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Asymmetric allele-specific expression in relation to developmental variation and drought stress in barley hybrids

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SUMMARY

In the present study, we analysed allele-specific expression (ASE) in the selfing species barley to assess the frequency of *cis*-acting regulatory variation and the effects of genetic background, developmental differences and drought stress on allelic expression levels. We measured ASE ratios in 30 genes putatively involved in stress responses in five hybrids and their reciprocals, namely *Hordeum spontaneum* 41-1/Alexis (HAl), *Hordeum spontaneum* 41-1/Arta (HAr), Sloop/WI3408 (SW), Tadmor/Sloop (TS) and Tadmor/WI3408 (TW). In order to detect *cis*-acting variation related to drought and developmental changes, the barley hybrids were grown under control and water-limited conditions, and leaf tissue was harvested at two developmental stages. The analysis demonstrated that more than half of the genes measured (63%) showed allelic differences in expression of up to 19-fold due to *cis*-regulatory variation in at least one cross by treatment/stage combination. Drought stress induced changes in allelic expression ratios, indicating differences between drought responsive *cis*-elements. In addition, ASE differences between developmental stages suggested the presence of *cis*-acting elements interacting with developmental cues. We were also able to demonstrate that the levels and frequency of allelic imbalance and hence differences in *cis*-regulatory elements are correlated with the genetic divergence between the parental lines, but may also arise as an adaptation to diverse habitats. Our findings suggest that *cis*-regulatory variation is a common phenomenon in barley, and may provide a molecular basis of transgression. Differential expression of near-isogenic members of the same gene family could potentially result in hybrid lines out performing their parents in terms of expression level, timing and response to developmental and environmental cues. Identification and targeted manipulation of *cis*-regulatory elements will assist in breeding improved crops with a better adaptation to changing environments.

Keywords: allele-specific expression, drought, barley, *cis*-acting variation, development.

INTRODUCTION

Genetic variation provides the basis for progress in plant breeding. In recent years, the genetic dissection of complex traits has focused on structural diversity in protein coding regions, under the assumption that diversification of protein function has driven the evolution of organismal form and function. However, recent studies suggest that regulation of gene expression accounts for a major part of natural genetic variation within and among species (Brem *et al.*, 2002; Levine, 2002; Brem and Kruglyak, 2005; Kliebenstein *et al.*, 2006; West *et al.*, 2007), and that even subtle changes in expression can significantly affect the phenotype (Wang *et al.*, 1999; Gompel *et al.*, 2005).

cis-acting elements residing in non-coding DNA sequences that influence transcription in an allele-specific manner have been identified as the major regulatory forces behind expression differences in human (Rockman and Wray, 2002; Yan *et al.*, 2002; Lo *et al.*, 2003; Pastinen *et al.*, 2004) and animal systems (Cowles *et al.*, 2002; Wittkopp *et al.*, 2004, 2008). In plants, evidence for the importance of regulatory variation for plant genetic adaptation has also

been obtained. Polymorphisms in a *cis*-regulatory region of the teosinte *branched1* gene have been implicated in the domestication of maize (Wang *et al.*, 1999), changes in the promoter region of the *ORFX* gene may have been associated with increases in fruit size during tomato domestication (Frary *et al.*, 2000; Cong *et al.*, 2002), and a major flowering time locus in maize has been mapped to a non-coding *cis*-regulatory element upstream of an *Ap2*-like transcription factor (Salvi *et al.*, 2007). However, comprehensive direct analyses of *cis*-acting elements are currently difficult, because the nature and position of all *cis*-regulatory sequences for any given gene are generally unknown. Analyses of allele-specific expression (ASE) provide an indirect measure for quantifying *cis*-regulatory effects by determining the relative proportions of alleles present in the transcript pool of heterozygous individuals. As both alleles in the heterozygote are expressed in the same cell and are exposed to common regulatory factors, genes exhibiting asymmetric allele expression are inferred to be controlled by *cis*-acting regulatory variation. Detection of ASE in heterozygous cells offers the advantage that the two alleles are compared under identical circumstances within a single individual genotype, providing an internal control for confounding factors such as differences in mRNA preparation and quality, and environmental and *trans*-acting factors.

Published studies quantifying *cis*-acting polymorphisms in plants have focused on outbreeding species such as poplar (Zhuang and Adams, 2007), and in particular maize (Guo *et al.*, 2003, 2004, 2008; Stupar and Springer, 2006; Springer and Stupar, 2007a; Stupar *et al.*, 2007), where the high frequency of *cis*-acting regulatory variation has been attributed to high levels of genetic diversity (Guo *et al.*, 2004, 2006; Birchler *et al.*, 2006; Springer and Stupar, 2007b). Detection of ASE indicates that two alleles exhibit *cis*-regulatory variation that is tissue-specific (Guo *et al.*, 2004; Stupar and Springer, 2006; Zhuang and Adams, 2007) or that results in differential responses to environmental (Guo *et al.*, 2004) or developmental cues (Adams and Wendel, 2005; Salvi *et al.*, 2007).

Here we report a high frequency of unequal allelic expression in barley, an inbreeding species. We show that the imbalance in reciprocal hybrids is influenced by genetic background and genetic divergence, developmental stages and drought stress. Knowledge of the frequency and existence of *cis*-acting regulatory variation in crop plants has

Table 1 List of genes assayed for allelic expression

Contig	GenBank accession number	Annotation
ABC00149	P26517	Glyceraldehyde 3-phosphate dehydrogenase
ABC00314	BAB08263	Putative ornithine aminotransferase
ABC00422	AAB18209.1	Chlorophyll *a*/*b* binding protein WCAB precursor
ABC00481	P49036	Sucrose synthase 2
ABC00600	CAA70817.1	Serine carboxypeptidase III precursor
ABC00871	P17990	Phospholipid transfer protein precursor
ABC00940	CAD12665.1	Putative fructose-1,6-biphosphate aldolase
ABC00949	AAB18209.1	Chlorophyll *a*/*b* binding protein WCAB precursor
ABC00953	NP_919208.1	Oxygen evolving enhancer 3 (PsbQ) family protein
ABC01249	CAA70175.1	R40g3 protein
ABC01741	AAO73223.1	Hypothetical protein
ABC02109	AAO65864.1	Putative actin-depolymerizing factor 3
ABC02112	CAA59485.1	Peroxidase (EC 1.11.1.7) 2 precursor
ABC02113	BAC83102.1	Peroxidase
ABC02329	CAD89604.1	12-oxophytodienoic acid reductase
ABC02924	AAB47996.1	Aldehyde dehydrogenase homologue Dha1
ABC02333	NP_190930.1	Putative pyrophosphate phosphohydrolase
ABC03154	CAA63659.1	Germin-like protein
ABC03204	BAB61039.1	Iron-deficiency induced gene
ABC03499	CAA47017.1	60 kDa jasmonate-induced protein
ABC04273	NP_910582.1	Ethylene-forming enzyme-like dioxygenase
ABC04900	AAO72389.1	Synaptobrevin-like protein
ABC05236	BAC83807.1	Putative thioredoxin
ABC05604	AAK92625.1	Putative phytoene dehydrogenase precursor
ABC05702	AAS00828.1	Extracellular calcium sensing receptor
ABC07787	AAR87222.1	Gibberellin-stimulated transcript 1-like protein
ABC08246	AAK27799	Putative amylase
ABC10029	AF348586	Putative heat shock protein
ABC15719	AAK50348.1	Putative protein kinase
ABC13238	AJ300144	Srg6 gene for stress-responsive gene protein 6

important implications for understanding how plants adapt to new environments. The identification of specific nucleotide changes that underlie differences in gene expression will also provide important tools for plant breeders to create new climate-relevant cultivars.

RESULTS

Assay development

The ASE assay in barley was developed to assess the frequency of *cis*-acting variation and to quantify the incidence of differences in ASE under drought stress and between the vegetative and generative stages (see analysed genes in Table 1 and Experimental procedures for details of the assay design, and Table S1 for details of primers, accession numbers and annotations). The hybrids used were *H. spontaneum* 41-1/Alexis (HAl), *H. spontaneum* 41-1/Arta (HAr), Sloop/WI3408 (SW), Tadmor/Sloop (TS) and Tadmor/WI3408 (TW). We thus examined (i) the deviation of allelic expression proportions in hybrid cDNA from the balanced allelic proportions in the hybrid DNA, and (ii) changes in the relative proportion of the two parental alleles in hybrid cDNA between two developmental stages and control and drought conditions (Tables 2 and 3). Three biological replicates consisting of three pooled plants, which were used to test for the reliability of expression data, showed a low standard error for the majority of genes (Table 2). In addition, we estimated allelic expression in two reciprocal hybrids in order to distinguish between allelic imbalance caused by regulatory variation (the same allele is more highly expressed in both reciprocal hybrids) and allelic expression differences caused by imprinting (the different alleles are more highly expressed in either of reciprocal hybrids depending on whether they are maternally or paternally inherited). A main effect of the cross direction was not detected.

Detection of unequal allele-specific expression

First we estimated the frequency and extent of *cis*-regulatory variation using model 1, which does not differentiate between developmental stage or drought treatment, but analyses significant deviation of the cDNA allele expression proportions from those of the hybrid genomic DNA (indicated by AI in Tables 2, 3 and S2). A total of 30 different genes were analysed, but the number of genes assayed per cross varied from 11 for HAr and SW to 27 for TS based on the presence of single nucleotide polymorphisms (SNPs) between the parental genotypes. The allelic expression proportions of 19 of 30 analysed genes (63%) deviated in at least one cross from those found in the hybrid DNA (Tables 2 and 3). In total, 38 of 82 gene/cross combinations (46%) were characterized by unequal allelic expression. Hybrids derived from a cross between Tadmor and WI3408 showed the highest level of *cis*-acting regulatory variation, with 61% of the genes showing an imbalance in allelic expression. On the other hand, hybrids derived from the two Australian cultivars Sloop and WI3408 showed imbalance for only 27% of the analysed genes. The majority of genes with asymmetric allele expression were differentially expressed in more than one cross (12 of 19 genes, Table 3). Of these, ABC00481, ABC00871, ABC00949, ABC02112, ABC02113, ABC02333 and ABC07787 showed allelic imbalance in all analysed crosses. With the exception of three gene/cross combinations, allelic proportions differed more than 1.5-fold ($\leq$0.4 or $\geq$0.6), and the expression differences varied more than fivefold for seven gene/cross combinations (Figure 1a and Table S2). In TS hybrids, for example, the Sloop allele of ABC07787 was expressed almost exclusively.

We were interested in evaluating the *cis*-regulatory properties of different haplotypes in different genetic backgrounds. Four genes, ABC00422, ABC00481, ABC00871 and ABC02329, were assayed in SW, TS and TW. An additional 20 genes were examined in at least two of the crosses SW, TS and TW, and seven genes in HAl and HAr, which allowed analysis of *cis*-acting haplotypes in at least two different genetic backgrounds. Allelic expression of the Hsp41-1 allele was consistent in the Alexis and Arta genetic background for all seven genes assayed in both crosses. In addition, of the 24 genes analysed in SW, TS and TW, 18 showed a consistent pattern of expression of the same parental genotype in at least two of the crosses (Table 2 and Figure 1a). For example, the Tadmor allele showed consistently lower expression in TS and TW hybrids for the genes ABC02113, ABC03499 and ABC07787. As the differences in allelic expression patterns suggest variation in the *cis*-regulatory regions, we looked for haplotype differences of the sequenced gene segments as an indicator of linkage disequilibrium with *cis*-acting elements, and were able to associate expression activity to a particular haplotype. For the genes ABC02113, ABC02333 and ABC03499, the identical haplotypes of Sloop and WI3408 differed from the Tadmor haplotype (Figure 1b). The correspondence of haplotype sequences and ASE was also confirmed in HAl and HAr crosses. The Hsp41-1 allele of ABC02113 had the same sequence as Tadmor, and, like the Tadmor allele, was always less expressed when combined with Alexis and Arta alleles, which shared a haplotype with Sloop and WI3408 (Figure 1a,b).

Effects of the developmental stage on allelic expression imbalance

The parental *cis*-regulatory haplotypes were found to respond differently to developmental cues, as indicated by significant effects of the developmental stages on the relative ASE of parental alleles within hybrid cDNA (Tables 2, 3 and S3). In total, 10 of 82 gene/cross combinations (12%)

Table 2 Least square means[a] and standard error of allelic expression ratios in five reciprocal crosses calculated for the various combinations of developmental stage (vegetative, generative) and treatments (drought, control) separately

Trait	Vegetative control	Vegetative drought	Generative control	Generative drought	AI[b]	Effect[c]
Hsp41-1/Alexis						
ABC00314	0.47 ± 0.01	0.50 ± 0.02	0.45 ± 0.01	0.48 ± 0.01		
ABC00422	0.49 ± 0.01	0.48 ± 0.02	0.00 ± 0.00	0.00 ± 0.00		S
ABC00600	0.47 ± 0.03	0.48 ± 0.03	0.48 ± 0.03	0.47 ± 0.03		
ABC00871	0.17 ± 0.03	0.10 ± 0.02	0.26 ± 0.05	0.03 ± 0.02	AI	T
ABC00949	0.80 ± 0.02	0.85 ± 0.02	0.82 ± 0.02	0.94 ± 0.01	AI	T
ABC00953	0.39 ± 0.01	0.38 ± 0.01	0.41 ± 0.01	0.40 ± 0.01	AI	
ABC01249	0.44 ± 0.01	0.46 ± 0.01	0.42 ± 0.02	0.45 ± 0.01		
ABC01741	0.70 ± 0.02	0.65 ± 0.04	0.56 ± 0.02	0.55 ± 0.03	AI	S
ABC02113	0.13 ± 0.04	0.26 ± 0.03	0.18 ± 0.03	0.38 ± 0.01	AI	T
ABC02329	0.58 ± 0.07	0.67 ± 0.04	0.42 ± 0.03	0.58 ± 0.05		S/T/S*I
ABC02924	0.47 ± 0.01	0.46 ± 0.01	0.50 ± 0.01	0.50 ± 0.01		
ABC03154	0.84 ± 0.02	0.73 ± 0.5	0.82 ± 0.03	0.68 ± 0.04	AI	
ABC03499	0.28 ± 0.01	0.19 ± 0.01	0.27 ± 0.01	0.16 ± 0.01	AI	T
ABC05604	0.46 ± 0.01	0.46 ± 0.01	0.47 ± 0.01	0.46 ± 0.01		
ABC15719	0.50 ± 0.02	0.48 ± 0.02	0.47 ± 0.01	0.50 ± 0.01		
Hsp41-1/Arta						
ABC00149	0.48 ± 0.01	0.50 ± 0.01	ND	ND		
ABC00314	0.41 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	AI	
ABC00871	0.32 ± 0.02	0.31 ± 0.01	0.34 ± 0.02	0.32 ± 0.01	AI	
ABC01249	0.54 ± 0.02	0.50 ± 0.01	0.51 ± 0.02	0.46 ± 0.01		
ABC02113	0.28 ± 0.01	0.33 ± 0.04	ND	ND	AI	
ABC02924	0.50 ± 0.01	0.48 ± 0.01	0.44 ± 0.02	0.50 ± 0.02		
ABC03499	0.47 ± 0.08	0.25 ± 0.06	0.41 ± 0.07	0.37 ± 0.06		
ABC05604	0.46 ± 0.01	0.48 ± 0.02	0.51 ± 0.02	0.48 ± 0.01		
ABC08246	0.53 ± 0.02	0.60 ± 0.01	0.74 ± 0.05	0.63 ± 0.01		S/S*T
ABC10029	0.18 ± 0.03	0.13 ± 0.01	0.16 ± 0.04	0.14 ± 0.01	AI	
ABC13238	0.49 ± 0.02	0.51 ± 0.03	0.54 ± 0.03	0.51 ± 0.03		
Sloop/WI3408						
ABC00422	0.40 ± 0.01	0.42 ± 0.06	0.47 ± 0.03	0.53 ± 0.06		
ABC00481	0.74 ± 0.04	0.72 ± 0.04	0.70 ± 0.04	0.75 ± 0.05	AI	
ABC00600	0.42 ± 0.03	0.45 ± 0.04	0.63 ± 0.03	0.66 ± 0.03		S
ABC00871	0.72 ± 0.05	0.74 ± 0.03	0.70 ± 0.02	0.86 ± 0.02	AI	T
ABC00953	0.50 ± 0.01	0.52 ± 0.02	0.50 ± 0.01	0.50 ± 0.01		
ABC02329	0.36 ± 0.03	0.21 ± 0.02	0.42 ± 0.01	0.28 ± 0.05		T
ABC03154	0.27 ± 0.02	0.31 ± 0.01	0.24 ± 0.01	0.26 ± 0.02	AI	
ABC04900	0.52 ± 0.01	0.52 ± 0.03	0.53 ± 0.01	0.52 ± 0.01		
ABC05604	0.51 ± 0.02	0.53 ± 0.01	0.53 ± 0.01	0.54 ± 0.01		
ABC13238	0.50 ± 0.02	0.44 ± 0.03	0.50 ± 0.01	0.51 ± 0.01		
ABC15719	0.52 ± 0.03	0.45 ± 0.04	0.50 ± 0.01	0.49 ± 0.01		
Tadmor/Sloop						
ABC00149	0.50 ± 0.01	0.50 ± 0.01	0.56 ± 0.01	0.54 ± 0.01		
ABC00314	0.58 ± 0.02	0.51 ± 0.03	0.68 ± 0.02	0.66 ± 0.02		S
ABC00422	0.85 ± 0.01	0.89 ± 0.01	0.80 ± 0.01	0.78 ± 0.02	AI	
ABC00481	0.87 ± 0.05	0.87 ± 0.04	0.82 ± 0.04	0.75 ± 0.03	AI	
ABC00600	0.25 ± 0.05	0.20 ± 0.03	0.27 ± 0.05	0.26 ± 0.06	AI	
ABC00871	0.79 ± 0.01	0.79 ± 0.01	0.65 ± 0.04	0.69 ± 0.03	AI	S
ABC00949	0.15 ± 0.02	0.13 ± 0.04	0.06 ± 0.01	0.03 ± 0.01	AI	S
ABC00953	0.53 ± 0.05	0.47 ± 0.06	0.56 ± 0.01	0.56 ± 0.02		
ABC01249	0.52 ± 0.01	0.51 ± 0.01	0.50 ± 0.01	0.49 ± 0.01		
ABC01741	0.43 ± 0.01	0.40 ± 0.01	0.47 ± 0.03	0.45 ± 0.05		
ABC02109	ND	ND	0.49 ± 0.01	0.45 ± 0.03		
ABC02112	0.41 ± 0.01	0.38 ± 0.04	0.35 ± 0.03	0.45 ± 0.04	AI	
ABC02113	0.17 ± 0.02	0.16 ± 0.04	0.26 ± 0.04	0.44 ± 0.05	AI	S
ABC02329	0.56 ± 0.02	0.20 ± 0.04	0.87 ± 0.04	0.90 ± 0.03		S/T/S*T
ABC02333	0.79 ± 0.03	0.86 ± 0.02	0.76 ± 0.03	0.82 ± 0.04	AI	
ABC02733	ND	ND	0.50 ± 0.06	0.53 ± 0.04		

Table 2 (Continued)

Trait	Vegetative control	Vegetative drought	Generative control	Generative drought	AI[b]	Effect[c]
ABC03154	ND	ND	0.49 ± 0.01	0.43 ± 0.02		
ABC03204	0.45 ± 0.02	0.49 ± 0.01	0.43 ± 0.01	0.42 ± 0.01		
ABC03499	0.40 ± 0.04	0.37 ± 0.02	0.31 ± 0.04	0.31 ± 0.01	AI	
ABC04900	0.46 ± 0.01	0.49 ± 0.01	0.50 ± 0.01	0.51 ± 0.03		
ABC05236	ND	ND	0.43 ± 0.02	0.49 ± 0.03		
ABC05604	0.49 ± 0.03	0.47 ± 0.03	0.55 ± 0.01	0.58 ± 0.01		
ABC06144	0.56 ± 0.01	0.56 ± 0.02	0.57 ± 0.02	0.53 ± 0.03		
ABC07787	0.03 ± 0.01	0.10 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	AI	
ABC10029	0.80 ± 0.04	0.57 ± 0.03	0.83 ± 0.03	0.67 ± 0.04	AI	T
ABC13238	0.43 ± 0.02	0.41 ± 0.01	0.38 ± 0.02	0.43 ± 0.02	AI	
ABC15719	0.65 ± 0.02	0.63 ± 0.01	0.64 ± 0.02	0.63 ± 0.01	AI	
Tadmor/WI3408						
ABC00149	0.47 ± 0.01	0.42 ± 0.01	0.46 ± 0.01	0.43 ± 0.01		
ABC00314	0.39 ± 0.04	0.33 ± 0.01	0.38 ± 0.01	0.35 ± 0.02	AI	
ABC00422	0.43 ± 0.02	0.39 ± 0.04	0.46 ± 0.02	0.47 ± 0.05		
ABC00481	0.95 ± 0.04	0.94 ± 0.06	0.96 ± 0.02	0.90 ± 0.07	AI	
ABC00871	0.74 ± 0.03	0.63 ± 0.02	0.69 ± 0.01	0.66 ± 0.02	AI	T
ABC01249	0.55 ± 0.02	0.56 ± 0.02	0.53 ± 0.01	0.50 ± 0.01		
ABC01741	0.37 ± 0.02	0.38 ± 0.01	0.38 ± 0.01	0.36 ± 0.02	AI	
ABC02112	0.33 ± 0.01	0.39 ± 0.01	0.27 ± 0.02	0.32 ± 0.04	AI	
ABC02113	0.17 ± 0.04	0.17 ± 0.08	0.26 ± 0.04	0.31 ± 0.03	AI	
ABC02329	0.53 ± 0.05	0.17 ± 0.05	0.54 ± 0.03	0.30 ± 0.10	AI	T
ABC02333	0.68 ± 0.01	0.73 ± 0.01	0.72 ± 0.01	0.71 ± 0.01	AI	
ABC03204	0.39 ± 0.01	0.43 ± 0.01	0.41 ± 0.01	0.41 ± 0.01	AI	
ABC03499	0.35 ± 0.03	0.37 ± 0.03	0.40 ± 0.04	0.36 ± 0.02	AI	
ABC05236	0.53 ± 0.02	0.55 ± 0.02	0.59 ± 0.01	0.59 ± 0.01		
ABC05702	0.50 ± 0.01	0.52 ± 0.01	0.49 ± 0.01	0.49 ± 0.01		
ABC06144	0.55 ± 0.01	0.58 ± 0.01	0.50 ± 0.06	0.56 ± 0.01		
ABC07787	0.15 ± 0.05	0.13 ± 0.01	0.09 ± 0.00	0.11 ± 0.01	AI	
ABC10029	0.55 ± 0.04	0.46 ± 0.03	0.46 ± 0.03	0.51 ± 0.04		

[a]Least square means of various combinations of developmental stages (vegetative, generative) × treatment (control, drought). Allele proportions are always given for the first parent in the cross designation. ND, not determined.
[b]AI indicates deviations of the allelic expression proportions from the allele proportions in F_1/RF_1 hybrid DNA, detection of *cis*-regulatory variation (see Experimental procedures, model 1).
[c]Effects are significant differences detected in the three-factorial ANOVA: *S,* developmental stage (vegetative versus generative); *T,* treatment (70% water capacity of the soil versus 10% water capacity of the soil); *S*T,* interaction effect between the developmental stage and the treatment; *S*I,* interaction effect between the developmental stage and the direction of the cross (F_1, RF_1) (see Experimental procedures, model 2).

showed a change in relative allelic expression between the vegetative and generative stages. The majority of significant effects were detected in TS and HAl hybrids, with 5/27 (19%) and 3/15 (20%) genes, respectively, showing allelic expression differences between the developmental stages. The incidence of changes in allelic expression was lower in the other crosses, with 1/11 (9%) genes in HAr and SW and no genes in TW. Changes in expression were observed in genes directly involved in photosynthesis, such as ABC00422 and ABC00949, and in genes putatively implicated in stress responses, such as ABC00314, ABC00871, ABC02113 and ABC02329. For example, in SW hybrids, we observed bi-directional allelic imbalance in the gene ABC00600, where the proportion of allelic expression was biased in favour of the WI3408 allele in the vegetative phase, and in favour of the Sloop allele in the generative stage (Figure 2a). The greatest change was observed in the gene ABC00422 in HAl, where parental alleles showed balanced expression in the vegetative stage, but the Alexis allele was exclusively expressed in the generative stage (Figure 2a). This was the only gene/cross combination that showed monoallelic expression.

The effects of drought stress on ASE

Allelic expression was analysed under two water regimes to characterize the frequency and modes of allelic regulatory variation responsive to drought. Changes in ASE were studied in adult plants exposed to a gradual reduction of available water at the vegetative and generative stages to mimic drought conditions in the field.

ANOVA revealed ten main effects for the factor treatment as calculated using general linear model 2. Consequently, 10/82 (12%) gene/cross combinations exhibited changes in relative allele expression between the control and the drought-

Table 3 Summary of results for allelic imbalance in five crosses and 30 genes

Genes	Hsp41-1/Alexis	Hsp41-1/Arta	Sloop/WI3408	Tadmor/Sloop	Tadmor/WI3408	Total	AI	Effects
ABC00149		x		x	x	3	0	0
ABC00314	x	AI		S	AI	4	2	1
ABC00422	S		x	AI	x	4	1	1
ABC00481			AI	AI	AI	3	3	0
ABC00600	x		S	AI		3	1	1
ABC00871	AI/T	AI	AI/T	AI/S	AI/T	5	5	4
ABC00940				x		1	0	0
ABC00949	AI/T			AI/S		2	2	2
ABC00953	AI		x	x		3	1	0
ABC01249	x	x		x	x	4	0	0
ABC01741	AI/S			x	AI	3	2	1
ABC02109				x		1	0	0
ABC02112				AI	AI	2	2	0
ABC02113	AI/T	AI		AI/S	AI	4	4	2
ABC02329	S/T/S*I		T	S/T/S*T	AI/T	4	1	7
ABC02333				AI	AI	2	2	0
ABC02924	x	x				2	0	0
ABC03154	AI		AI	x		3	2	0
ABC03204				x	AI	2	1	0
ABC03499	AI/T	x		AI	AI	4	3	1
ABC04900			x	x		2	0	0
ABC05236				x	x	2	0	0
ABC05604	x	x	x	x		4	0	0
ABC05702					x	1	0	0
ABC06144				x	x	2	0	0
ABC07787				AI	AI	2	2	0
ABC08246		S/S*T				1	0	2
ABC10029		AI		AI/T	x	3	2	1
ABC13238		x	x	AI		3	1	0
ABC15719	x		x	AI		3	1	0
Total	15	11	11	27	18	82		
AI (%)	7 (47%)	4 (36%)	3 (27%)	13 (48%)	11 (61%)		38	
Effects	8 (53%)	2 (18%)	3 (27%)	8 (33%)	2 (11%)			23
Total number of genes	15 (100%)	6 (55%)	6 (54%)	21 (81%)	13 (72%)			

The table shows which genes/cross combinations were analysed. Analysed gene/cross combinations with balanced allele expression are indicated with an x. Gene/cross combinations that exhibited deviation from the balanced allelic proportions in the hybrid DNA are indicated by AI. Gene/cross combinations that showed a significant change in allelic expression between developmental stages (vegetative versus generative) are indicated by S, those between treatments (control versus drought) are indicated by T, those between stages and treatments are indicated by S*T, and those between developmental stages and cross direction (F_1, RF_1) are indicated by S*I.

treated plants (Tables 2, 3 and S3). The number of genes exhibiting changes in ASE between control and drought conditions differed between crosses, ranging from none in HAr to 5/15 (33%) in the hybrids derived from Hsp41-1 and Alexis (Tables 2, 3 and S3). The majority of genes with changes in allelic expression upon drought stress also showed deviation from equimolar allele expression under control conditions. Stress effects varied from decreasing the imbalance in allelic expression (ABC10029 in TS hybrids) to further increasing it (ABC03499 in HAI) (Table 2 and Figure 2b). Only the gene ABC02329 showed a balanced allelic expression under control conditions in SW and TW, while expression of the WI3408 allele under drought conditions was significantly increased relative to the second parent in both crosses (Figure 2b).

The GLM model also detected interaction effects between developmental stage and drought treatment; changes in ASE upon drought stress differed between the two developmental stages (Tables 2 and 3). One of the genes affected was ABC02329 in the TS hybrid (Figure 2c). In the vegetative stage, alleles of control plants exhibited equimolar expression levels, which were altered upon drought treatment in favour of the Sloop allele. In the generative stage, by contrast, both stressed and non-stressed plants showed similar expression profiles, with elevated expression levels of the Tadmor allele.

Relationship between genetic divergence and ASE variation

We were interested in determining whether there is a correlation between the levels of genetic divergence between parents and the frequency of *cis*-acting regulatory variation

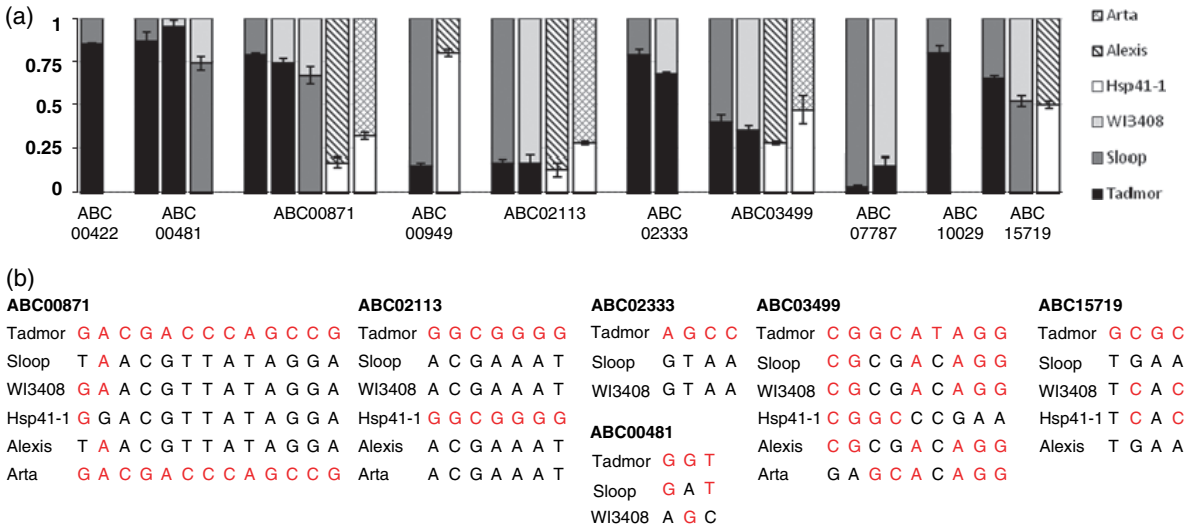

Figure 1. *cis* regulatory variation in barley.
(a) Relative quantification of allele-specific expression across various genetic backgrounds. Genes were assayed for allele-specific expression in leaf tissue harvested from plants at the vegetative stage of reciprocal hybrids derived from different parental lines (Tadmor, Sloop, WI3408, Hsp41-1, Alexis and Arta) grown under control conditions (70% AWC). The relative allele proportion is shown on the *y* axis. Data are from Table 2.
(b) Allele-specific haplotypes of sequenced fragments for some of the analysed genes.

detected in hybrids. Parental genotypes were sampled from different gene pools, with one representative from wild barley, two landraces and three cultivars, and crosses were performed between closely related parents (Sloop, WI3408) as well as between parents with high levels of genetic divergence (Hsp41-1, Alexis). In addition, parental genotypes were chosen from different environments, with Hsp41-1, Tadmor, Arta and WI3408 being well-adapted to drought-prone environments, and Sloop and in particular Alexis better adapted to more favourable humid environments. This allowed us to assess whether parents from similar eco-geographic regions and different gene pools showed similar patterns of adaptive regulatory variation.

The genetic distance of the parents was determined using 28 SSR markers and correlated with (i) the proportion of genes per cross showing significant effects as calculated using GLM models 1 and 2, (ii) the proportion of genes per cross showing a significant deviation from equal allele expression (ANOVA model 1), and (iii) the proportion of genes exhibiting significant changes between either developmental stages, treatments or interactions between both factors (number of significant effects in ANOVA model 2 excluding the effects due to imprinting) (Figure S1). The proportion of genes per cross with biased allele expression ratios indicate the percentage of genes displaying *cis*-regulatory variation independent of developmental stage and treatment. The number of significant effects and changes in ASE caused by the developmental stage and/or treatment indicate additional *cis*-regulatory variation responsive to stress and development. Genetic distance analysis showed the greatest divergence between Hsp41-1 and Alexis, followed by Tadmor and Sloop, Tadmor and WI3408, Hsp41-1 and Arta, and finally Sloop and WI3408. The highest proportion of imbalanced genes was detected for HAI, TS and TW, crosses that show the greatest genetic distance. Accordingly, the lowest proportion of genes with imbalanced allelic expression was detected for the cross SW, whose parents were characterized by low levels of genetic variation. We found a high correlation coefficient (0.83) between the genetic distance of parental genotypes and the accumulated effects from models 1 and 2 (Figure S1a). A lower correlation coefficient of 0.44 was detected between the genetic distance and the proportion of genes showing deviation from the balanced allelic expression as calculated using model 1 (Figure S1b). Comparison of genetic distance between parents with the number of significant effects per cross as calculated in GLM model 2 showed an even lower positive correlation coefficient of 0.32 (Figure S1c).

DISCUSSION

ASE and *cis*-acting variation in F_1 hybrids

This study examined allele-specific expression and changes in ASE under drought and different developmental stages in an inbreeding crop species. Our results indicate that *cis*-acting regulatory variation is a common phenomenon in barley, as it is associated with allelic expression imbalances in 63% of all genes (19/30) tested across five different crosses. These findings are supported by the results of a recent genome-wide barley eQTL analysis involving the double haploid population of Steptoe and Morex (Potokina *et al.*, 2008). The authors estimated that more than half of all genes were regulated in *cis*, and that *cis*-regulated genes

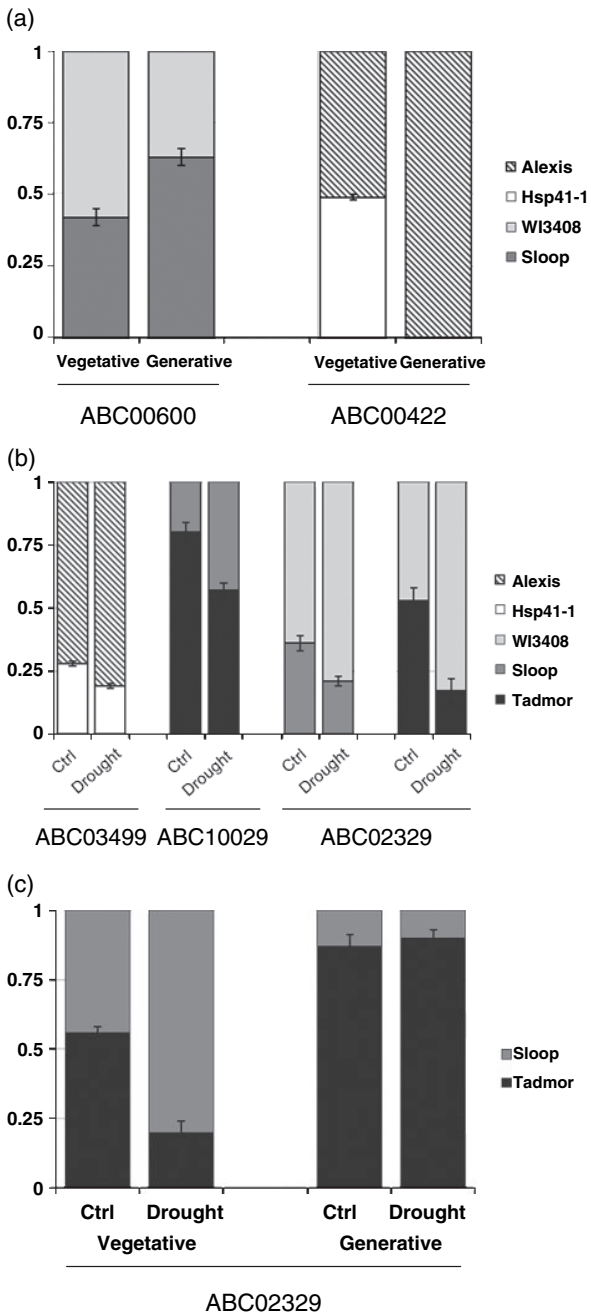

Figure 2. Effects of the developmental stage and/or drought treatment on *cis*-regulatory variation. Relative quantification of allele-specific expression across vegetative and generative stages under control (70% AWC) and drought (10% AWC) conditions in reciprocal hybrids for genes ABC00600 in SW hybrids and ABC00422 in HAI hybrids (a), ABC03499 in HAI hybrids, ABC10029 in TS hybrids, and ABC02329 in TW and SW hybrids (b), and ABC02329 in TS hybrids (c). The relative allele proportion is shown on the *y* axis. Data are from Table 2.

showed greater expression differences than those regulated in *trans*. However, we observed considerable variation in the proportion of the genes regulated in *cis*, ranging from 27 to 61%, when considering each genotype separately. The differences in expression were also influenced by developmental stage and/or drought treatment. Comparison of the results from the statistical models 1 and 2 demonstrated that the number of gene/cross combinations showing imbalanced allele expression independent of developmental stage and environmental conditions was considerably higher than the number of gene/cross combinations displaying changes in allelic expression ratios between developmental stages and/or different environmental treatments. This indicates that ASE in the analysed genes/crosses was presumably influenced by a large number of factors other than those tested, such as tissue, temperature, radiation etc.

The frequencies of *cis*-regulatory variation in barley detected in this study are comparable with those previously detected in other plant species. For maize, Springer and Stupar (2007a) demonstrated that 43–53% of the 316 analysed genes (depending on the cross) showed unequal allelic expression. A subsequent genome-wide ASE analysis using massively parallel signature sequencing showed that 60% of the genes in the maize hybrid meristems exhibited differential allelic expression (Guo *et al.*, 2008). Similarly, a survey of ASE in 30 genes in *Populus* inter-specific F_1 hybrids revealed allelic expression imbalance in 57% of the genes in leaves and stems. The present study revealed a maximum of a 19-fold difference in expression levels (ABC07787 in the TS hybrids, Table 2). Monoallelic expression was observed for only one gene in one hybrid (ABC0422 in HAI hybrids during the generative stage). The levels of allelic imbalance detected in this study were thus as high as, or even higher than, those found in maize and poplar. In Arabidopsis, which, like barley, is an inbreeding plant species, the levels and in particular the frequencies of allelic imbalance detected were lower than in maize (Kiekens *et al.*, 2006, de Meaux *et al.* 2005). De Meaux *et al.* (2005) found up to threefold expression differences within various Arabidopsis species for a chalcone synthase gene. A genome-wide analysis of ASE differences based on a diallele design showed that only 7% of the genes, likely to carry allelic polymorphisms, are responsible for at least 1.5-fold allelic expression differences in a total of ten diploid hybrids (Kiekens *et al.*, 2006). Different approaches to measure ASE may influence the detection of and hence the percentage of genes showing *cis*-acting variation. The low frequency of ASE detected by Kiekens *et al.* (2006) probably underestimates the true proportion of genes that harbor *cis*-regulatory variants in Arabidopsis because of the conservative threshold applied for classifying differential allelic expression, and because only one developmental stage was examined under a particular environmental condition. In addition, different reproductive strategies (inbreeding versus outcrossing), different historical selection pressures (i.e. domestication), differences in genome plasticity or differences in the levels of sequence variation (both nucleotide substitutions as well as structural variants) may

determine the frequencies and levels of *cis*-acting variation and their functional relevance. In maize and poplar, the high frequencies of *cis*-acting regulatory variation have been attributed to high levels of genetic diversity, and were proposed as a potential basis for heterosis (Birchler *et al.*, 2006; Springer and Stupar, 2007b; Zhuang and Adams, 2007). Maize inbred lines can differ essentially in the composition of intergenic regions because of the presence of different types of retroelements (Brunner *et al.*, 2005). Similarly, in barley, a comparison of the *rph7* locus in Morex and Cepada Capa demonstrated a high level of sequence divergence, with neither the type of repetitive elements nor their insertion positions conserved between the two cultivars (Scherrer *et al.*, 2005). The recent burst of transposition activity that seems to characterize many plant genomes studied to date may be responsible for a high level of structural variation that may result in *cis*-regulatory variation (Morgante *et al.*, 2007).

Interestingly, in the present study, two paralogous genes analysed in TS showed contrasting allelic expression, with a 6 times higher expression of the Tadmor allele of ABC0422 and a 6 times higher expression of the Sloop allele of ABC0949 (Figure 1a). ABC0422 and ABC0949 both encode chlorophyll *a*/*b* binding proteins that differ in ten amino acids in the non-conserved regions of the protein. Ancient gene duplications in the grass genomes have generated large gene families, and paralogous genes with redundant functions may act as buffers. The differential expression of these nearly isogenic paralogues could potentially result in barley lines outperforming their parents in terms of expression level, expression timing/duration, and response to developmental and environmental cues, thus providing adaptation to different environments and during domestication and genetic improvement through breeding (Emrich *et al.*, 2007).

The selection of genes under investigation may also influence the detection of *cis*-acting variation. In the present study, assays were designed for genes that are differentially expressed under abiotic stress. It has been shown that stress-related genes are subject to accelerated rates of amino acid substitutions according to the hypothesis of adaptive Darwinian evolution (Frankel *et al.*, 2003). Our findings suggest that the intergenic regulatory regions of these stress-related genes may also be characterized by more rapid changes, thus increasing the frequencies of *cis*-acting variation. Results obtained in maize support these results, with genes relevant for stress tolerance exhibiting the highest levels of *cis*-acting variation (Guo *et al.*, 2004).

ASE varies between developmental stages

In 12% of all gene/cross combinations, allelic expression ratios were significantly different between the vegetative and the generative stage. Most of these changes were detected in TS and HAI hybrids, which are derived from parental lines showing the largest differences in flowering time (data not shown), and differing with respect to their vernalization requirement and response to photoperiod. The genes affected were primarily involved in the photosynthesis (ABC00422, ABC00949), in protection of the photosynthetic apparatus (ABC02113) and in stress signalling (ABC02329). Changes in allelic expression were also detected in ABC00600 and ABC08246, genes that are involved in carbohydrate metabolism and proteolysis and are thus responsible for remobilization of resources and energy. Microarray data have demonstrated that both genes were strongly induced upon drought stress (Guo *et al.*, 2009), and the availability of stored resources may be important under biotic stress conditions, when the rate of photosynthesis is reduced. ABC08264 and ABC02329 also showed interaction effects between developmental stage and drought treatment, and four genes (ABC00871, ABC00949, ABC02113 and ABC02329) that showed an effect for developmental stage also showed significant effects between different treatments in different crosses. These findings suggest that the development of the plant and the stress response are not independent. Senescence, for example, may play a role for both factors, treatment and development stage, as seen in the effects for ABC02113. Drought stress applied at the vegetative and generative stage resulted in different expression regulation, presumably because different *cis*-elements or combinations of *cis*-elements are responsible for governing drought response in the vegetative and generative stages.

ASE varies under different soil moisture regimes

Changes in ASE were studied in adult plants exposed to a gradual reduction of available water at the vegetative and generative stages to mimic drought conditions in the field by slowly reducing water over a period of approximately 7–10 days. Changes in relative allele expression between the control and drought-treated plants were observed in 12% of all gene/cross combinations involving six different genes. The majority of effects were detected in HAI hybrids, which are derived from the parents from the most contrasting environments with respect to water availability, indicating the adaptive role of *cis*-regulatory variation. Hybrids derived from Hsp41-1 and Arta, which both grow in the same drought-prone environments, did not show any changes in allelic expression upon drought stress. This lack of *cis*-acting variation suggests that both parental alleles show similar expression patterns as an adaptation to the same environmental conditions. Genes responsible for stress protection (ABC02113, ABC03499 and ABC10029), stress signalling (ABC02329) and lipid transport (ABC0871) showed changes, indicating that differential expression of these stress-related genes may affect drought adaptation of the barley line. Guo *et al.* (2004) observed changes in ASE in maize hybrids subjected to drought and high-density planting stress. Interestingly, the gene with the strongest differences in ASE between different environments was also a lipid transfer protein, which is known to respond to stress. However, a

functional relationship between allelic expression differences in these genes and phenotypic performance remains to be established.

ASE in different genetic backgrounds

In order to evaluate regulatory properties of different haplotypes in different genetic backgrounds, the relative expression of parental alleles assayed in at least two different crosses was compared. When we looked for haplotype differences of the sequenced gene segments as a marker of linked polymorphisms in *cis*-acting elements, we were able to associate allelic expression activity to a particular haplotype. These correlations between haplotypes and linked *cis*-acting variation indicate that differences in expression patterns could be forecast based on haplotypes. However, although for the majority of the genes the pattern of ASE corresponded to haplotype qualities, for a few of them this correspondence could not be confirmed. These cases could reflect the proportion of genes whose expression is influenced by *trans*-acting factors that act differentially on the *cis*-acting regulatory elements of the haplotypes under consideration (*cis*-specific *trans*-regulation). In addition, the available sequence information only included gene segments, and haplotype differences could not be scored for the corresponding *cis*-regulatory regions.

The analysis of different haplotypes in different genetic backgrounds also suggested that *cis*-acting variation may not always be additive, as seen, for example, in gene ABC00871. Based on the ASE results in TS and TW, a 1.5-fold higher expression of the Sloop over the WI3408 allele would have been expected, but a threefold difference was actually observed in SW; however, this showed variation across the control and drought treatments (Figure 1a). Tao *et al.* (2007) showed that ASE of keratin-1 (KRT1) in human white blood cells results from the haplotypic combinations and interactions of five *cis*-regulatory elements, showing that *cis*-regulatory variation acts as a complex trait.

Genetic distance and ASE variation

We were interested in analysing whether there is a correlation between the levels of *cis*-acting variation in hybrids and the genetic relationship and/or eco-geographic origin of the parents. The analysis showed a positive correlation between genetic distance and the proportion of genes with ASE differences as compared to the hybrid genomic DNA, indicating that *cis*-acting polymorphisms accumulate proportionally to the divergence of the genomes (Figure S1). Similar results were obtained in *Drosophila*, where the mean percentage of regulatory divergence explained by *cis*-regulatory differences was 35% within species and 64% between species (Wittkopp *et al.*, 2008). However, the correlation between genetic distance and the proportion of genes showing changes in ASE between developmental stages and upon drought stress was considerably lower, suggesting that the occurrence of *cis*-regulatory variation is not only associated with genetic distance, but also with adaptation to different environments. The cross between Hsp41-1 and Arta, for example, with a rather high genetic distance, had a low number of significant effects of development or treatment, presumably because both parents are adapted to similar environmental conditions. By contrast, hybrids derived from Sloop and WI3408, with a low genetic distance but different drought adaptation, exhibited a higher relative number of genes with changes in ASE ratios between both developmental stages and treatments. These findings are in line with the different adaptive strategies of both parents, despite similar genetic backgrounds.

In order to test the hypothesis that barley domestication may have involved large changes in *cis*-variation, we included a wild barley accession in our analysis. However, the two different crosses with wild barley displayed very different levels of *cis*-acting variation, suggesting that the largest differences in *cis*-elements do not occur between domesticated and wild barley, but between lines with high genetic distances and adaptation to different environments. Nevertheless, we need to take into consideration that the correlation analysis was only performed for the five different crosses with different genes analysed in each cross. In addition, the number of genes and hence the significant effects observed were rather low in some crosses (HAr and SW). Therefore, correlation coefficients should be viewed with caution and may only indicate a trend: the amount of *cis*-regulatory variation increases with genetic distance, as also seen in Figure S1, where the accumulated effects from models 1 and 2 and genetic distance show a high correlation coefficient of 0.83.

CONCLUSIONS

Plant adaptation through natural selection or breeding is achieved by fine-tuning of dynamic processes such as reproduction, development and stress tolerance. This fine-tuning may be more readily realised through gradual changes in gene regulation rather than protein structure, which is generally more static. Indeed, for decades, evolutionary biologists have argued that changes in regulatory sequences, and in particular in *cis*-regulatory sequences, constitute an important part of the genetic basis for adaptation. In the present study, we have demonstrated that ASE differences may play an important role in barley and accumulate with genetic divergence and possibly with adaptation to different environments. In addition, changes in ASE between different water regimes and developmental stages indicated the presence of *cis*-regulatory elements that are responsive to drought and developmental cues. Future comparative studies using information from Arabidopsis and rice will help to identify these *cis*-acting elements in barley. Further studies are required to link the natural variation in regulatory regions and the associated expression

differences with phenotypic performance. Detailed genetic maps of *cis*-acting elements and their effects can then be exploited in order to breed better-adapted varieties.

EXPERIMENTAL PROCEDURES

Plant materials and experimental design

Six barley genotypes were used to generate reciprocal hybrids. The parental genotypes were selected to represent different germplasm pools, including wild barley accession Hsp41-1, two landrace selections, Tadmor and Arta, two Australian cultivars, Sloop and WI3408, and a German barley cultivar Alexis. The wild barley accession *Hordeum vulgare* ssp. *spontaneum* Hsp41-1 was selected for its adaptation to severe drought (Baum *et al.*, 2003). Tadmor and Arta are Syrian landraces that are well adapted to the driest sites of the country (Weltzien, 1988). The Australian genotype WI3408 is a malting barley with good adaptation to dry environments in Western Australia. Sloop is an Australian malting barley that is more susceptible to drought than WI3408. Finally, Alexis is a German malting barley tat is adapted to the Middle European climate. Tadmor, Sloop and WI3408 were used to generate all possible F_1 and RF_1 crosses, which are designated as TS for Tadmor × Sloop (Sloop × Tadmor), TW for Tadmor × WI3408 (WI3408 × Tadmor) and SW for Sloop × WI3408 (WI3408 × Sloop). In addition, two reciprocal crosses Arta × Hsp41-1 (Hsp41-1 × Arta) and Alexis × Hsp41-1 (Hsp41-1 × Alexis) were generated, abbreviated as HAr and HAl, respectively.

Three vernalized seedlings of the same cross were transplanted into a 3.0 L pot (15 cm in height and 16 cm in diameter) filled with 2.2 kg of sterilized field soil, which contained about 6% water. Field capacity, wilting point and the available water content (AWC) of the soil were measured in the soil laboratory of the International Center for Agricultural Research in the Dry Areas (ICARDA) (Tel Hadya, Aleppo, Syria, http://www.icarda.org). Control and drought conditions corresponded to 70% and 10% AWC in the soil, respectively (Doorenbos and Pruit, 1977).

The pot experiment was arranged in a randomized complete-block design with the two treatments (well-watered and drought stress) applied at two developmental stages, vegetative and generative. Three plants per pot were planted in three replications for each combination of cross (F_1, RF_1) by stage (vegetative, generative) and treatment (control, drought). Each cross was thus planted in 24 pots, each with three plants, amounting to a total of 72 plants. The plants were grown under 16 h daylight at 28°C and an 8 h dark period at 20°C under controlled conditions in a greenhouse at ICARDA. The drought treatment was started by withholding water at two developmental stages, the vegetative stage (4–5 leaves) and the generative stage (post-anthesis). The soil moisture in pots under well-watered and drought stress conditions was maintained by the required amounts of water by weighing the pots and watering the plants every day. The relative water content was measured in two fully expanded leaves as described by Teulat *et al.* (1997), and plants with similar relative water contents were selected for RNA extraction. From these plants, the second leaf from the top was harvested at day 3 after the AWC in the soil reached 10%, and the leaves from the three plants were pooled. Immediately after collection, the sampled leaf material was placed into liquid nitrogen and stored at −80°C.

Nucleic acid extraction and cDNA synthesis

For the crosses TS and SW, two separate total RNA extractions from the same leaf material (technical replicates) were performed, while for the three remaining crosses only one RNA extraction for each of the three biological replicates consisting of three pooled plants was used for the assays. Nucleic acid extraction and cDNA synthesis were performed as described by Salvi *et al.* (2007).

Allele-specific expression assay

Fifty genes showing expression changes under abiotic stress (Rostoks *et al.*, 2005) and exhibiting a high number of SNPs in the associated EST sequences were selected from the barley SNP database (http://bioinf.scri.ac.uk/barley_snpdb/). In addition, 20 genes responsive to drought were chosen on the basis of a microarray experiment comparing expression changes under drought in three barley genotypes (Guo *et al.*, 2009). Genic segments of these genes were amplified across six genotypes, namely Alexis, Arta, Hsp41-1, Sloop, Tadmor and WI3408 using the primer pairs indicated in Table S1, and direct sequencing of the products was performed in order to identify polymorphisms between lines. Base calling and sequence assembly were performed using Phred and Phrap (Ewing and Green, 1998; Ewing *et al.*, 1998). SNPs were identified using PolyPhred (Nickerson *et al.*, 1997) and confirmed by manual examination of sequence assemblies in Consed (Gordon *et al.*, 1998). Thirty genes selected for the allelic expression analysis had at least one transcribed SNP in one of the five crosses. PCR primers that flanked the marker polymorphism were designed using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer_3www.cgi). Single base extension (SBE) primers were designed with a minimum length of 18 nucleotides. All PCR and SBE primer sequences are listed in Table S1. PCR amplifications, primer extension reactions and detection on an Applied Biosystems 3730 DNA analyser (http://www.appliedbiosystems.com/) were performed as described by Salvi *et al.* (2007).

Calibration

Parental mixes of genomic DNA were prepared in proportions of 0.05:0.95, 0.1:0.9, 0.25:0.75, 0.5:0.5, 0.75:0.25, 0.9:0.1 and 0.95:0.05, and SBE reactions on these templates were run alongside those on cDNAs and three genomic samples obtained from each of the F_1 and RF_1 hybrids. The genomic parental mixes allowed the construction of a titration curve by regressing the observed peak ratios onto the expected peak ratios and forcing the curve through 0 and 1. For the majority of genes, the standard titration curve was best modelled by a second-degree polynomial equation. The observed cDNA peak ratios were calibrated by solving the titration curve equation for the expected proportion. The obtained proportions were then normalized on the basis of the peak height ratio measurements obtained from SBE on hybrid genomic DNA (three replications each for F_1 and RF_1), representing a perfect 50:50 proportion of the two alleles. The final allelic expression proportions were adjusted by subtracting the calibrated F_1/RF_1 mean from the calibrated cDNA data and adding 0.5.

Statistical analyses

First we investigated whether the allelic expression proportions of the hybrid cDNA deviated from those of genomic F_1 and RF_1 samples without distinguishing between the different cross directions, developmental stages and treatments. The calibrated allele proportions of the parental DNA mixes were tested for significant differences from those of the hybrid DNA. In the absence of significant differences, the results from the parental mixes (multiplied by the inverse of their mix proportions) were used together with the hybrid allele proportions for comparisons with cDNA; in the case of significant differences, only the data obtained for hybrid DNA were used for these comparisons.

In order to determine the deviation of the cDNA allele expression proportions from those of the hybrid genomic DNA, a one-way ANOVA was computed using the following fixed model in the SAS general linear model (GLM) procedure:

$$Y_{ij} = \mu + A_i + \varepsilon_{ij} \quad (1)$$

where Y_{ij} corresponds to the ASE expression ratios, and the factor A_i contains two levels (for cDNA and genomic DNA). Least-squares estimates of means of the hybrid DNA allele proportions and the cDNA allele expression proportions were calculated within the GLM procedure. The estimate statement was used to calculate the differences between hybrid DNA allele and cDNA allele expression proportions.

In the second step, we tested for effects of cross direction, developmental stage and treatment on allelic expression proportions without using measurements of the genomic hybrid DNA. A three-way ANOVA was performed using the following fixed model in the SAS general linear model (GLM) procedure (SAS version 9.1, SAS Institute, 2003):

$$Y_{ijkm} = \mu + I_i + S_j + T_k + IS_{ij} + IT_{ik} + ST_{jk} + IST_{ijk} + \varepsilon_{ijkm} \quad (2)$$

where Y_{ijkm} corresponds to the ASE ratios, and I_i, S_j and T_k correspond to the fixed effects of the cross direction (F_1, RF_1), the developmental stage (vegetative, generative) and the treatment (control, drought), respectively, and IS_{ij}, IT_{ik}, ST_{jk} and IST_{ijk} are the corresponding interaction effects. The variance explained by the different effects was calculated by dividing the sums of the squares of *I*, *S*, *T* and the interaction effects by the total sums of the squares. Within model 2, least square means were calculated for each combination of gene, cross direction, developmental stage and treatment. Significant effects in models 1 and 2 were determined using a false discovery rate of 0.05 (Benjamini and Yekutieli, 2005). The analyses were performed for each of the five crosses separately.

Correlation between genetic distance and proportion of genes showing allelic imbalance

The parental genotypes were genotyped using 28 SSR markers (Table S4) with the objective of calculating genetic distance coefficients. PCR amplifications were performed as described by Ramsay *et al.* (2000), Costa *et al.* (2001) and Rostoks *et al.* (2005), and PCR fragments were separated on an Applied Biosystems 3100 DNA analyser.

The data from the six parental lines and 28 SSR markers were used to compute pairwise simple matching coefficients (Sokal and Michener, 1958). Correlation coefficients were calculated between the simple matching coefficients and (i) the proportion of genes per cross showing allelic imbalance across both developmental stages and treatments (significant effects in model 1), (ii) the proportion of significant effects in model 2, the proportion of changes in ASE between developmental stages and treatments (and interactions), and (iii) the added effects from (i) and (ii).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Correlation of genetic distance between parents of the derived hybrids and ASE.

Table S1. Sequencing primers, PCR amplification primers and SBE primers for every gene and fragment analysed, and SNPs targeted for the ASE analysis in the various barley lines.

Table S2. Significant deviations of the cDNA allele expression ratios from the hybrid DNA allele ratios calculated for every cross and gene separately.

Table S3. Results of the three-factorial ANOVA.

Table S4. SSR markers used for calculating the genetic distance between the six parental genotypes Alexis, Arta, Hsp41-1, Sloop, Tadmor and WI3408.

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