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A genome-wide analysis of the *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES* (*AS2/LOB*) gene family in barley (*Hordeum vulgare* L.)*#

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Abstract: *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES* (*AS2*/*LOB*) genes are a family of plant specific transcription factors, which play an important role in the regulation of plant lateral organ development and metabolism. However, a genome-wide analysis of the *AS2*/*LOB* gene family is still not available for barley. In the present study, 24 *AS2-like* (*ASL*)/*LOB domain* (*LBD*) genes were identified based on the barley (*Hordeum vulgare* L.) genome sequence. A phylogenetic tree of ASL/LBD proteins from barley, *Arabidopsis*, maize, and rice was constructed. The *ASL*/*LBD* genes were classified into two classes, class I and class II, which were divided into five and two subgroups, respectively. Genes homologous in barley and *Arabidopsis* were analyzed. In addition, the structure and chromosomal locations of the genes were analyzed. Expression profiles indicated that barley *HvASL/LBD* genes exhibit a variety of expression patterns, suggesting that they are involved in various aspects of physiological and developmental processes. This genome-wide analysis of the barley *AS2/LOB* gene family contributes to our understanding of the functions of the *AS2/LOB* gene family.

Key words: Barley, *AS2/LOB* gene family, Phylogenetic tree, Expression pattern
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1 Introduction

The plant-specific *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES* (*AS2*/*LOB*) gene family contains transcription factors which play an important role in the regulation of plant lateral organ development. *AS2-like* (*ASL*)/*LOB domain* (*LBD*) genes are also involved in the regulation of anthocyanin and nitrogen metabolism (Rubin *et al*., 2009; Majer and Hochholdinger, 2011; Koppolu *et al*., 2013; Xu *et al*., 2016). *ASL*/*LBD* genes encode a protein containing a conserved amino acid domain of unknown function, termed the AS2/LOB domain.

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AS2/LOB domain recognized a 6-bp GCGGCG consensus motif and interacts with a specific basic helix-loop-helix (bHLH) protein (Husbands *et al*., 2007). According to the structure of the AS2/LOB domain in the N-terminus, the *AS2/LOB* gene family can be divided into two classes, class I and class II. Class I contains a conserved CX2CX6CX3C zinc finger-like motif and an LX6LX3LX6L leucine zipper-like coiled-coil motif (Shuai *et al*., 2002). There are also two conserved blocks, the C block and GAS block, in the AS2/LOB domain of the class I proteins (Husbands *et al*., 2007). However, class II *ASL*/*LBD* genes have only a conserved zinc finger-like domain (Shuai *et al*., 2002).

In *Arabidopsis*, 43 *AS2/LOB* gene family members have been identified (Shuai *et al*., 2002; Matsumura *et al*., 2009). Based on a genome scan of the published genome sequence and protein sequence similarity in different species, 35 rice and 44 maize *ASL*/*LBD* genes have been reported. Expression analysis suggests that these genes are transcribed in a wide variety of tissues and organs (Yang *et al*., 2006; Zhang *et al*., 2014). Lateral organs of a higher plant are initiated from small cell groups on the flanks of the dome-shaped shoot apical meristem (SAM) (Borghi *et al*., 2007). Maintenance of an active shoot meristem requires expression of homeobox *KNOX* genes, such as SHOOT MERISTEMLESS (STM) of *Arabidopsis*, which are excluded from organ primordia (Long *et al*., 1996). *AS1* is expressed in organ initials and physically interacts with *AS2*, which encodes nuclear protein with the plant-specific AS2/LOB domain, to repress *KNOX* gene expression, thus guiding primordia towards differentiation (Ori *et al*., 2000; Semiarti *et al*., 2001; Byrne *et al*., 2002; Guo *et al*., 2008). The AS1-AS2 complex also represses the *ETTIN* gene directly and the *ETTIN* and Auxin Response Factor 4 (*ARF4*) genes indirectly through trans-acting short-interfering RNA (tasiR)-ARF in adaxial-abaxial specification of *Arabidopsis* leaves (Iwasaki *et al*., 2013). *AS2* is also expressed in the adaxial parts of leaf primordia and young floral organs (Iwakawa *et al*., 2007; Keta *et al*., 2012). In cooperation with *AS1* and *JAGGED* (*JAG*), it restricts the boundary cells in the floral organs. Genetic analysis showed that *AS1*, *AS2*, and *JAG* genes function in the sepal and petal primordia to repress boundary-specifying genes (*CUC1*, *CUC2*, and *PETAL LOSS*) to promote normal development of the organs (Xu *et al*., 2008). Normal maize ears are unbranched and tassels have long branches only at their base. However, the *ramosa2* (*ra2*) mutant of maize results in increased branching, with short branches replaced by long indeterminate ones (Bortiri *et al*., 2006). Function analysis showed that *ra2* encodes the AS2/LOB domain transcription factor which determines the fate of stem cells in branch meristems of maize (Bortiri *et al*., 2006). Similarly, *Vrs4* is the ortholog of maize *RAMOSA2* in barley. Genetic mapping and mutant analysis revealed that *Vrs4* controls spikelet determinacy and row-type in barley (Koppolu *et al*., 2013).

Plant root development is also affected by *ASL*/*LBD* genes, including lateral root development in *Arabidopsis*, rice, and maize (Inukai *et al*., 2005; Liu *et al*., 2005; Okushima *et al*., 2007; Taramino *et al*., 2007). *AtARF7* and *AtARF19* regulate lateral root formation as transcriptional activators of early auxin response genes in *Arabidopsis thaliana* (Okushima *et al*., 2007). Further analysis revealed that *AtARF7* and *AtARF19* directly regulate the auxin-mediated transcription of *AtASL18/LBD16* and *AtASL16/LBD29* in roots (Okushima *et al*., 2007). Overexpression of *AtASL18/LBD16* and *AtASL16/LBD29* induces lateral root formation in the absence of *AtARF7* and *AtARF19*. In addition, *AtASL20/LBD18* in conjunction with *AtASL18/LBD16* functions in the initiation and emergence of lateral roots as a downstream regulator of *AtARF7* and *AtARF19* (Lee *et al*., 2009). These data suggest that *ASL*/*LBD* genes mediate lateral root formation in *Arabidopsis* by different molecular pathways. In maize, conventional genetic approaches have led to the identification of *ASL*/*LBD* genes that function in plant growth and development. For example, the *rtcs* (rootless concerning crown and seminal roots, *Zmlbd2*) mutant is impaired in the initiation of the embryonic seminal roots and the post-embryonic shoot-borne root system (Taramino *et al*., 2007). The *RTCL* (*RTCS-like*, *ZmLBD43*) gene is a paralog of *RTCS*, which displays spatio-temporal expression patterns in roots that are highly correlated with those of the *RTCS* gene (Taramino *et al*., 2007). Both RTCS and RTCL proteins are auxin-responsive genes involved in the early events that lead to the initiation and maintenance of seminal and shoot-borne root primordia formation. Both act as transcription factors and bind to downstream

promoters of *ASL*/*LBD* genes (Taramino *et al*., 2007). Taken together, these results suggest that *ASL*/*LBD* genes function in lateral and seminal root initiation and emergence, as well as shoot-borne root primordia formation.

To date, only a subset of the *AS2*/*LOB* gene family has been systematically analyzed based on genome sequencing databases (Iwakawa *et al*., 2002; Shuai *et al*., 2002; Yang *et al*., 2006, Wang *et al*., 2013; Zhang *et al*., 2014). Several *ASL*/*LBD* genes associated with mutant phenotypes involving many aspects of plant development, including embryo, root, leaf, and inflorescence development, have been functionally characterized (Majer and Hochholdinger, 2011). Therefore, a major focus of this study was to gain a better understanding of the barley *AS2*/*LOB* gene family and its expression pattern in different tissues. Barley is one of the world's earliest domesticated and most important plant crops. It has been used as animal fodder, as a source of fermentable material for beer, and as a component of various health foods. However, the barley *AS2*/*LOB* gene family has not been characterized in detail. Recently, physical, genetic, and functional sequence maps of the barley genome have been published which can be used to screen the *AS2*/*LOB* gene family (International Barley Genome Sequencing Consortium *et al*., 2012). In the present study, we provide detailed information on the genomic structure, chromosomal locations, sequence homology, and expression patterns of barley *ASL*/*LBD* genes. A phylogenetic tree of *ASL*/*LBD* genes in barley, *Arabidopsis*, maize, and rice was also constructed, which will help future studies aimed at elucidating the vital roles of *HvASL*/*LBD* genes in barley developmental processes.

2 Materials and methods

2.1 Sequence database searches

Multiple database searches were performed to collect all members of the barley *AS2*/*LOB* gene members. Barley sequence data were sourced from the Morex assembly (International Barley Genome Sequencing Consortium *et al*., 2012) and National Center for Biotechnology Information (NCBI) database. We used the BLAST programs (TBLASTN and BLASTN) available on the Institute of Plant Genetics and Crop Plant Research (IPK) barley genome database and NCBI barley expressed sequence tag (EST) database. As a query sequence, we used the amino acid sequence of the AS2/LOB domain from *Arabidopsis thaliana*, rice (*Oryza sativa* L.), and maize (*Zea mays* L.) *ASL/LBD* genes (http://planttfdb.cbi.pku.edu.cn). To increase the extent of the database search results, we also performed database searches using amino acid sequences of some members of the barley *AS2/LOB* gene family as query sequences to confirm completion of the collection. All hits with expected (*E*) values less than 1.0 were retrieved and the non-redundant sequences were examined for the presence of the conserved AS2/LOB domain using domain analysis programs Pfam (protein family; http://pfam.sanger.ac.uk) and SMART (simple modular architecture research tool; http://smart.embl-heidelberg.de) with the default cutoff parameters (Letunic *et al*., 2012; Punta *et al*., 2012). All the ASL/LBD proteins contained full length sequence.

Isoelectric points and protein molecular weights were obtained with the help of the proteomics and sequence analysis tools on the ExPASy proteomics server (http://expasy.org) (Artimo *et al*., 2012). Putative promoter sequences (2000 bp upstream from the 5' UTR region) of *HvASL/LBD* genes were obtained from the draft barley genome sequence (International Barley Genome Sequencing Consortium *et al*., 2012) and a search for hormone responsive elements was performed using the PlantCARE database (Lescot *et al*., 2002).

2.2 Chromosomal location and structure of the *HvASL/LBD* genes

Chromosomal locations and genomic sequences were retrieved from the barley genome database that was downloaded from the IPK database. All genes were mapped to the chromosomes with MapDraw software (Liu and Meng, 2003). The exon/intron structures were constructed using GSDS (gene structure display server; http://gsds.cbi.pku.edu.cn) (Hu *et al*., 2015). The HvASL/LBD proteins were named sequentially according to AS2/LOB domain blast results (Iwakawa *et al*., 2002) and their placement in the barley chromosomes, respectively.

2.3 Sequence analysis and construction of the phylogenetic tree

Full-length amino acid sequences of *ASL/LBD* genes identified in *Arabidopsis*, maize, rice, and barley were aligned using the Clustal X 1.83 program with default pairwise and multiple alignment parameters (Husbands *et al*., 2007). The phylogenetic tree was constructed based on this alignment result using the neighbor joining (NJ) method in MEGA Version 6 (Tamura *et al*., 2013) with the following parameters: Poisson correction, pairwise deletion, uniform rates, and bootstrap (1000 replicates). Conserved motifs were investigated by multiple alignment analyses using MEME Version 3.0 (Bailey and Elkan, 1994).

2.4 Expression analysis of the *HvASL/LBD* genes

Gene expression data from the cultivar "Morex" were obtained by making use of the barley genome database (http://apex.ipk-gatersleben.de/apex/f?p=284:10:6281639160219::NO). Eight tissues of "Morex" (three biological replications each) earmarking sequential stages of the barley life cycle were selected for deep RNA sequencing (RNA-seq) (International Barley Genome Sequencing Consortium *et al*., 2012). The tissues comprised: 4-d embryos dissected from germinating grains, roots, and shoots from seedlings (10 cm shoot stage), young developing inflorescences (5 mm), developing inflorescences (1.0–1.5 cm), developing tillers at the six-leaf stage (the third internode), and developing grains at 5 and 15 d post-anthesis (DPA) (bracts removed). The expression patterns are presented as heat maps in green/yellow/red coding, which reflected the fragments per kb of transcript per million mapped reads (FPKM), with red indicating high, yellow medium, and green low expression levels.

3 Results

3.1 Identification of the *AS2*/*LOB* family genes in barley

To identify the *AS2*/*LOB* gene family members in barley, BLAST searches of the barley databases were performed using the AS2/LOB domain (DUF260 domain) of the *Arabidopsis*, maize, and rice proteins as a query sequence, and then SMART and Pfam tools were used to check the domains. Twenty-four genes were identified as possibly encoding the AS2/LOB domain (Tables 1 and S1). There were 4, 2, 5, 10, 1, 1, and 1 genes located on chromosomes 1H to 7H (Table 1), respectively. The gene identifier, chromosome position, length of coding sequence, length of amino acid sequence, molecular weight, and p*I* (isoelectric point) are detailed in Table 1. The identified HvASL/LBD proteins had from 177 (HvASL10/LBD22) to 378 (HvASL7/LBD10) amino acids, a protein mass from 18.89 kD (HvASL11/LBD13) to 40.99 kD (HvASL7/LBD10) and protein p*I*s ranging from 4.55 (HvASL7/LBD10) to 10.46 (HvASL19/LBD17).

3.2 Phylogenetic and gene structure analysis of the *ASL/LBD* genes

To clarify the phylogenetic relationships among the 24 barley ASL/LBD proteins, alignment analyses were performed using full length amino acid sequences of the HvASL/LBD proteins. The alignment indicated that the HvASL/LBD proteins were identified as two monophyletic subfamilies (class I and class II) (Fig. 1), including 19 and 5 *HvASL*/*LBD* genes, respectively, and 5 sister pairs of paralogous *ASL/LBD* genes (*HvASL1/LBD1* and *HvASL10/LBD22*, *HvASL2/LBD2* and *HvASL6/LBD9*, *HvASL3/LBD3* and *HvASL8/LBD11*, *HvASL4/LBD4* and *HvASL9/LBD16*, *HvASL15/LBD21* and *HvASL17/LBD24*). Sister pairs of paralogous *ASL*/*LBD* genes (10/24, 41.67%) had high bootstrap support (bootstrap >90%).

Structural analyses provide valuable information concerning duplication events when interpreting phylogenetic relationships within gene families. Thus, we analyzed the exon/intron structures of the *AS2*/*LOB* family genes (right panel in Fig. 1). In barley, the number of exons ranged from 1 within 10 genes (*HvASL4/LBD4*, *HvASL20/LBD6*, *HvASL22/LBD7*, *HvASL5/LBD8*, *HvASL7/LBD10*, *HvASL18/LBD15*, *HvASL13/LBD18*, *HvASL14/LBD19*, *HvASL24/LBD20* and *HvASL10/LBD22*) to 5 within a single gene (*HvASL19/LBD17*). The remaining *HvASL/LBD* genes had 2 (*HvASL1/LBD1*, *HvASL2/LBD2*, *HvASL3/LBD3*, *HvASL21/LBD5*, *HvASL6/LBD9*, *HvASL12/LBD12*, *HvASL11/LBD13*, *HvASL23/LBD14*, *HvASL9/LBD16*, *HvASL15/LBD21*, *HvASL16/LBD23* and *HvASL17/LBD24*) or 3 (*HvASL8/LBD11*) exons. Some members within the same subgroup shared a similar intron/exon structure and gene length

Table 1 *HvAS2/LOB* family genes in barley

Gene name	Accession No.[a]	Chromosome position	Coding sequence length (bp)[c]	Amino acid length (aa)	Mass (Da)	p*I*	*Arabidopsis* homologous gene[d]
HvASL1/LBD1	MLOC_54949.1	1H:55.1146901608579 cM	567	189	20469.5	6.77	*AtASL5/LBD12*
HvASL2/LBD2	MLOC_61156.1	1H:62.3229461756374 cM	783	261	26835.9	7.80	*AS2*
HvASL3/LBD3	MLOC_56075.1	1H:unknown	708	236	24713.3	8.48	*AtASL11/LBD15*
HvASL4/LBD4	MLOC_68570.1	1H:unknown	645	215	22002.7	6.60	*AtASL8/LBD1*
HvASL21/LBD5	MLOC_11838.1	2H:55.5949008498584 cM	711	237	24494.1	8.22	*AtASL41/LBD39*
HvASL20/LBD6	MLOC_58304.1	2H:57.9320113314448 cM	906	302	32907.7	5.97	*AtASL36/LBD42*
HvASL22/LBD7	AK373051	3H:39.3767705382436 cM	846	282	29712.1	6.50	*AtASL36/LBD42*
HvASL5/LBD8	Contig_2547112[b]	3H:39.51713869975 cM	771	257	26458.1	7.68	*AtASL4/LOB*
HvASL6/LBD9	AK373607	3H:108.42776203966 cM	777	259	26578.9	8.12	*AS2*
HvASL7/LBD10	MLOC_81908.1	3H:142.209631728045 cM	1134	378	40990.6	4.55	*AtASL31/LBD7*
HvASL8/LBD11	MLOC_16076.3	3HL:unknown	876	292	31051.7	7.16	*HvASL18/LBD15*
HvASL12/LBD12	MLOC_52276.7	4H:0.77903682719547 cM	676	225	24941.7	4.90	*AtASL16/LBD29*
HvASL11/LBD13	MLOC_57082.1	4H:3.47025495750708 cM	534	178	18889.1	9.09	*AtASL6/LBD4*
HvASL23/LBD14	MLOC_51325.1	4H:51.4041247262572 cM	684	228	23435.0	7.18	*AtASL41/LBD39*
HvASL18/LBD15	MLOC_66372.1	4H:51.4041247262572 cM	903	301	32446.3	7.48	*AtASL35/LBD9*
HvASL9/LBD16	MLOC_73009.1	4H:51.4041247262572 cM	672	224	24122.9	6.00	*AtASL8/LBD1*
HvASL19/LBD17	MLOC_78342.2	4H:51.4164305949008 cM	768	256	27514.4	10.46	*AS2*
HvASL13/LBD18	MLOC_10783.1	4H:99.0793201133144 cM	552	184	19261.8	6.77	*AtASL16/LBD29*
HvASL14/LBD19	MLOC_10784.1	4H:99.0793201133144 cM	852	284	30404.2	7.38	*AtASL16/LBD29*
HvASL24/LBD20	MLOC_65651.1	4HL:unknown	864	288	30197.7	7.98	*AtASL36/LBD42*
HvASL15/LBD21	MLOC_55239.1	4HL:unknown	768	256	26591.2	7.85	*AtASL20/LBD18*
HvASL10/LBD22	MLOC_5148.1	5H:46.5277777777777 cM	531	177	19670.9	7.37	*AtASL5/LBD12*
HvASL16/LBD23	AK368515	6H:117.988668555241 cM	636	212	21897.7	7.72	*AtASL18/LBD16*
HvASL17/LBD24	MLOC_20803.1	7H:69.1096591753137 cM	684	228	24764.2	6.50	*AtASL20/LBD18*

[a] Barley gene model primary accession number (International Barley Genome Sequencing Consortium *et al.*, 2012). [b] Barley Morex Contig (International Barley Genome Sequencing Consortium *et al.*, 2012). [c] All the ASL/LBD proteins contained full-length sequence, Morex_contig_63283 contained partial amino acid of ASL/LBD protein which was not further analysed in the present study. [d] The *Arabidopsis* genes were generated from previous reports (Shuai *et al*., 2002; Matsumura *et al*., 2009)

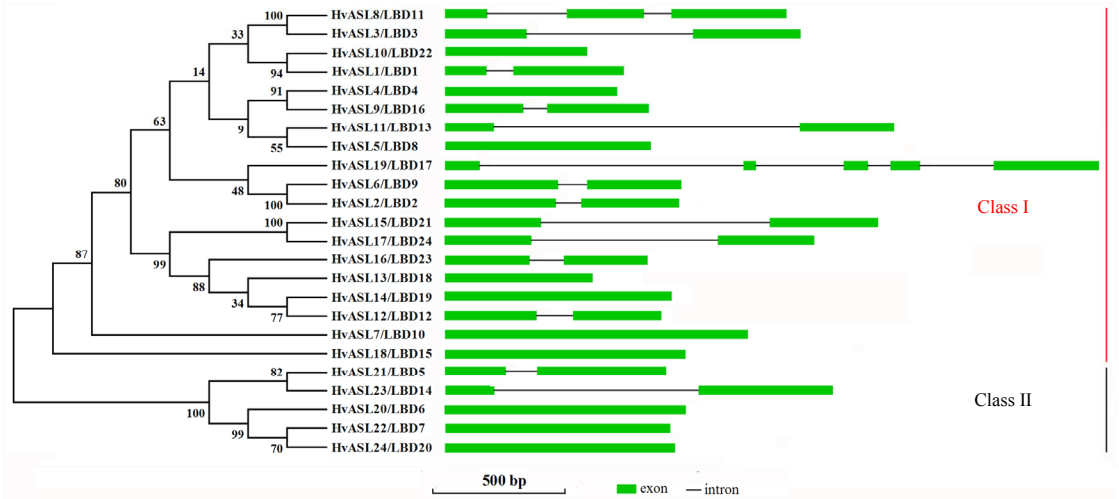


Fig. 1 Phylogenetic tree and gene structure analysis of the HvASL/LBD proteins

(*HvASL2*/*LBD2* and *HvASL6*/*LBD9*, *HvLBD21*/*24* and *HvLBD6*/*7*/*20*). The conserved intron/exon structure in each subgroup supported their close evolutionary relationship and the stated classification of subfamilies.

To compare the evolutionary patterns of barley HvASL/LBD proteins with those of other plants and then group them into established subfamilies, a phylogenetic tree was generated using *Arabidopsis*, maize, rice, and barley full length protein sequences (Fig. 2). The tree suggests two major classes of *ASL*/*LBD* genes, characterized by the presence (class I) or absence (class II) of functional leucine-zipper-like domains (Shuai *et al*., 2002). Class I and class II were subdivided into 5 and 2 groups, respectively. In class I, the subgroups Ia to Ie included 45, 9, 37, 23, and 7 genes, respectively, while the class II group comprised 11 genes in subgroup IIa and 14 genes in subgroup IIb. Subgroups Ia and Ic were the largest (45 and 37 *ASL*/*LBD* genes, respectively), and included 9 and 6 barley *ASL*/*LBD* genes, respectively. Subgroups Ib, Id, and Ie contained 2, 1, and 1 barley *ASL*/*LBD* genes, respectively. In addition, there were 11 *ASL*/*LBD* genes in subgroup IIa, of which 2 were from barley, and 14 in subgroup IIb, of which 3 were from barley (Fig. 2). Interestingly, most of the *Arabidopsis* (dicot) ASL/LBD proteins clustered separately from those of maize, rice, and barley (monocots) (Fig. 2). For example, *AtASL39*/*LBD37*, *AtASL40*/*LBD38*, and *AtASL41*/*LBD39* clustered independently from *ASL*/*LBD* genes in barley (*HvASL21*/*LBD5* and *HvASL23*/*14*), maize (*ZmLBD7*, *ZmLBD13*, and *ZmLBD14*) (Zhang *et al*., 2014), and rice (*Os07g40000.1*, *Os03g33090.1*, and *Os03g41330.1*) (Yang *et al*., 2006), indicating an evolutionary dichotomy of *ASL*/*LBD* genes between dicot and monocot plants.

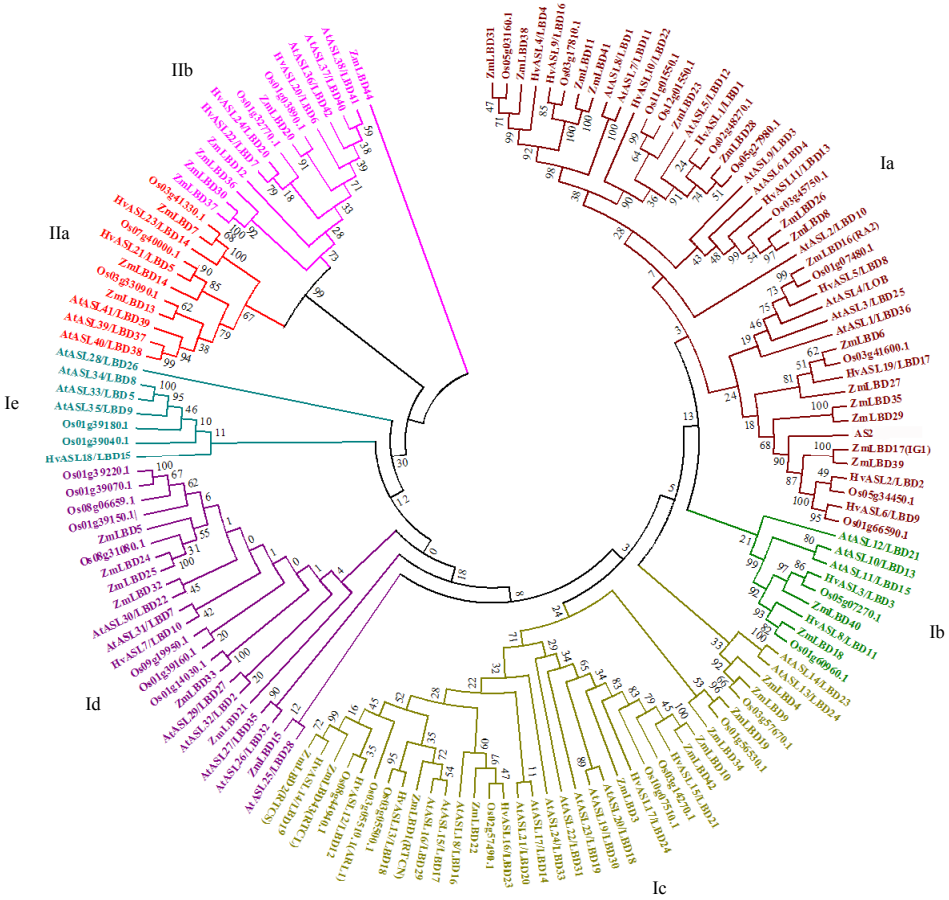


Fig. 2 Phylogenetic analysis of *ASL*/*LBD* genes in *Arabidopsis*, maize, rice, and barley

The gene names used for *AtASL/LBDs*, *ZmLBDs* were according to Matsumura *et al.* (2009) and Zhang *et al.* (2014); the rice gene ID was derived from *Oryza sativa* subsp. *indica* (Yang *et al.*, 2006). AtLBD34 does not contain a complete AS2/LOB domain and was therefore omitted from the phylogenetic tree

3.3 Chromosomal location analysis of *HvASL/LBD* genes

Twenty-four genes were located among the 7 barley chromosomes (Table 1, Fig. 3). Chromosome 4 contained the most (10) *HvASL/LBD* genes. Four genes were identified in 1H and five in 3H. Two *HvASL/LBD* genes were distributed in 2H, while only one was located on each of chromosomes 5H, 6H, and 7H. Further investigation showed that some members were clustered together within chromosomes (Fig. 3), including *HvASL22/LBD7* and *HvASL5/LBD8*, *HvASL23/LBD14*, *HvASL18/LBD15*, *HvASL9/LBD16*, and *HvASL19/LBD17*, and *HvASL13/LBD18* and *HvASL14/LBD19*. Precise genetic distances were mapped for a total of 19 genes for the corresponding chromosome. No positional information was found for the genes *HvASL3/LBD3*, *HvASL4/LBD4*, *HvASL8/LBD11*, *HvASL14/LBD19*, or *HvASL24/LBD20*.

3.4 Sequence alignment and conserved motifs of *HvASL/LBD* genes

In *Arabidopsis*, the *ASL/LBD* genes have a conserved AS2/LOB domain in the N-terminus of the proteins, and there are two conserved blocks, the C and GAS blocks, in the AS2/LOB domain of the class I proteins. In the present study, sequence alignment results showed that *HvASL/LBD* genes also contained the C and GAS blocks in the AS2/LOB domains (Fig. 4a). A conserved CX2CX6CX3C zinc finger-like domain was detected within all HvASL/LBD proteins, while an LX6LX3LX6L leucine zipper-like domain was found in only 8 of the class I *ASL/LBD* genes (Fig. 4b). However, none of the class II proteins were predicted to form coiled-coil structures.

3.5 Expression profiles of the *HvASL/LBD* genes at different developmental stages from RNA-seq data

To investigate the potential functions of the *HvASL/LBD* genes in barley development, we searched the deep RNA sequencing (RNA-seq) data from eight tissues of the cultivar "Morex" (International Barley Genome Sequencing Consortium *et al*., 2012). The tissues represent stages of the barley life cycle from germinating grain to the maturing caryopsis. Transcripts from all genes were detected by RNA sequencing except those from the *HvASL5/LBD8* gene. The *HvASL5/LBD8* gene, also named the *Six-rowed spike4* (*Vrs4*)/*HvRAMOSA2* (*HvRA2*), is an ortholog of the maize inflorescence architecture gene *RAMOSA2* (*RA2*). In situ hybridization analyses revealed that the expression of *HvRA2* was first detected during the double ridge stage. At the triple-mound stage, *HvRA2* mRNA signals were abundant all over the lateral spikelet primordia with weaker expression in the central spikelet primordia. At the glume primordium stage, *HvRA2* mRNAs were detected in both the central and lateral spikelets. Transcript levels of *HvRA2* in developing spikes at the triple mound and glume primordium stages were

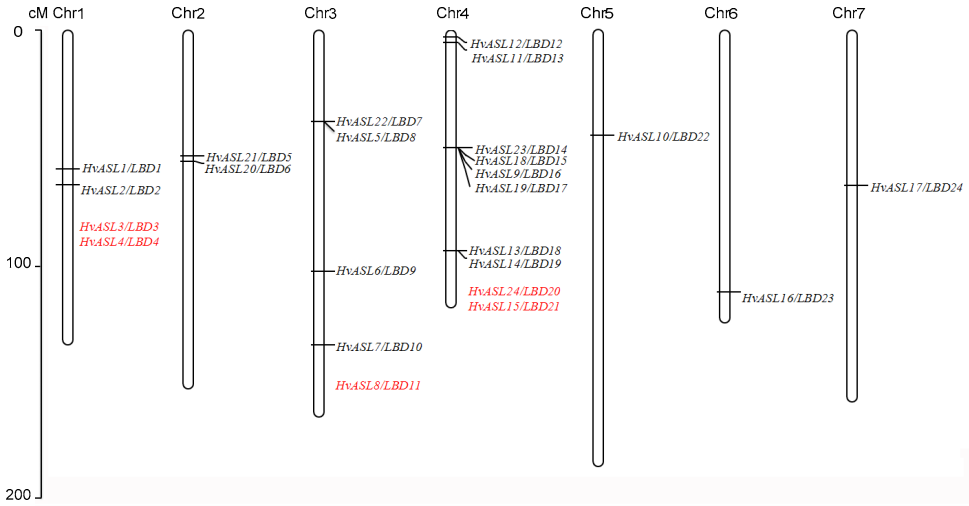


Fig. 3 Chromosomal location analysis of the *AS2/LOB* gene family in barley

The red color identifies *HvASL/LBD* genes for which position information was not found (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

higher than those during later stages (Koppolu *et al*., 2013). Further analysis revealed that transcripts of eleven genes (*HvASL2/LBD2*, *HvASL21/LBD5*, *HvASL20/LBD6*, *HvASL22/LBD7*, *HvASL6/LBD9*, *HvASL8/LBD11*, *HvASL11/LBD13*, *HvASL23/LBD14*, *HvASL18/LBD15*, *HvASL19/LBD17*, and *HvASL15/LBD21*) could be detected in the eight tissues (Fig. 5). Transcripts of *HvASL17/LBD24* were detected only in developing grain bracts removed at 5 DPA. Transcripts of *HvASL24/LBD20* predominantly present in developing grain bracts removed at 15 DPA, but there was slight expression in roots, young developing inflorescences, and developing tillers (six-leaf and third internode stage) (Fig. 5). These expression patterns could contribute to functional analysis of *ASL/LBD* genes in barley.

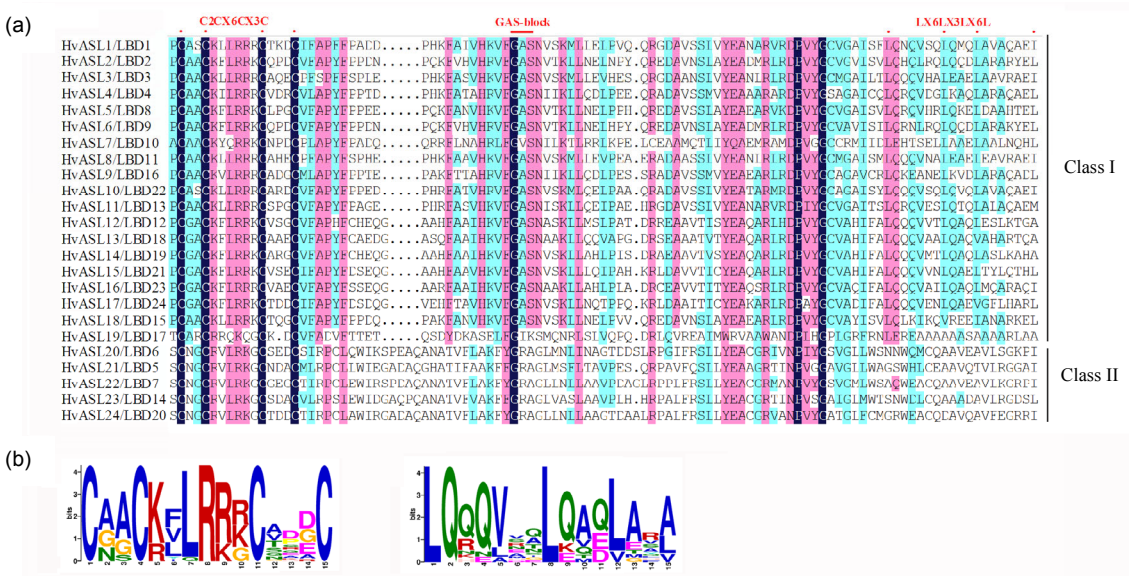


Fig. 4 Conserved domains of *HvAS2/LOB* gene family

(a) AS2/LOB domain element of barley HvASL/LBD proteins. The red dots indicate residues that are conserved in the AS2/LOB domain. (b) The CX2CX6CX3C zinc finger-like domain sequence logos (left) and LX6LX3LX6L leucine zipper-like domain sequence logos (right) (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

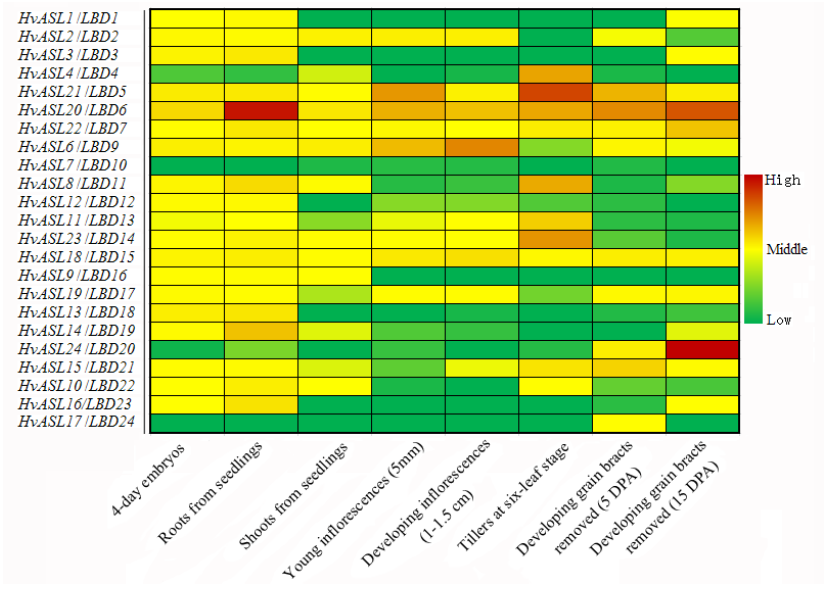


Fig. 5 Expression profiles of *HvASL/LBD* genes (except *HvASL5/LBD8*) in different tissues of barley

Expression patterns are presented as heat maps in green/yellow/red coding, reflecting the fragments per kb of transcript per million mapped reads (FPKM) with red indicating high, yellow medium, and green low expression levels (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

4 Discussion

4.1 Characterization of the barley *AS2*/*LOB* gene family

The plant-specific *AS2*/*LOB* gene family has a potential role in plant-specific processes (Iwakawa *et al*., 2002; Shuai *et al*., 2002; Matsumura *et al*., 2009; Rubin *et al*., 2009). Based on plant genome sequencing, 43 *ASL*/*LBD* genes have been identified in *Arabidopsis* (Iwakawa *et al*., 2002; Shuai *et al*., 2002; Matsumura *et al*., 2009), 35 in rice (Yang *et al*., 2006), and 44 in maize (Zhang *et al*., 2014). In the present study, 24 *HvASL/LBD* genes were identified from among 79 379 high- and low-confidence annotated genes listed by the International Barley Genome Sequencing Consortium *et al*. (2012). Each of them has notable features with a conserved AS2/LOB domain. Remarkably, barley had fewer *ASL/LBD* genes than *Arabidopsis*, maize, or rice (Iwakawa *et al*., 2002; Shuai *et al*., 2002; Yang *et al*., 2006; Zhang *et al*., 2014). This may indicate that other *HvASL/LBD* genes existing in the unknown genomic regions or chromosome duplication events may have restricted barley evolutionarily expansion.

4.2 Phylogenetic analysis and evolution of barley *ASL/LBD* genes

Previous phylogenetic analyses have revealed the evolutionary relationships of the ASL/LBD proteins among *Arabidopsis*, rice, and maize, dividing the *AS2/LOB* gene family into class I and class II (Iwakawa *et al*., 2002; Shuai *et al*., 2002; Yang *et al*., 2006; Matsumura *et al*., 2009; Majer and Hochholdinger, 2011; Zhang *et al*., 2014). In the present study, a phylogenetic tree was constructed using *Arabidopsis*, rice, maize, and barley ASL/LBD full amino acid sequences. Recently, several *ASL*/*LBD* genes associated with mutant phenotypes involved in many aspects of plant development, including embryo, root, leaf, and inflorescence development, have been functionally characterized (Majer and Hochholdinger, 2011). Maize *Ra2* encodes an AS2/LOB domain transcription factor, and the *ramosa2* (*ra2*) mutant has increased branching with short branches replaced by long indeterminate ones (Bortiri *et al*., 2006). In the present study, the barley gene *HvASL5*/*LBD8* (*Vrs4*/*HvRa2*) was found to be the ortholog of maize *Ra2*. Mutant analysis suggested that *Vrs4/HvRa2* was a central player in establishing the inflorescence architecture of barley spikes and in determining yield potential and grain number (Koppolu *et al*., 2013). The *ASL*/*LBD* genes *CRL1* (*ARL1*) in rice and *RTCS* in maize (Fig. 2) are close relatives of *AtASL16*/*LBD29* and are involved in the formation of monocot specific crown roots (Hetz *et al*., 1996; Inukai *et al*., 2005; Liu *et al*., 2005; Taramino *et al*., 2007). The barley genes *HvASL12*/*LBD12* and *HvASL14*/*LBD19* are close to these genes in the phylogenetic tree (Fig. 2, Table 1), and auxin-responsive elements were also detected in the promoters (Fig. S1). This indicates that *HvASL12*/*LBD12* and *HvASL14*/*LBD19* may be involved in the formation of crown roots in barley. The *asymmetric leaves2* (*as2*) mutant generated abnormal leaves from petioles in a bilaterally asymmetric manner in *Arabidopsis* (Semiarti *et al*., 2001), and domain swapping between AS2 and other members of the family showed that the AS2/LOB domain of AS2 was specific for the function of the *AS2* gene (Matsumura *et al*., 2009). In addition, The AS2/LOB domain protein encoded by *IG1* (*ZmLBD1*) is very similar to that of *AS2* of *Arabidopsis*, which also displays abnormal leaf morphology (Evans, 2007). In the present study, *HvASL2/LBD2* was the closest orthologous gene of *AS2*, suggesting that the function of *HvASL2/LBD2* should be further investigated. Detecting close phylogenetic relationships and identifying orthologs between monocots and dicots can contribute to the prediction of *ASL*/*LBD* gene function in plants (Matsumura *et al*., 2009; Majer and Hochholdinger, 2011).

4.3 Expression analysis of *ASL/LBD* genes on plant growth and development

ASL/LBD proteins play a crucial role in defining organ boundaries and are involved in almost all aspects of plant development, including embryo, root, leaf, and inflorescence development (Byrne *et al*., 2000; Borghi *et al*., 2007; Majer and Hochholdinger, 2011; Xu *et al*., 2016). Several *Arabidopsis* class I members have been implicated in plant development. *ASL*/*LBD* gene member *AS2* regulates symmetric flat leaf formation by repression of cell proliferation in the adaxial domain (Semiarti *et al*., 2001; Iwakawa *et al*., 2007; Iwasaki *et al*., 2013). Analysis by in situ hybridization showed transcripts of *AS2* accumulated

in the entire leaf primordium at an early stage (Semiarti *et al*., 2001; Iwakawa *et al*., 2007). The *AS2* gene was highly expressed in a sample from shoot apices and was involved in leaf adaxial-abaxial polarity (Iwakawa *et al*., 2002; Iwasaki *et al*., 2013). In barley, *HvASL2/LBD2* was the closest homolog of *AS2*. It was highly expressed in shoots from seedlings, indicating that barley *ASL*/*LBD* genes may have a similar function in plant development.

In plants, different aspects of root development are affected by *ASL*/*LBD* genes, including lateral and shoot-borne root development in *Arabidopsis*, rice, and maize (Inukai *et al*., 2005; Liu *et al*., 2005; Okushima *et al*., 2007; Taramino *et al*., 2007). *AtARF7* and *AtARF19* regulate lateral root formation as transcriptional activators of early auxin response genes in *Arabidopsis thaliana*. Target-gene analysis revealed that *AtARF7* and *AtARF19* directly regulate the auxin-mediated transcriptions of *AtASL18/LBD16* and *AtASL16/LBD29* in roots, respectively. Overexpression of *AtASL18/LBD16* and *AtASL16/LBD29* induces lateral root formation in the absence of *AtARF7* and *AtARF19*. In addition, *AtASL20/LBD18* in conjunction with *AtASL18/LBD16* functions in the initiation and emergence of lateral roots as a downstream regulator of *AtARF7* and *AtARF19*. These data suggest that *ASL/LBD* genes mediate lateral root formation in *Arabidopsis* by different molecular pathways. In barley, the closest homologous genes of *AtASL18/LBD16*, *AtASL16/LBD29*, and *AtASL20/LBD18* were *HvASL16/LBD23*, *HvASL13/LBD18*, and *HvASL17/LBD24*, respectively. Expression profile analysis revealed that *HvASL17/LBD24* was relatively highly expressed in 4-d embryos after germination, and in roots and shoots from seedlings (10-cm shoot stage). This suggests that expression analysis may explain the conserved function in this gene family.

Besides being developmental regulators, a reverse genetic characterization of group IIa genes showed that *AtASL39/LBD37*, *AtASL40/LBD38*, and *AtASL41/LBD39* mediate the repressive effect of N/NO_3^- on anthocyanin biosynthesis and further affect N-responsive genes and N metabolism (Rubin *et al*., 2009). In the present study, *HvASL21/LBD5* and *HvASL23/LBD14* were expressed in all selected tissues, and highly expressed in developing tillers at the six-leaf third internode stage. The potential roles of the *HvASL21/LBD5* and *HvASL23/LBD14* should be further investigated.

Compliance with ethics guidelines

Bao-jian GUO, Jun WANG, Shen LIN, Zheng TIAN, Kai ZHOU, Hai-ye LUAN, Chao LYU, Xin-zhong ZHANG, and Ru-gen XU declare that they have no concfict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Table S1 Amino acid sequence of the *ASL*/*LBD* genes in barley

Fig. S1 Promoter analysis of *HvASL12/LBD12* and *HvASL14/LBD19* genes

中文概要

题　目：大麦 ***ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES*（*AS2*/*LOB*）基因家族的全基因组分析**

目　的：从大麦全基因组中鉴定 *AS2*/*LOB* 基因家族，并进行基因进化、基因结构、染色体定位以及组织、表达分析，为大麦 *ASL*/*LBD* 基因进一步功能研究与鉴定奠定基础。

创新点：首次在大麦全基因组水平上分析 *AS2*/*LOB* 基因家族，并对部分基因的功能进行预测和分析。

方　法：利用大麦基因组数据库，通过生物信息学手段，鉴定大麦 *AS2*/*LOB* 基因家族成员；采用 MEGA6 软件进行系统进化树分析；利用 GSDS 及 MapDraw 工具进行基因结构及染色体定位分析；利用已有的大麦 RNAseq 数据进行组织表达谱分析。

结　论：通过全基因组分析，大麦 *AS2*/*LOB* 家族基因包括 24 个成员，在进化上分为两大类，7 个亚家族，分布于大麦 7 条染色体上，组织表达模式具有多样性，与已经报道的 *ASL*/*LBD* 基因具有良好的同源性。这些信息为大麦 *AS2*/*LOB* 基因家族的功能分析奠定了基础。

关键词：大麦；*AS2*/*LOB* 基因家族；进化树；表达模式