Molecular genetic studies on *ASYMMETRIC LEAVES2* (AS2) of *Arabidopsis*: Insight into the function of the AS2 protein

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Summary: The *ASYMMETRIC LEAVES2* (AS2) gene is involved in leaf development along adaxial-abaxial, medial-lateral and proximal-distal axes in *Arabidopsis*. The AS2 gene belongs to the AS2/*LATERAL ORGAN BOUNDARIES* (LOB) family. There are 42 members of the AS2/LOB family in the *Arabidopsis* genome. Many recent studies revealed that AS2/LOB family genes are involved in various physiological processes including the regulation of development and metabolism in plants. The approximately 100 amino acid AS2/LOB-domain, which defines this family, is highly conserved. Conserved regions include the C-motif, an internal region containing an invariant glycine residue, and a leucine-zipper-like motif, in the case of class I proteins. The conserved AS2/LOB-domain of AS2 cannot be functionally replaced by those of other members of the family. AS2 acts in a same pathway as that of AS1, which is an ortholog of ROUGH SHEATH2 of maize and PHANTASTICA of snapdragon. Recent molecular genetic studies for the role of AS2 provided important information. The regulation of gene expression and leaf development by AS2 is genetically linked with the regulations based on chromatin level, post-transcriptional level, protein metabolism and cell proliferation. These novel insights contribute to a better understanding of the role of AS2/LOB family genes for plant morphogenesis and physiological responses.

Key words: ASYMMETRIC LEAVES2 (AS2), LOB, *Arabidopsis*, leaf development, AS2/LOB-domain

AS2/LOB family

The *ASYMMETRIC LEAVES2* (AS2) gene was isolated as a gene involved in the formation of leaf shape and venation pattern in *Arabidopsis* (Rédei and Hirono 1964, Semiarti et al. 2001, Iwakawa et al. 2002). The *LATERAL ORGAN BOUNDARIES* (LOB) gene was isolated as a gene whose transcripts were enriched in the boundary region between the shoot apical meristem

(SAM) and leaf primordia of *Arabidopsis* (Shuai et al. 2002). AS2 and LOB share a common domain that is conserved in proteins throughout the plant kingdom, namely the AS2/LOB-domain (Fig. 1). This domain is found in the N-terminal halves of the AS2 and LOB proteins (Iwakawa et al. 2002, Shuai et al. 2002). There are 42 genes encoding AS2/LOB-domain containing proteins in the *Arabidopsis* genome; these genes are

Figure 1 Structure of AS2 and the AS2/LOB-domain. Four Cys residues composing the C-motif are shaded by yellow. The conserved Gly and Pro residues are shaded by pink. The hydrophobic residues composing the leucine-zipper like motif are shaded by green. PCK1: Peacock1 (Nakazawa et al. 2003). At: *Arabidopsis*. Zm: maize.

classified as belonging to a gene family named as *AS2/LOB* family. The 41 members other than *AS2* were named as *AS2-LIKE1* to *41* (*ASL1-41*). Alternatively the 41 members other than *LOB* were named as *LOB-DOMAIN1-33* (*LBD1-33*) and *LBD35-42*. Here *LBD34* is not included in the family, as it might be a pseudogene because the conserved glycine (Gly) residue described below is not found in the predicted amino acid sequence. In rice, 35 genes belonging to the *AS2/LOB* family have been reported (Yang et al. 2006). Among 42 members of the *Arabidopsis AS2/LOB* family genes, 36 members (*AS2* and *ASL1-35*) are categorized as class I and 6 members (*ASL36-42*) as class II, on the basis of the amino acid sequences of their *AS2/LOB*-domains.

Many recent studies revealed that *AS2/LOB* family genes are involved in various physiological events including the regulation of development and metabolism in plants. *ASL1/LBD36* has a partially redundant function with *AS2* to repress class1-*KNOX* expression in flower development (Chalfun-Junior et al. 2005). *ASL3/LBD25* is involved in auxin-responses and photomorphogenesis (Mangeon et al. 2011). *ASL9/LBD3* appears to regulate cytokinin-dependent development (Naito et al. 2007). *ASL16/LBD29*, *ASL18/LBD16* and *ASL20/LBD18* are auxin-regulated and required for lateral root initiation (Okushima et al. 2007). *ASL19/LBD30* and *ASL20/LBD18* act as positive regulators of xylem development (Soyano et al. 2008). *ASL19/LBD30* is also required for proper cell division in embryogenesis and for the development of cotyledons (Borghi et al. 2007). *ASL29/LBD27* is essential for the asymmetric division and the consequent development of the male gametophyte (Oh et al. 2010). *ASL39/LBD37*, *ASL40/LBD38* and *ASL41/LBD39* regulate the expression of anthocyanin biosynthesis genes depending on the nutrient (Rubin et al. 2009, Albinsky et al. 2010). In maize, loss-of-function mutations in genes that encode proteins with an *AS2/LOB* domain, namely, *indeterminate gametophyte1* (*ig1*) and *ramosa2* (*ra2*), affect the development of the embryo sac and inflorescence branching patterns, respectively (Bortiri et al. 2006, Evans 2007). The *Crown rootless1/Adventitious rootless1* (*Cr11/Arl1*) gene of rice,

and the *rootless concerning crown and seminal roots* (*rtcs*) gene of maize encode proteins with *AS2/LOB* domains that exhibit high degrees of identity, in terms of their respective amino acid sequences, with those of *ASL16/LBD29* and *ASL18/LBD16* of *Arabidopsis* (Inukai et al. 2005, Liu et al. 2005, Taramino et al. 2007). The common involvement in auxin responses and lateral root formation indicate functional conservation between the *Arabidopsis*, rice, and maize proteins.

AS2/LOB-domain

Because *AS2/LOB* family genes are involved in various aspects of plant developmental and physiological responses, biochemical characterization of the *AS2/LOB*-domain is important to develop a better understanding of the nature of plant development and physiological responses. The C₂C₆C₃C sequence (C: cysteine; x: unconserved; positions from 10 to 24 in *AS2*) is completely conserved in the N-terminal region of the *AS2/LOB*-domain of all the predicted proteins belonging to the *AS2/LOB* family of *Arabidopsis* (Fig. 1). The sequence was named the C-motif. A leucine-zipper like sequence is conserved in the C-terminal region of *AS2/LOB*-domain belonging to class I *AS2/LOB* family. Class II *AS2/LOB*-domain proteins appear to lack the functional leucine-zipper motif. The amino acid sequence of the internal region between the C-motif and the leucine-zipper like motif is also conserved. Especially, the Gly and Pro residues at positions 46 and 80, respectively, in *AS2* are conserved through all members of *AS2/LOB* family in *Arabidopsis* (Iwakawa et al. 2002, Shuai et al. 2002). In the amino acid sequence of the mutant *AS2* protein encoded by the *as2-5* mutant allele, the conserved Gly is replaced by a Glu residue, suggesting that the conserved Gly residue has an essential role (Semiarti et al. 2001, Iwakawa et al. 2002).

The amino acid sequence of the C-terminal region of each *AS2/LOB* family protein is variable and might confer functional specificity to each protein. Indeed, the mutant *AS2* protein encoded by the *as2-4* mutant allele is truncated so that it lacks a complete C-terminus and its full function. However, the conserved *AS2/LOB*-domain

also confers a specific function to the AS2 protein, as the AS2/LOB-domain of AS2 cannot be functionally replaced by those of other members of the family (Matsumura et al. 2009). This result suggests that the conserved AS2/LOB-domain of each member of the AS2/LOB family plays a role in specifying the function of each protein. Similarly, the C-terminus of LOB/ASL4 functions to stabilize or enhance protein-protein interactions rather than to specify the interaction *in vitro* (Husbands et al. 2007).

The recombinant LOB/ASL4, AS2, ASL6/LBD4, ASL9/LBD3, ASL18/LBD16 and ASL38/LBD41 proteins, members of phylogenetically distinct groups within the family that are functionally diverse, are capable of binding DNA with the consensus sequence of 5'-GCGGCG-3' *in vitro* (Husbands et al. 2007). These data indicate that AS2/LOB proteins have the same or similar DNA-binding activity, suggesting that recognition of DNA sequence is not sufficient to confer functional specificity to each member of AS2/LOB family. One example is the specific partner for protein-protein interactions. In the case of LOB/ASL4, the AS2/LOB-domain mediates not only DNA-binding but also homodimerization and the interaction with another protein, bHLH048.

Protein-protein interaction between AS1 and AS2

The phenotype of *asymmetric leaves1 (as1)* mutants resembles that of *as2* mutants (Rédei, 1965, Semiarti et al. 2001). The *AS1* gene encodes the MYB-domain containing protein, which is an ortholog of ROUGH SHEATH2 (RS2) of maize and PHANTASTICA (PHAN) of snapdragon (Byrne et al. 2000, Timmermans et al. 1999, Tsiantis et al. 1999, Waites et al. 1998). Green fluorescent protein (GFP)-fused RS2 (RS2-GFP) or PHAN (PHAN-GFP) can functionally replace AS1 and restore the phenotype of *as1* mutants, suggesting that RS2 and PHAN are evolutionally conserved functional orthologs of AS1 (Theodoris et al. 2003, Nurmberg et al. 2007). The *as1* mutation does not enhance the phenotype of *as2* mutants and vice versa (Serrano-Cartagena et al. 1999). Thus, AS1 and AS2 act in a same pathway. When AS1 and AS2 were co-expressed in a single cell,

both proteins were co-localized as speckled bodies (AS2-bodies) adjacent to nucleoli (Ueno et al. 2007). Recombinant AS1 and AS2 proteins can interact *in vitro* (Xu et al. 2003). Based on these results, it is believed that AS1 and AS2 proteins function in a heteromeric protein complex. However, the evidence of their interaction *in vivo* has not yet been shown. The accumulation pattern of transcripts of *AS1* is not same as that of *AS2* (Iwakawa et al. 2007). In addition, an AS2-independent function of *AS1* was reported. AS1 directly represses transcription of jasmonic acid (JA)-responsive genes and regulates the plant immune response, an activity that does not depend on the endogenous AS2 gene (Nurmberg et al. 2007). It remains elusive whether the interaction between AS1 and AS2 proteins is essential for the developmental roles of both proteins.

Transcriptional regulation by AS1 and AS2

Leaves develop along three axes – proximal-distal, adaxial-abaxial and medial-lateral. Starting as a bulge on the flank of the SAM, the newly initiated leaf becomes asymmetric. The meristem provides leaves with an inherent sidedness, with the adaxial side adjacent to the meristem and the proximal end attached to the meristem (Fig. 2). Polarization along these axes eventually leads to the asymmetric distribution of cell types in the mature leaf that is critical for the physiology of the plant. For

Figure 2 Establishment of adaxial-abaxial polarity in a developing leaf. The leaf newly initiated from the SAM is initially radial, and lacks characteristics of adaxial domain when it is incipient primordium. Adaxial identity is obtained during development. AS2, AS1 and other redundant factors are involved in this step. SAM: shoot apical meristem (large circle). lp: leaf primordium (small circle). ad: adaxial domain. ab: abaxial domain

example, the adaxial side of the leaf blade often consists of cells specialized for light harvesting while the abaxial side contains cells involved in gas exchange to maximize photosynthesis.

The *as1* and *as2* mutants are deficient in leaf development along adaxial-abaxial and medial-lateral axes (Semiarti et al. 2002, Xu et al. 2003). Conversely, overexpression of *AS2* also affects the adaxial-abaxial polarity of the leaf (Lin et al. 2003). Transcripts of genes related to the abaxial domain, *KANADII* (*KANI*), *KAN2*, *ETTIN* (*ETT*)/*AUXIN RESPONSE FACTOR3* (*ARF3*), *FILAMENTOUS FLOWER* (*FIL*) and *YABBY5* (*YAB5*) are highly or ectopically accumulated in *as1* or *as2* mutant plants (Garcia et al. 2006, Huang et al. 2006, Iwakawa et al. 2007, Takahashi et al. 2008, Xu et al. 2006).

One of the earliest indications of leaf development is the downregulation at the site of incipient primordia of class1-*KNOX* genes, which are specifically expressed in the meristem. Through *KNOX* gene repression, a switch from an indeterminate to a determinate fate occurs in the small group of cells that will be the immediate precursor of the leaf primordium (Moon and Hake 2010). The *AS2* protein directly represses the transcription of *BREVIPEDICELLUS* (*BP*)/*KNAT1* and *KNAT2* in the presence of *AS1* (Guo et al. 2008). The accumulation of transcripts and activation of promoters of *BP*, *KNAT2* and *KNAT6*, all belonging to class1-*KNOX* genes, are ectopically found in leaves of *as1* and *as2* mutants (Ori et al. 2000, Semiarti et al. 2001). However, the ectopic expression of class1-*KNOX* genes in leaves does not distort leaf initiation, but affects leaf patterning along the proximal-distal axis, in part by altering levels of gibberellin (Ikezaki et al. 2010, Ramirez et al., 2009, Sakamoto et al. 2001). These findings indicate that *AS1* and *AS2* are also involved in patterning leaf development along the proximal-distal axis. Studies of *blade on petiole* (*bop*) of *Arabidopsis*, a mutant with defects in proximal-distal identity, are consistent with this idea. The *bop* mutants accumulate class1-*KNOX* transcripts in the leaf and the BOP complex directly and positively regulates the transcription of *AS2* (Ha et al. 2003, Jun et al. 2010).

Although defects in adaxial-abaxial polarity of

leaves in *as1* and *as2* mutants were not due to the ectopic expression of class1-*KNOX* genes (Ikezaki et al. 2010), ectopic class1-*KNOX* expression is capable of affecting the adaxial-abaxial polarity of floral organs in *Arabidopsis*. The class1-*KNOX* genes have the potential to be involved in the establishment of adaxial-abaxial polarity in leaves in another species, tobacco. *ARF6* and *ARF8*, in parallel with *AS1* and *AS2*, repress the class1-*KNOX* genes in *Arabidopsis*. Ectopic expression of class1-*KNOX* genes affects the elongation and adaxial-abaxial polarity of floral organs through altered JA biosynthesis in *arf6 arf8* double mutants (Tabata et al. 2010). *NTH15* of tobacco, which is related to *SHOOT MERISTEMLESS* of *Arabidopsis*, is predominantly transcribed in the SAM and the adaxial domains of leaf primordia. The antisense repression of *NTH15* causes plants deficient in the development of adaxial domains of leaves (Tamaoki et al. 1997). Therefore, it appears that ectopically expressed class1-*KNOX* genes might be able to affect adaxial-abaxial polarity of leaf-like organs.

In the presence of *AS1*, a main role for the *AS2* protein is the transcriptional repression of several genes including class1-*KNOX* genes and genes related to leaf polarity, as described above. It is indeed reported that the C-terminal region of the *AS1* protein has the activity of transcriptional repression (Guo et al. 2008). It was, however, reported that either the N- or C-terminal half of maize *RS2* protein could partially restore the phenotype of *Arabidopsis as1* mutants (Theodoris et al. 2003). In addition, microarray analysis revealed that the transcript level of over 289 genes was possibly upregulated by *AS1* and *AS2* directly or indirectly (Takahashi et al. 2008). We cannot exclude the possibility that *AS2* or the *AS1-AS2* complex act as a transcriptional activator. The identification of direct target genes of *AS1* and *AS2* will be a crucial step to elucidate the biological and biochemical roles of these proteins. Furthermore, considering the results derived from molecular genetic studies (Table 1), we may assume that the mechanism underlying the transcriptional regulation by *AS1* and/or *AS2* is novel or that *AS1* and/or *AS2* also appear(s) to play a role other than in transcriptional regulation.

Table 1 The mutation that enhance the phenotype of *as1* or *as2* mutants

Mutant	Gene product or its role	Reference
<Gene silencing>		
<i>rdr6</i>	ta-siRNA pathway	Garcia et al. 2006, Li et al. 2005
<i>sgs3, ago7</i>	ta-siRNA pathway	Xu et al. 2006
<Protein metabolism or cell proliferation>		
<i>rpl5, rpl9, rpl10a, rpl28a, rpl24b</i>	ribosomal proteins	Pinon et al. 2008, Yao et al. 2008
<i>pt2a, rpt4a, rpt5a, rpn1a, rpn9a, pad1, pbe1</i>	26S proteasome	Huang et al. 2006
<i>atate1, 2</i>	N-end rule	Graciet et al. 2009
<i>ae7</i>	DUF59 protein	Yuan et al. 2010
<i>an3</i>	GIF1	Horiguchi et al. 2010, Wang et al. 2011
<chromatin-related>		
<i>hira</i> (kd)	chromatin assembly factor	Phelps-Durr et al. 2005
<i>pkl</i>	chromatin remodeling factor	Ori et al. 2000
<i>hdt1, 2</i> (kd)	HDAC	Ueno et al. 2007
kd: knock-down		

Gene silencing pathway

Gene silencing pathways are complex and partially overlapping, but at least three basic classes can be distinguished: post-transcriptional gene silencing (PTGS) mediated by small interfering RNA (siRNA), silencing mediated by microRNAs, and transcriptional gene silencing (TGS) mediated by siRNA-directed methylation of DNA and histone proteins. MicroRNA- and trans-acting siRNA- (ta-siRNA-) mediated pathways post-transcriptionally regulate the expression of endogenous genes and consequently plant development. In plants, PTGS is an important antiviral response that suppresses viral gene expression through siRNA-mediated viral RNA degradation. To counteract the host PTGS response, viruses encode various suppressors of RNA silencing.

Recent studies revealed that AS1 and AS2 play roles in RNA silencing pathways. AS1 and AS2 have redundant functions in the ta-siRNA pathway to regulate adaxial-abaxial polarity in leaves (Garcia et al. 2006, Li et al. 2005, Xu et al. 2006). The accumulation of microRNA165 (miR165) and miR166 (miR165/166), which negatively regulate *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *CORONA* (*CNA*) and *ATHB8*, is negatively regulated by AS1 and AS2 (Li et al. 2005, Ueno et al. 2007). Interestingly, the levels of miR165/166 precursors are unaffected by

gain- or loss-of-function of AS2 (Ueno et al. 2007). In addition, a viral silencing suppressor, when overproduced in *Arabidopsis* plants, represses the accumulation of miR165/166. β C1, an RNA silencing suppressor encoded by the ORF located on DNA β of the *Tomato yellow leaf curl China virus* (*TYLCCNV*), interacts with AS1 and partially mimics the function of AS2 including the repression of miR165/166 and leaf morphogenesis (Yang et al. 2008). Because of the biochemical similarities between PTGS and the endogenous miRNA pathway, viral suppressors also interfere with the latter, thereby causing alterations in plant development (Kasschau et al. 2003). Although it has not yet been known whether the regulation is direct or not, AS1 and AS2 might post-transcriptionally regulate the expression of miR165/166.

Protein metabolism and cell proliferation

Genes encoding the factors involved in protein synthesis or degradation pathways genetically interact with the *AS1* and *AS2* genes. Mutations in genes encoding several subunits of the ribosome enhance the phenotype of *as1* or *as2* mutant plants (Pinon et al. 2008, Yao et al. 2008). It is, however, not yet known whether the function of ribosome itself is related to that of AS1 and AS2. The genetic interaction between the genes related to ribosomal RNA and *as1* or *as2* would give us important information.

On the other hand, mutations in genes encoding several subunits of the 26S proteasome also enhance the phenotype of *as1* or *as2* mutant plants (Huang et al. 2006). In addition, it was reported that arginyl-tRNA-protein transferases, which are involved in N-terminal amino acid residues-dependent protein degradation, also have a partially redundant function with AS1 (Graciet et al. 2009).

A possible explanation for why mutations disrupting protein synthesis or degradation pathways enhance the phenotype of *as1* and *as2* mutants is that proper cell proliferation is required for leaf polarity establishment. The *as1/2 enhancer7* (*ae7*) mutation disrupting the gene encoding DUF59 (domain of unknown function 59) protein enhances the phenotype of *as1* and *as2* mutants (Yuan et al. 2010). Mutation in *ANGUSTIFOLIA3* (*AN3*), which encodes GRF-INTERACTING FACTOR1 (GIF1), also enhances the phenotype of *as1* and *as2* mutants (Horiguchi et al. 2010, Wang et al. 2011). The *AE7* and *AN3* genes are involved in the promotion of cell proliferation. The switch from cell proliferation to differentiation often coincides with the switch from the mitotic cell cycle to the endoreduplication cycle during leaf development (Boudolf et al. 2004, Ishida et al. 2010). The ploidy level is high in the *an3* mutants compared to that of wild-type plants (Wang et al. 2011). Proper cell division and consequent polarized cell proliferation might be important for the establishment of leaf polarity. Cell proliferation and the expression of *AS1* might be partially regulated by a common mechanism. CINCINNATA-like (CIN-like) TCP family proteins are transcription factors involved in cell proliferation and consequent leaf shape formation (Nath et al. 2003, Palatnik et al. 2003). Koyama et al (2010) reported that TCP3, one of CIN-like TCPs, directly activated the transcription of *AS1*.

Chromatin related factors

Factors related to chromatin regulation genetically interact with the *AS1* or *AS2* genes. A reduction in *HIRA* function or the loss-of-function *pickle* (*pk1*) mutation enhances the phenotype of *as1* and *as2* plants (Ori et al. 2000, Phelps-Durr et al. 2005). The HIRA protein is a chromatin assembly factor that can physically interact with AS1 (Phelps-Durr et al. 2005). The *PKL* gene encodes a CHD3 ATP-dependent chromatin remodeling factor (Ogas et al. 1999). Mi-2, a mammalian homolog of PKL, is a component of a protein complex that includes histone deacetylases (HDACs, Ahringer 2000), suggesting that some genes for HDACs may also genetically interact with *as1* or *as2*. Indeed, treatment with inhibitors of HDAC activity enhanced leaf polarity defects in the *as1* and *as2* mutant plants. The responsible genes, *HDT1/HD2A* and *HDT2/HD2B* (*HDT1/2*), were identified by an RNAi-library screening method (Fig. 3). The knock-down of *HDT1/2* enhances the phenotype of *as2* mutants (Ueno et al. 2007). *HDT1/2* are *Arabidopsis* homologs of maize HD2 (Dangl et al. 2001), which was originally isolated by biochemical purification from germinated embryos of maize (Lusser et al. 1997). What is the overlapping role among *HDT1/2* and *AS2*? The *HDT1/2* pathway may be related to ribosome or cell proliferation. *HDT1* is involved in the transcriptional

Figure 3 Schematic model for screening of an RNAi-library to functionally characterize individual members of a gene family of interest.

repression of rDNA genes (Lawrence et al. 2004). The mRNA and activity levels of HD2 are relatively high during germination of maize embryos, in which cell proliferation is vigorous (Georgieva et al. 1991, Lusser et al. 1997). The expression levels of HDT1/2 are high in shoot apices and region in which cell proliferation occurs, and reduced by the *an3* mutation (Horiguchi et al. 2010, Wu et al. 2000, Zhou et al. 2004). Alternatively, nuclear structure may possibly be related to the function of HDT1/2 or AS2. The predominant accumulation of HD2, HDT1 and HDT2 proteins is found in nucleoli (Lawrence et al. 2004, Lusser et al. 1997, Zhou et al. 2004). The function of the nucleolus remains largely unknown. It is notable that the predominant accumulation of AS1 and AS2 proteins is observed in AS2-bodies, which are physically near nucleoli, and that not only leaf polarity but also the repression of miR165/166 is affected by the inhibition of HDACs in the *as2* mutant background (Ueno et al. 2007). Investigating such a phenotype might be a clue to dissect the molecular function of HDT1/2 and AS2.

Many recent studies revealed that members of the AS2/LOB family are involved in various aspects of plant growth and development. The biochemical characteristics of the AS2/LOB-domain proteins are of raising importance. The AS2/LOB family proteins act as transcriptional regulators. AS2 is not only one of key regulators in leaf development but also the best model molecule for AS2/LOB family proteins. Again, genetic studies for *as1* and *as2* mutants suggest that the mechanism underlying the transcriptional regulation by AS1 and/or AS2 is novel or that AS1 and/or AS2 may also have additional role besides that of transcriptional regulation.

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