumi S, Mori N, Atanassov A, Nakamura C (2000) New members of a cold-responsive group-3 *Lea/Rab*-related *Cor* gene family from common wheat (*Triticum aestivum* L.). *Genes Genet Syst* 75: 179-188

Tsunewaki K (1966) Comparative gene analysis of common wheat and its ancestral species. II. Waxiness, growth habit and awnedness. *Jpn J Bot* 19: 175-229

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc Natl Acad Sci USA* 106: 17588-17593

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K and Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci USA* 97: 11632-11637

Vágújfalvi A, Crosatti C, Galiba G, Dubcovsky J, Cattivelli L (2000) Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost-tolerant and frost-sensitive genotypes. *Mol Genet Genomics* 263: 194-200

Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. *Mol Genet Genomics* 269: 60-67

Vágújfalvi A, Aprile A, Miller A, Dubcovsky J, Delugu G, Galiba G, Cattivelli L (2005) The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat. *Mol Genet Genomics* 274: 506-514

Van Leene J, Hollunder J, Eeckhout D, Persiau G, Van De Slijke E, Stals H, Van Isterdael G, Verkest A, Neirynck S, Buffel Y, De Bodt S, Maere S, Laukens K, Pharazyn A, Ferreira PCG, Eloy N, Renne C, Meyer C, Faure JD, Steinbrenner J, Beynon J, Larkin JC, Van de Peer Y, Hilson P, Kuiper M, De Veylder L, Van Onckelen H, Inzé D, Witters E, De Jaeger G (2010) Targeted interactomics reveals a complex core cell cycle machinery in *Arabidopsis thaliana*. *Mol Syst Biol* 6: e397

Van Slageren MW (1994) Wild Wheats: A Monograph of *Aegilops* L. and Amblyopyrum (Jaub. & Spach) Eig (Poaceae); Wageningen Agricultural University, Wageningen; pp326-344

Veisz O, Sutka J (1990) Frost resistance studies with wheat in natural and artificial conditions. In: Panayotov I, Pavlova S (eds) Proceedings of the international symposium on cereal adaptation to low temperature stress, Albena, Bulgaria, pp. 12-17

Vitulo N, Albiero A, Forcato C, Campagna D, Dal Pero F, Bagnaresi P, Colaiacovo M, Faccioli P, Lamontanara A, Simkova H, Kubalakova M, Perrotta G, Facella P, Lopez L, Pietrella M, Gianese G, Dolezel J, Giuliano G, Cattivelli L, Valle G, Stanca AM (2011) First survey of the wheat chromosome 5A composition through a next generation sequencing approach. *PLoS ONE* 6: e26421

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. *J Hered* 93: 77-78

Walker-Simmons M (1988) Enhancement of ABA responsiveness in wheat embryos by high temperature. *Plant Cell Environ* 11: 769-775

Wang H, Qi Q, Schorr P, Cutler AJ, Crosby WL, Fowke LC (1998) ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. *Plant J* 15: 501-510

Wang JR, Zhang L, Wei YM, Yan ZH, Baum BR, Nevo E, Zheng YL (2007) Sequence polymorphisms and relationships of dimeric α-amylase inhibitor genes in the B genomes of *Triticum* and S genomes of *Aegilops*. *Plant Sci* 173, 173:1-11

Wang S, Basten CJ, Zeng ZB (2011) Windows QTL cartographer 2.5. Department of Statics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

Wang J, Luo MC, Chen Z, You FM, Wei Y, Zheng Y, Dvorak J (2013) *Aegilops tauschii* single nucleotide polymorphisms shed light on the origins of wheat D-genome genetic diversity and pinpoint the geographic origin of hexaploid wheat. *New Phytol* 198: 925-937

Werner JD, Borevitz JO, Uhlenhaut NH, Ecker JR, Chory J, Weigel D (2005) FRIGIDA-independent variation in flowering time of natural *Arabidopsis thaliana* accessions. *Genetics* 170: 1197-1207

Wu FQ, Xin Q, Cao Z, Liu ZQ, Du SY, Mei C, Zhao CX, Wang XF, Shang Y, Jiang T, Zhang XF, Yan L, Zhao R, Cui ZN, Liu R, Sun HL, Yang XL, Su Z, Zhang DP (2009) The magnesium-chelatase H

subunit binds abscisic acid and functions in abscisic acid signaling: new evidence in Arabidopsis. *Plant Physiol* 150: 1940-1954

Xue GP, Loveridge CW (2004) *HvDRF1* is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. *Plant J* 37: 326-339

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low temperature or high-salt stress. *Plant Cell* 6: 251-264

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci* 10: 88-94

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

Yamamoto T, Nagasaki H, Yonemaru J, Ebana K, Nakajima M, Shibaya T, Yano M (2010) Fine definition of the pedigree haplotypes of closely related rice cultivars by means of genome-wide discovery of single-nucleotide polymorphisms. *BMC Genomics* 11: 267

Yano M (2001) Genetic and molecular dissection of naturally occurring variation. *Curr Opin Plant Biol* 4: 130-135

Yin P, Fan H, Hao Q, Yuan X, Wu D, Pang Y, Yan C, Li W, Wang J, Yan N (2009) Structural insights into the mechanism of abscisic acid signaling by PYL proteins. *Nature Struct Mol Biol* 16: 1230-1236

You FM, Huo N, Deal KR, Gu YQ, Luo MC, McGuire PE, Dvorak J, Anderson OD (2011) Annotation-based genome-wide SNP discovery in the large and complex *Aegilops tauschii* genome using next-generation sequencing without a reference genome sequence. *BMC Genomics* 12: 59

Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) Cold induction of Arabidopsis *CBF* genes involves multiple ICE (Inducer of *CBF* Expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiol* 133: 910-918