

*Full Length Research Paper*

# Cloning, localization and phylogenetic analysis of barley putative APETALA 2/ethylene responsive element binding protein (AP2/EREBP) genes

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Eight putative barley AP2/EREBP genes were cloned based on the barley HarvEST database. The introns in the translated regions of these genes were all located at the front of the conserved AP2 domain. Among the eight genes, DNA sequences of hv.18885 and hv.17070 were the same between Steptoe and Morex. Of the other six genes, there was very high ratio of 'G' and 'C' bases in the variant DNA sequence regions of three genes between Steptoe and Morex. Thus, only three genes were successfully located on the chromosomes. hv.8737 and hv.15732 were mapped to the short arm of chromosome 7H and hv.2871 to a major quantitative trait loci (QTL) region conferring adult spot blotch tolerance on the short arm of chromosomes 3H. Phylogenetic analysis showed that putative plant AP2/EREBP genes in the AP2 subfamily were completely separated from those in other subfamilies. The putative AP2/EREBP genes in RAV and Soloist subfamilies were clustered together, while those in the ERF and CRT/DRE subfamily were clustered together. Among the main branch-groups of each subfamily in plant, those from wheat, barley and rice had the tendency to cluster together when compared with those from Arabidopsis.

**Key words:** AP2/EREBP, clone, map, phylogenetic, barley.

## INTRODUCTION

AP2/EREBP (APETALA 2/ethylene responsive element binding protein) genes are characterized by the presence of the AP2-DNA binding domain which is about 60 amino

acids in length (Okamuro et al., 1997). They are involved in various aspects of plant growth and development such as flower development, hormone signal transduction and cellular differentiation (Wilson et al., 1996; Karim et al., 2009; Krizek, 2009). Meanwhile, they are known to be involved in responses to biotic, pathogenic and environmental stresses (Choi et al., 2002; Yi et al., 2004; Feng et al., 2005; Jung et al., 2007).

Among the more than 2000 transcription factors identified in the Arabidopsis thaliana genome (<http://plantfdb.cbi.edu.cn/>), about nine percent belong to the AP2/EREBP family (Dietz et al., 2010). The AP2/EREBP genes are classified into five subfamilies including

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**Abbreviations:** AP2/EREBP, APETALA 2/ethylene responsive element binding protein; ERF, ethylene response factors; CRT/DRE, C-repeat binding/dehydration-responsive element-binding factors; RAV, ABI3/VP1; DH, doubled haploid; NJ, neighbour-joining; QTL, quantitative trait loci.

**Table 1.** PCR primers designed from conservative regions in the putative barley *AP2/EREBP* genes.

Unigene	Primer	Temperature (°C)	Material	NCBI Number	Length (bp)
<i>hv.17070</i>	F: CGATTGCGAGCTCTAGATACC	55	Morex	HQ647351	3324
	R: CAAAAGAAACACCAAACAGAGAAG		Steptoe	HQ647352	3324
<i>hv.15734</i>	F: CGGCGTGGTATCAGCGGC	60	Morex	HQ647357	2252
	R: ACAGGGTGCTTCCCAATCTCC		Steptoe	HQ647358	2264
<i>hv.7703</i>	F: TCTCGTGGGCTCAAGTCTTCTCT	55	Morex	HQ647355	948
	R: GCATTGTGATAGTCGTGCTCG		Steptoe	HQ647356	951
<i>hv.17532</i>	F: GCGCACGCCACTTGTACAC	58	Morex	HQ647353	841
	R: CAAAACGAGGAAGGAAGGAGG		Steptoe	HQ647354	841
<i>hv.8737</i>	F: CTCGTGTTCTCWTCTCCATAGC	60	Morex	HQ647361	1165
	R: ACAATACAAACCAACCAATATCAG		Steptoe	HQ647362	1165
<i>hv.18885</i>	F: CCCTTCCTCCCTCCTCTTCC	58	Morex	HQ647359	1662
	R: AGCTTCAGGTTGCAGATAATTTG		Steptoe	HQ647360	1662
<i>hv.2871</i>	F: TCTTCCACACCGACGGCATCT	55	Morex	HQ647363	614
	R: TGCTCGGGAACAGACAC		Steptoe	HQ647364	614
<i>hv.15732</i>	F: GGCAACTGCTGCTGGTGAAT	58	Morex	HQ647365	822
	R: ACCCAGCAACCTCAATTCC		Steptoe	HQ647366	822

response factors (ERF), C-repeat binding/dehydration-responsive element-binding factors (CRT/DRE), ABI3/VP1 (RAV), AP2 and Soloist (Sakuma et al., 2002; Gutterson and Reuber, 2004). The AP2/EREBP genes in species such as poplar, rice, barley and wheat have been studied by comparing them to those of *A. thaliana* (Nakano et al., 2006; Zhuang et al., 2008, 2010, 2011).

Barley (*Hordeum vulgare* L.), one of the major food and feed crops in the world, is more widely adaptable to unfavourable growth conditions such as cold, dry and saline environments (Jung et al., 2007). A few AP2/EREBP genes (HvRAF, HvCBF1, HvCBF2 and HvCBF3) have been characterized in barley (Choi et al., 2002; Xue, 2003; Xue and Loveridge, 2004; Jung et al., 2007). Clustering, amino acid sequences analysis, and expression assessment of barley putative AP2/EREBP genes had been done by Zhuang et al. (2011). However, gene structure, chromosomal localization, and phylogenetic analysis of barley AP2/EREBP genes were not reported. In this study, we identified the DNA sequences of eight putative barley AP2/EREBP genes, cloned and mapped several genes to chromosomes, and constructed a phylogenetic tree of putative AP2/EREBP

genes in Arabidopsis, rice, wheat and barley.

## MATERIALS AND METHODS

### Plant materials and DNA isolation

A doubled haploid (DH) population of barley, produced by a modified bulbosum method from a cross between Steptoe and Morex (Chen and Hayes, 1989), was used in this study. One hundred and fifty lines and their parents were grown in a greenhouse and genomic DNA was extracted from single plants following the CTAB procedure (Sambrook et al., 1989).

### Cloning and sequencing the putative barley AP2/EREBP genes

To obtain genomic DNA sequences of the putative barley AP2/EREBP genes, PCR primers (Table 1) were designed using barley EST sequences from the HarVest database and tested against the two parents of the mapping populations, Steptoe and Morex. The PCR reaction volume was 25 µl, containing approximately 0.2 µg template DNA, 2.5 units Taq DNA polymerase with high fidelity (Takara Bio, Inc., Kyoto, Japan), 0.3 µM each primer (Table 1), 200 µM each of dNTP (Takara Bio, Inc., Japan), 1.5 mM MgCl<sub>2</sub>, and 1× PCR buffer. PCR amplifications were carried out in a PTC-240 thermocycler (Genetic Technologies, MJ

**Table 2.** Specific primers designed from SNPs in the putative barley *AP2/EREBP* genes.

Unigene	Primer	Temperature (°C)	Material	Length (bp)
<i>hv.15732</i>	GAATATGACTGTGCCTCTGA	58	Morex	0
	ACCCAGCAACCTCAATTCC		Steptoe	806
<i>hv.2871</i>	TCTTCCACACCGACGGCATCT	55	Morex	0
	CCATGCCTCGGCCAAAGACA		Steptoe	409
<i>hv.8737</i>	CTCGTGTTCCTCWTCTCCATAGC	58	Morex	0
	CCTACACCGCCGACCGTTAA		Steptoe	1070

The bold letters with underline at the 3' end means extra mismatched nucleotides.

Research, USA). The PCR reaction conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55 to 60°C for 60 s and 72°C for 1.5 min, with a final extension at 72°C for 7 min. Amplified products were directly visualized by electrophoresis using 1.5% agarose gels. The PCR products were purified, cloned and sequenced in both directions by BGI Life Tech Co., Ltd, Shanghai, China.

The intron/exon structure was determined by blasting cDNA sequence based on the EST sequences from barley HarvEST and NCBI databases and each gene DNA sequence was cloned with DNAMAN 5.2.2 (<http://www.lynnon.com>).

#### Linkage mapping of the putative barley *AP2/EREBP* genes

DNA sequences between the two barley cultivars Steptoe and Morex were compared by blast with DNAMAN 5.2.2 (<http://www.lynnon.com>) (NCBI No: HQ647351-HQ647366, eight genes from two different cultivars). Based on differences of DNA sequences between Steptoe and Morex, PCR primers (Table 2) were designed to allow the detection of base substitutions between these two cultivars. The primers of five other genes were not successfully designed due to same DNA sequences between Steptoe and Morex or very high ratio of 'G' and 'C' bases in the regions with base substitutions or indels between the two cultivars. The 150 DH lines derived from a cross between these two cultivars were then genotyped with the markers developed, and their map locations were determined by aligning their genotypic profiles with those existing genotypic data of the mapping population ([http://wheat.pw.usda.gov/ggpages/map\\_shortlist.html](http://wheat.pw.usda.gov/ggpages/map_shortlist.html)) (Kleinhofs et al., 1993; Rodrigues et al., 2006; Stein et al., 2007). Linkage maps were constructed using Map Manager Version 3.0 (Lincoln et al., 1993) and drawn with the winQTLCart 2.5 (Wang et al., 2007).

#### Phylogenetic analysis

Phylogenetic trees of putative *AP2/EREBP* genes had been constructed in some plant species, such as Arabidopsis, rice, wheat and barley (Nakano et al., 2006; Dietz et al., 2010; Zhuang et al., 2010, 2011). Based on these phylogenetic trees, one random gene from each main cluster in each main branch-group of the five subfamilies was used for multiple protein sequence alignments. Putative *AP2/EREBP* gene without conserved *AP2* domain or absence in some branch-group of each subfamily in these plant species were not used in this study. The sequences analysis was carried out using DNAMAN 5.2.2 (<http://www.lynnon.com>) and results obtained were used for phylogenetic analysis. Phylogenetic

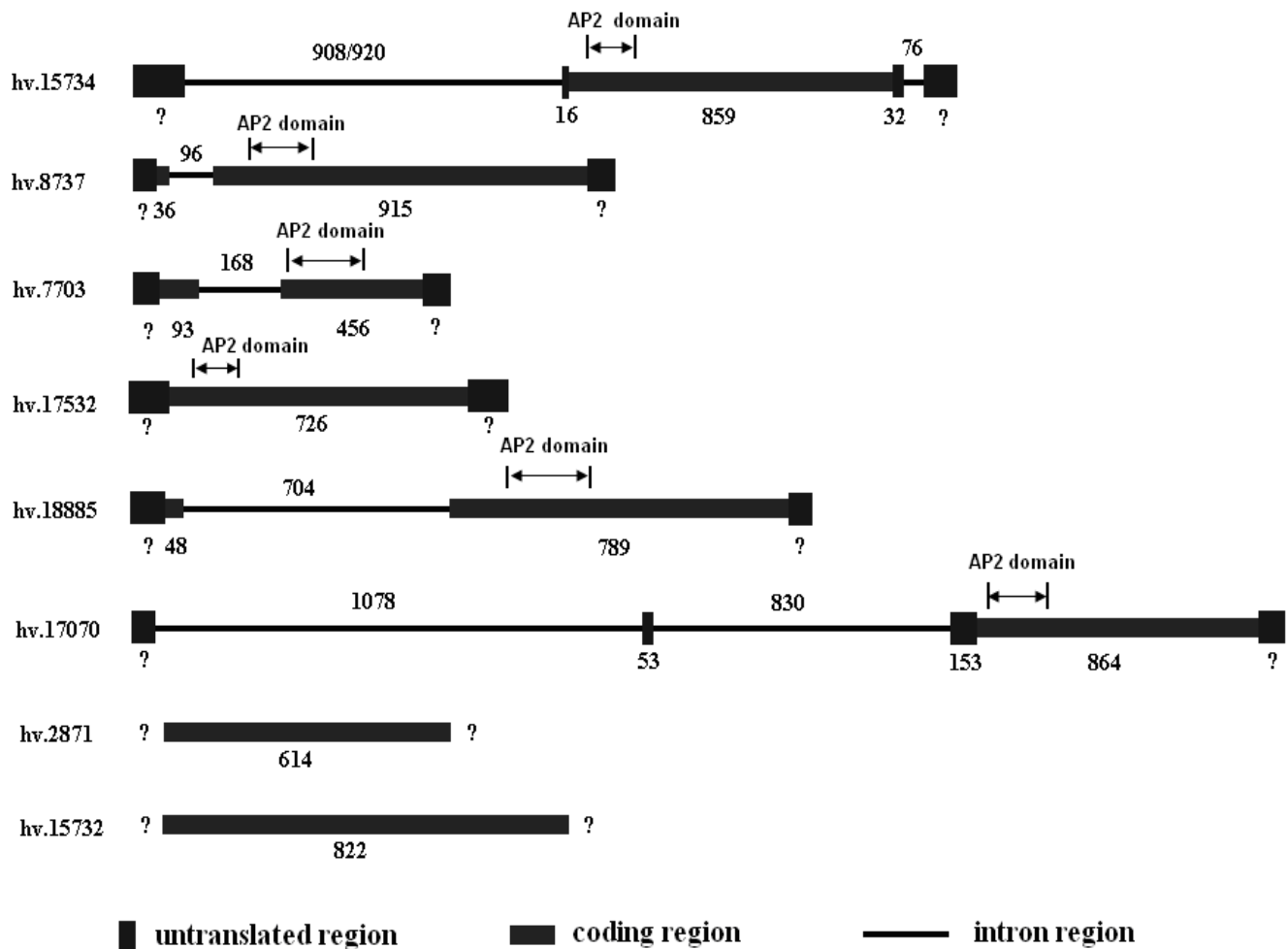
trees were constructed using the Neighbour-Joining (NJ) method based on MEGA 4 (Tamura et al., 2007). Secondary structures of predicted proteins were analysed using Lasergene Version 8.0 (Allex, 1999).

## RESULTS

### Putative *AP2/EREBP* genes cloned from barley

The unigene code of the HarvEST database of barley was used except for *hv.8737* as an identical code for this gene did not exist in the NCBI database. A total of eight barley DNA sequences were obtained. Of these, six (*hv.15734*, *hv.8737*, *hv.7703*, *hv.17532*, *hv.18885* and *hv.17070*) contained intact exons, and the remaining two (*hv.2871* and *hv.15732*) were partial gene sequences (Figure 1). These eight genes belonged to either the DREB or the ERF subfamily. For the six genes containing intact exons, *hv.8737*, *hv.18885* and *hv.7703* possessed one intron in the translated region, while *hv.17532* and *hv.15734* contained no intron in the translated region. *hv.17070* and *hv.15734* possessed two introns in the untranslated region.

The intron sizes of the putative barley *AP2/EREBP* genes ranged from 76 to 1078 bp. All the introns in the translated region were located in the front of the conserved *AP2* domain. Of the eight putative barley *AP2/EREBP* gene sequences, differences between Morex and Steptoe were found for neither *hv.17070* nor *hv.18885*. Differences in base substitutions or indels for the other six were identified in the translated or untranslated regions. For instance, there were four base substitutions and two indels in intron I of *hv.15734* between Morex and Steptoe (HQ647357 and HQ647358). Among the six putative *AP2/EREBP* genes with base substitutions or indels between Morex and Steptoe, only two (*hv.8737* and *hv.15732*) had different amino acid sequences. For *hv.8737*, the residue was 'G' at position 293 for Morex but 'V' for Steptoe. For *hv.15732*, the residue was 'G' at position 97 for Morex but 'E' for Steptoe. However, the difference in amino acid



**Figure 1.** Exon/intron structures of eight putative barley *AP2/EREBP* genes. The lengths of the introns each gene was shown above the each gene-figure, while the lengths of untranslated and coding regions were shown under the each gene-figure. Fragment length of Steptoe is the front in two numbers parted by "/", while that of Morex is the latter. Rectangle: exon; line: intron; '?': unknown number of DNA base.

sequences between Steptoe and Morex did not result in the change of their secondary structures by analysis using Lasergene Version 8.0.

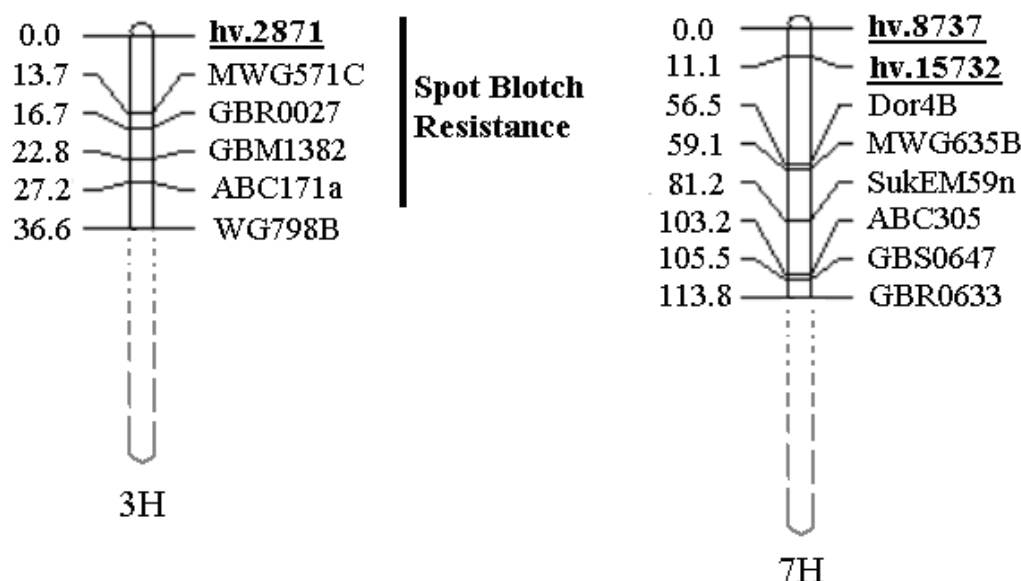
#### Mapping barley putative *AP2/EREBP* genes

Locus-specific primers were successfully designed for three of the putative barley *AP2/EREBP* genes based on the differences of DNA sequences between Steptoe and Morex (Table 2). Sequence differences for two (hv.18885 and hv.17070) of the other five genes were not present between these two varieties and those for the remainder (hv.15734, hv.7703 and hv.17532) contained very high ratio of 'G' and 'C' bases in the regions with base substitutions or indels. One of these genes for which primers were obtained, hv.2871, was mapped at the

distal end of the short arm of chromosome 3H with a distance of 13.7 cm from the marker MWG571C. The other two genes, hv.8737 and hv.15732, were both mapped on the distal end of the short arm of chromosome 7H. The distance between these two genes was 11.1 cm, and, hv.8737 was more distally located (Figure 2).

#### Phylogenetic tree of putative *AP2/EREBP* genes in plant

Putative *AP2/EREBP* genes in plant could be clearly divided into five subfamilies, which were consistent with the denoted group related to *APETALA2* (Figure 3). The putative *AP2/EREBP* genes in the *AP2* subfamily were completely separated from others, whereas those in the



**Figure 2.** Map locations of the putative barley *AP2/EREBP* genes with MAPMAKER (LOD=3.0). The genotypes of each putative *AP2/EREBP* gene in this mapping population were compared with mapping data (SNP, SSR, RFLP, genes and SSAP). The map location of putative *AP2/EREBP* genes is underlined. The dark bar on chromosomes (3H) indicate quantitative trait locus (QTL) from barley cv Steptoe × barley cv Morex mapping population (Bilgic et al., 2005).

RAV and Soloist subfamilies were clustered together. Those in the ERF and CRT/DRE subfamilies were also clustered together. Among the main branch-groups of each subfamily in plant, those from wheat, barley and rice had the tendency to cluster together as compared to those from Arabidopsis. However, it was found that two putative *AP2/EREBP* genes (ta.50437 and ta.56957) in CRT/DRE subfamily from wheat were not grouped together with others in CRT/DRE subfamily.

## DISCUSSION

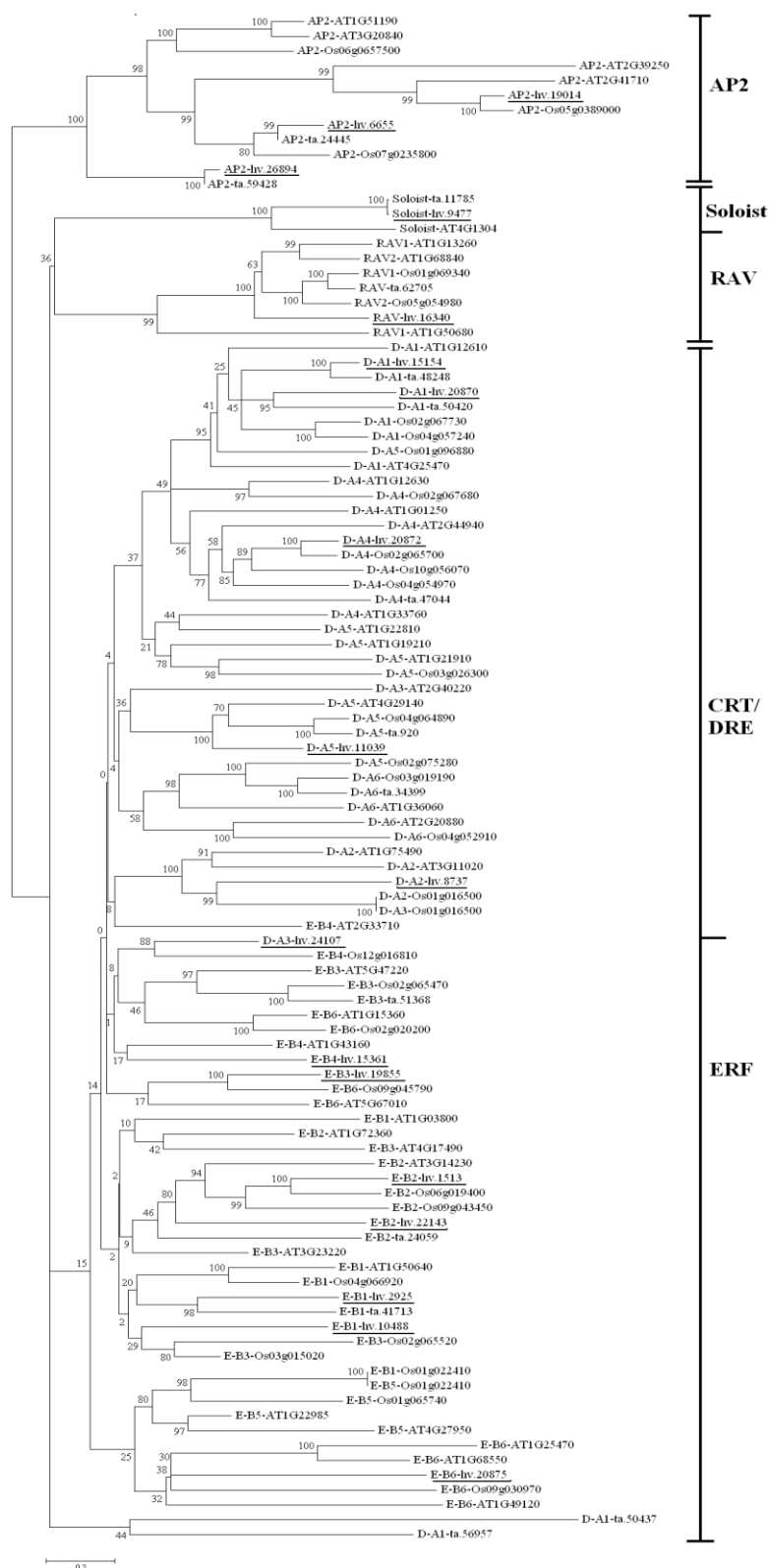
Previous scientists analyzed and clustered the 147 *AP2/EREBP* genes in Arabidopsis, 164 in rice and 117 in wheat (Sakuma et al., 2002; Nakano et al., 2006; Riano et al., 2007; Zhuang et al., 2011), and only 53 in barley (Zhuang et al., 2011). Of these four species, genes belonging to the Soloist subfamily have not been detected in rice. Though the numbers of the *AP2/EREBP* genes differ among the four species, the percentage of genes in each of the subfamilies (excluding those for the Soloist subfamily) was similar (Zhuang et al., 2010). As the barley genome is approximately 40 times larger than that of Arabidopsis and 10 times larger than that of rice (Arumuganathan and Earle, 1991), it is thus expected that additional *AP2/EREBP* genes could exist in barley.

Though sizes and cleavage sites of introns of putative barley *AP2/EREBP* genes cloned were varietal, the locations of introns in regulated region were all on the

front of conserved *AP2* domain, not inside. The characteristic of intron was also found in those universal stress proteins in plant (Li et al., 2010). The reason may be to avoid disappearance of conserved *AP2* domain when false cleavage of introns occurred.

The three putative barley *AP2/EREBP* genes mapped in this study were located on two different chromosome regions. On the basis of previous quantitative trait loci (QTL) mapping of the 'Morex × Steptoe' population, linkage analysis showed that only hv.2871 falls into a major QTL region conferring adult spot blotch tolerance on chromosome 3H (Bilgic et al., 2005). The mapping results could also supply a molecular basis for developing candidate genes related with biotic or abiotic tolerance and development of plant lines by combining QTL results obtained in the future. Additionally, the three genes could be used for Marker-Assisted Selection after identifying their biological function. On the basis of the phylogenetic tree obtained for Arabidopsis, wheat, rice and barley, the putative *AP2/EREBP* genes in monocots were clustered together in main branch-groups of each subfamily by comparing with those in dicots. It is inferred that there were species specificities in each subfamily of the putative *AP2/EREBP* genes, which also is proved by similar clustering tendency of each subfamilies of Arabidopsis, wheat, barley and rice.

On the basis of clustering results, the molecular evolution of the *AP2/EREBP* genes in *AP2* subfamily might be independent, whereas those between RAV and Soloist subfamilies might be related with each other as



**Figure 3.** Phylogenetic tree constructed with the Neighbour-Joining method and Poisson correction of the AP2/EREBP in *Arabidopsis*, rice, wheat and barley. D, E, R, AP2 and Soloist represent CRT/DRE-, ERF-, RAV-, AP2- and Soloist-types, respectively. The barley AP2/EREBP genes are underlined.

well as those between ERF and CRT/DRE subfamilies. Additionally, ta.50437 and ta.56957 were separated from other genes of CRT/DRE subfamily in Arabidopsis, wheat, rice and barley, which may be caused by different genotype. It is inferred that there were some specific AP2/EREBP genes in CRT/DRE subfamily of hexaploid wheat.

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