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ASYMMETRIC LEAVES2-LIKE1 gene, a member of the AS2/LOB family, controls proximal–distal patterning in *Arabidopsis* petals

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Abstract

The formation and the development of the floral organs require an intercalate expression of organ-specific genes. At the same time, meristem-specific genes are repressed to complete the differentiation of the organs in the floral whorls. In an *Arabidopsis* activation tagging population, a mutant affected in inflorescence architecture was identified. This gain-of-function mutant, designated *downwards siliques1* (*dsl1-D*), has shorter internodes and the lateral organs such as flowers are bending downwards, similar to the loss-of-function *brevipedicellus* (*bp*) mutant. The affected gene in *dsl1*-D appeared to be *ASYMMETRIC LEAVES2-LIKE1 (ASL1)/LATERAL ORGAN BOUNDARIES* domain gene 36 (*LBD36*), which is a member of the ASYMMETRIC LEAVES2 (AS2)/LATERAL ORGAN BOUNDARIES (LOB) domain gene family. Analysis of the loss-of-function mutant *asl1/lbd36* did not show morphological aberration. Double mutant analysis of *asl1/lbd36* together with *as2*, the *ASL1/LBD36* closest homologue, demonstrates that these two members of the AS2/LOB family act partially redundant to control cell fate determination in *Arabidopsis* petals. Moreover, molecular analysis revealed that overexpression of *ASL1/LBD36* leads to repression of the homeobox gene *BP,* which supports the model that an antagonistic relationship between ASL/LBD and homeobox members is required for the differentiation of lateral organs.

Introduction

During the past two decades, studies on the molecular control of flower development were very popular and successful. Enormous progress has been made in the understanding of reproductive organ development and the homeotic genes specifying organ identity. Nevertheless, due to its complex nature our knowledge is fragmentary and virtually absent when it concerns the differentiation of the floral organs.

An *Arabidopsis* flower is composed of four delineated organ types organised in symmetric whorls, which contain from the outermost to the innermost whorl four sepals, four petals, six stamens and two fused carpels. Flower formation is initiated in the floral meristem, where cells are initially undifferentiated (Weigel and Jürgens, 2002) and subsequently, their fate is determined by the action of homeotic genes belonging to the well-known ABC classes (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Floral organs, like leaves, are considered lateral organs, because they arise as differentiated structures from the flanks of a meristem. Lateral organs display polarity, which is determined by its initial relationship to the meristem, the apical or floral meristem. This polarity can be defined as adaxial–abaxial and proximal–distal. The adaxial side of the lateral organ anlagen is closest to the meristem, whereas the abaxial surface is located to the periphery (Eshed *et al.*, 2001; Tasaka, 2001; Bowman *et al.*,

2002). Several genes are involved in the establishment of polarity either in leaves or in floral organs and they have been used to study cell polarity in *Arabidopsis* and other plant species. In *Arabidopsis*, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (McConnell *et al*., 2001) are key regulators of this process. Mutations in these genes alter cells of the abaxial surface into an adaxial identity. Members of the *KANADI* gene family are also important factors in abaxial cell fate, because both loss and gain-of-function mutants show strong aberrant phenotypes in abaxial cell identity (Eshed *et al*., 2001; Kerstetter *et al*., 2001). A double mutation in *FIL* (*FILAMENTOUS FLOWER*) and *YAB3* (*YABBY3*), both members of the YABBY gene family, leads to a more pronounced loss of polar differentiation of tissues in lateral organs, and the formation of abnormal meristems, which is attributed to the derepression of *KNOX* genes (Siegfried *et al*., 1999).

Genes of the *KNOTTED1* homeodomain, or KNOX family, such as *SHOOTMERISTEMLESS* (*STM*) and *BREVIPEDICELLUS* (*BP)*; also known as *KNAT1,* are required for the maintenance and growth of the shoot apical meristem (SAM) and they are downregulated when organ primordia develop (Lincoln *et al*., 1994; Long *et al*., 1996).

It has been reported that *ASYMMETRIC LEAVES2 (AS2)*, a member of the AS2 gene family (Iwakawa *et al*., 2002) that is also described as LATERAL ORGAN BOUNDARIES (LOB) domain-gene family (Lin *et al*., 2003), is important to repress the *KNOX* genes *BP, KNAT2* and *KNAT6* in leaves, by acting alone or in combination with *ASYMMETRIC LEAVES1 (AS1)* (Byrne *et al*., 2000, 2002; Ori *et al*., 2000; Semiarti *et al*., 2001). *AS1* is a member of a small MYB-related gene family, which also contains the maize *ROUGHT SHEATH2* (*RS2*) and the *Antirrhinum PHANTASTICA* (*PHAN*) gene. Expression studies revealed that these *AS1*-like genes are able to repress *KNOX* genes in leaves and by that they retain the differentiated state in the lateral organs (Schneeberger *et al*., 1998; Timmermans *et al*., 1999; Tsiantis *et al*., 1999; Byrne *et al*., 2000). The maize gene *RS2*, which is the putative orthologue of the *Arabidopsis AS1* gene, acts in the establishment of a blade-sheath boundary in maize leaves (Schneeberger *et al*., 1998). The *rs2* mutant displays disorganised differentiation of the blade-sheath boundary and furthermore, aberrant vascular patterning and the generation of semi-bladeless leaves (Schneeberger *et al*., 1998). In the *as1* mutant there are multiple bundles of elongated cells extending from the petiole into the leaf blade (Byrne *et al*., 2000).

Recent experiments indicated that *AS2* is involved in lateral organ polarity. Ectopic expression of *AS2* under the control of the CaMV *35S* promoter, resulted in adaxial–abaxial abnormalities in leaves (Lin *et al*., 2003), although in the *as2* loss-of-function mutant, polarity is not affected (Semiarti *et al*., 2001; Iwakawa *et al*., 2002; Xu *et al*., 2003). Changes comprise leaf lobbing and leaflet-like structures appearing on the leaves (Semiarti *et al*., 2001; Iwakawa *et al*., 2002; Xu *et al*., 2003). AS1 and AS2 are positive regulators of the founding member of the LOB family: the *LOB* gene (Byrne *et al*., 2002; Shuai *et al*., 2002). Based on its expression pattern, it has been postulated that *LOB* plays a role in the establishment of boundaries between the meristem and the differentiated lateral organs (Shuai *et al*., 2002). Other members of this AS2/LOB family have only been characterised by gain-of-function mutation (Nakazawa *et al*., 2003), which revealed remarkably similar phenotypes.

Here we describe the analysis of a gain-of-function mutant *downwards siliques1* (*dsl1-D*) that was found in an activation tagging population. The phenotype of *dsl1-D* is reminiscent with the phenotype of the loss-of-function *bp* mutant (Douglas *et al*., 2002; Venglat *et al*., 2002) and gain-of-function of a few members of the AS2/LOB family. Further analysis showed that this *bp*-like phenotype is due to the negative regulation of *BP* by the overexpression of *ASL1 (LBD36)*. In addition, mutant analysis of a knockout mutant of *asl1* revealed that this AS2/LOB-domain gene acts redundantly with *AS2* (*LBD6*) in establishing boundaries in the *Arabidopsis* floral organs. A model describing the relationship between the AS2/*LOB* family members and the *BP* gene will be discussed.

Materials and methods

Screening activation tagging population

The stable *En-I* (*Spm*) transposon population (ecotype Wassilewskija (WS-3)) (Marsch-Martinez

et al., 2002) was screened visually in the greenhouse for flower and silique aberrations. The selected mutant was selfed and a F1 segregating population was raised to check the inheritance.

Southern blot analysis

Genomic DNA from 24 F1 segregating plants and from the parental mutant was isolated (Pereira and Aarts, 1998) and approximately 300 ng of DNA was digested with restriction enzyme *Eco*RI. Equal loading of DNA was verified by ethidium bromide staining. DNA was eletrophoresed in a 1.0% (w/v) agarose gel in 1× TBE (1.0 M Tris, 0.9 M boric Acid, 0.01 M EDTA), blotted onto Hybond N+ membrane (Amersham Pharmacy Biotech) following the normal manufacturer's instructions. A 1.3 kb PCR fragment was used as probe, amplified from the 5′ end of the *BAR* gene to the 3′ end of the right transposon junction (Marsch-Martinez *et al*., 2002) and labelled by random oligonucleotide priming (Gibco BRL®).

Identification of the activated gene

To identify the putative activated gene, genomic DNA was used to isolate flanking DNA fragments of the *En-I* elements using a modified thermal asymmetric interlaced-PCR (TAIL-PCR) method, as previously described (Marsch-Martinez *et al*., 2002). Flanking DNA sequences were compared to the *Arabidopsis* database using BLASTN. The 4 × *35S* enhancers present in the AIE were located between two genes, 2 kb downstream of gene At5g66860 and about 5 kb upstream of gene AT5g66870. The two predicted open reading frames were amplified using the following forward and reverse primers for At5g66860 and At5g66870, respectively: PRI839F: 5′-TGAGAT GGCGAAATGGTGGC-3′, PRI840R: 5′-AGA CTTATTTTATGCTTGAACAG-3′, PRI 841F: 5′-AAATGGCGTCTTCAAGCTCTC CTTG-3′, PRI842R: 5′-AGATTAACCAATGACATTCCT TCTACCC-3′. Expression was analysed by Northern blot hybridisation with approximately 5 μg of total RNA, isolated from rosette leaves (RL), cauline leaves (CL), young flower buds (top of the inflorescence) (CF) and open mature flowers (OF), from *Arabidopsis thaliana* plants, ecotype WS-3. RNA was isolated using the Qiagen® RNA isolation mini kit, denatured with 1.5 M glyoxal, and fractionated on 1.4% agarose gel (w/v). The two genes described above were used as probes for hybridisation. The probes were labelled by random oligonucleotide priming (Gibco BRL®) and blots were hybridised and washed as described previously (Angenent *et al*., 1992). To test for equal loading, RNA quantities were checked by ethidium bromide staining of the gel.

Constructs

For the confirmation of the overexpressed gene, full-length cDNA of the predicted *ASL1*/*LBD36* gene (At5g66870) was cloned, using the GATEWAY™ cloning technology (Gibco BRL®). Approximately 0.2 μg of total RNA from closed flowers was used as template for the first RT-PCR reaction, following the supplier's instructions. The predicted *ASL1*/*LBD36* cDNA fragment was obtained by PFU proofreading polymerase (Stratagene), using gene specific primers, above described as At5g66870Forw and At5g66870Rev. Terminal 'A' ends were made with Super Taq polymerase (Stratagene) with approximately 1.5 units for 30′ at 72 °C to be subcloned in pGEMT-EASY® (Promega). After subcloning in pGEMT-EASY®, a *ASL1*/*LBD36* cDNA fragment with GATEWAY™ sites (underlined) was obtained using the following PCR-primers: PRO060: 5′-GGGGA CAAGTTTGTACAAAAAAGCAGGCTATGG CGTCTTCAAGCTCTCCTTGCGCAGCT-3′ and PRO061: 5′- GGGGACCACTTTGTACAAGAA AGCTGGGTAGATTAACCAATGCAATTCCT TCTACCC-3′. Subsequently, the fragment was cloned into pDONR207 vector (Gibco BRL®) and recombined to the binary vector pGD625, establishing pARC082. The vector pGD625 was generated by cloning the CaMV*35S* expression cassette from pGD120 (Immink *et al*., 2002), as an *Asc*I/*Pac*I fragment in the blunted *Xba*I site of pBINPLUS (van Engelen *et al*.,1995).

For the *pASL1::GUS* construct, the putative 1.8 kb *ASL1*/*LBD36* promoter fragment was amplified from genomic DNA of ecotype Columbia. PCR was carried out with Taq plus precision polymerase enzyme (Stratagene) using the following primers including GATEWAY™ sites (underlined): PRO095: 5′-GGGGACAAGTTTG TACAAAAAAGCAGGCTCGCGTGAACGTG TCCTTATCATATAAGCAACC-3′ and PRO096: 5′-GGGGACCACTTTGTACAAGAAAGCTGG

GTTTTTTTTGTCTCTTTTCAGACTTGAGAG CCT-3′, located just upstream of the ATG start. The obtained fragment was subcloned in vector pDONR207 (Gibco BRL®) and recombined to the binary vector PBGWFS7, resulting in pARC 201.

The *BP* fragment that was used as probe was amplified using the following primers: PRO025: 5′-ATGGAAGAATACCAGCATGACAACAGC-3′ and PRO026: 5′-GGCAGTCCAAGTAAGCT TGTAGGAGGG-3′.

Plant transformations

All constructs used were transformed to *Agrobacterium tumefaciens* strain GV3101 and introduced into *Arabidopsis* plants ecotype Columbia (Col-0) by the floral dip method (Clough and Bent, 1998). Selection for transformants was done on MS medium containing Kanamycin at 50 mg l^{-1}, with seeds being surface sterilized (http://plantpath.wisc.edu/~afb/vapster.html), and resistant plants were transferred to greenhouse. For selection for BASTA resistance, seeds were vapor surface-sterilized (http://plantpath.wisc.edu/~afb/vapster.html) and grown directly on soil in the greenhouse. Seven days after germination (DAG) resistant plants were selected by spraying twice with a solution of 1:1500 (v/v) Finale® (Gluphosinate ammonium, 150 g l^{-1}, Aventis), with an interval of 2 days between the sprayings.

Knockout insertion mutants

The *asl1*/*lbd36* mutant (WS-3) was obtained via the Wisconsin Facility Service (Sussman *et al*., 2000). The putative knockout mutant was confirmed via segregation analysis and also by PCR using gene specific primer PRI841 and T-DNA vector left border primer JL-270 (5′-TTTCTCC ATATTGACCATCATACTCATTG-3′. This primer combination was also used for homozygosity identification. The insertion position was confirmed by sequencing the left flanking sequence of the single T-DNA insertion (BigDye™ sequencing kit, Applied Biosystems) using the JL-270 primer. It revealed that the insertion was located downstream of the predicted AS2/LOB domain, at approximately one third of the open reading frame (position 138 of the predicted protein product).

T2 seeds of the *as2* mutant were obtained from the GABI-Kat FST population (Li *et al*., 2003), line ID 044C07, ecotype Col-0. Plants were grown under normal greenhouse conditions. To confirm the insertion in the gene, the following primer combination was used: PRO139 5′- TATAGTTT TCTCATCACCAAGCGA-3′ (*AS2*-specific) and T-DNA left border primer PRO140 5′- CCCATT TGGACGTGAATGTAGACAC-3′. The insertion in the open reading frame is located at amino acid position 40 of the predicted protein product, at approximately one third of the AS2/LOB domain.

Microscopy and histology

Scanning Electron Microscopy (SEM) was performed as described previously by Kater *et al*. (2000).

Histology and light-microscopy were performed as described by Angenent *et al*. (1993). Tissue preparation and histochemical staining (with phloroglucinol–HCl (1% (wt/vol) phloroglucinol in 6 N HCl) for lignin detection were performed as described by Mele *et al*. (2003). Petals were cleared in Hoyer's solution as described by Liu and Meinke (1998) and examined as described by Angenent *et al*. (1993).

Results

Isolation of downwards siliques1 (dsl1-D): a dominant, gain-of-function mutant

To obtain flower developmental mutants, a population containing the *En-I* maize transposon-based activation tagging system (Marsch-Martinez *et al*., 2002), was screened for morphological mutants. A mutant affected in inflorescence architecture was identified and designated *downwards siliques1* (*dsl1-D*) (Figure 1). In the *dsl1-D* mutant, flowers and siliques bend downwards and the pedicels are reduced in length (Figure 1C and D). The inflorescence of *dsl1-D* is more compact than wild type inflorescences due to a reduction of the internode length (Figure 1B and D). As a consequence of the reduced internode length, the overall plant height is approximately half the height of a wild type WS-3 plant. The *dsl1-D* mutant phenocopies activation tagged *Arabidopsis* plants, *iso-3D* and *iso-4D* that appeared to be

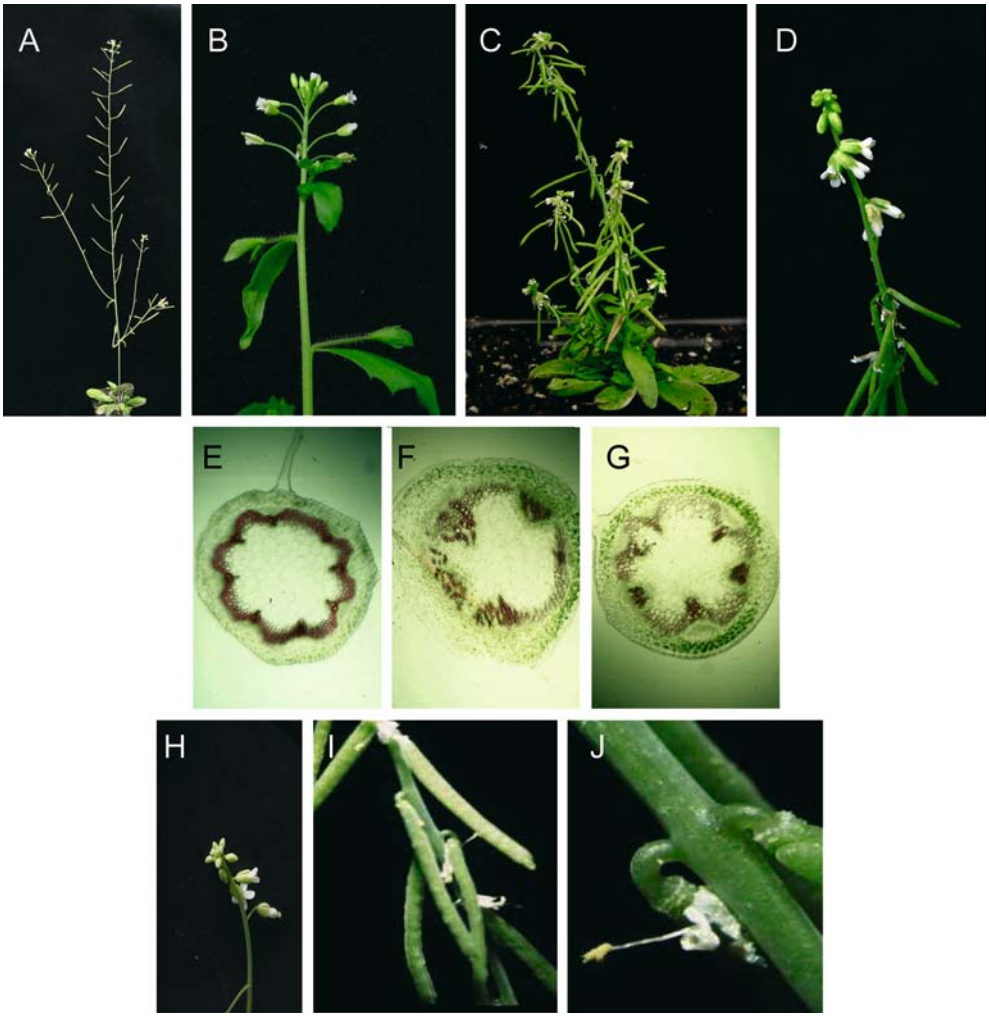

Figure 1. Comparison of phenotypes of the gain-of-function mutant *dsl1-D* and wild type plants. (A) Adult wild type Col-O plant. (B) Inflorescence of a wild type plant. (C) *dsl1-D* adult mutant plant (D) Inflorescence of the *dsl1-D* mutant plant, with flowers and siliques bending downwards. (E)–(G) Histological analysis of fresh stem sections taken at the basis of the inflorescence at comparable developmental stages. The red/brown colour represents lignin, which is deposited around the vascular bundles. (E) Wild type stem showing a thin continuous ring of lignin. (F) *dsl1-D* mutant stem. Lignification in the *dsl1-D* mutant is irregular and leaves gaps in the lignin ring. (G) *bp* mutant plant with a comparable lignification pattern as present in the *dsl1-D* mutant. (H)–(J) Phenotypes of *35S::ASL1* plants, which is similar to the *dsl1-D* mutant phenotype. (H) Inflorescence of a *35S::ASL1* line, showing flowers bending downwards. (I) Siliques of a *35S::ASL1* plant. (J) Close-up of the internode region of a *35S::ASL1* plant showing a short pedicel.

allelic mutants caused by activation of the *ASL1/LBD36* gene (Nakazawa *et al*., 2003).

Histological analysis of stems of *dsl1-D* and wild type plants revealed a change in lignin deposition in the mutant. An irregular pattern of lignin accumulation resulting in gaps in the lignin ring is observed in the mutant (Figure 1E–G). The lignin deposition also started much earlier in *dsl1-D* plants (results not shown). This difference in lignification depended on the position of the stems that were used for analysis, because sections at the basis of *dsl1-D* stems revealed similar lignin deposition as observed for wild type stems (results not shown). The lignin deposition in *dsl1-D* was comparable with the pattern present in the loss-of-function mutant *brevipedicellus (bp)* (Figure 1G, (Mele *et al*., 2003)).

To analyse the inheritance of the *downwards silique1* trait, the *dsl1-D* mutant was selfed and the progeny was analysed, revealing a 3:1 ratio

between mutant and wild type phenotype. This demonstrated the dominant nature of the *dsl1-D* mutant caused by a single transposon insertion.

Identification of the affected gene

The *dsl1-D* mutant was obtained by transposon-based activation tagging using quadruple cauliflower mosaic virus (CaMV) *35S* enhancers as activator (Marsch-Martinez *et al*., 2002). First, Southern blot analysis was performed to determine whether single or multiple transposon insertions were present in the genome of the *dsl1-D* plant. This segregation analysis confirmed that the mutation is caused by a single activation I element (AIE) (data not shown). Subsequently, TAIL-PCR was used to amplify the flanking DNA sequences of the insert (Liu *et al*., 1995; Liu and Whittier, 1995; Tsugeki *et al*., 1996). Sequence comparison of the obtained flanking regions with the *Arabidopsis* genome sequence, revealed that the 4 × *35S* enhancers present in the AIE were positioned between two annotated genes, with a distance to the translation start sites of approximately 2 kb, for gene At5g66860, and about 5 kb for gene At5g66870, respectively. A schematic presentation of the insertion in the genome is given in Figure 2A. The 4 × *35S* enhancers in the AIE are in the same direction as the predicted transcription of the upstream gene. Expression levels of the candidate genes were checked by RNA gel blot hybridisation, using tissues from RL, stems (S), top of the inflorescence with young closed flowers (CL) and OF from mutant and wild type Col-0 and WS-3 plants (Figure 2B). Compared to the wild type samples, increased expression levels of the downstream gene (At5g66870) was observed in *dsl1-D* mutant plants, suggesting that the overexpression of this gene in the mutant caused the *dsl1-D* phenotype. Surprisingly, the expression of the upstream gene located approximately 2 kb from the *35S* enhancers appeared to be not affected in this mutant. This contrasts to previous reports where T-DNA activation tagging resulted in

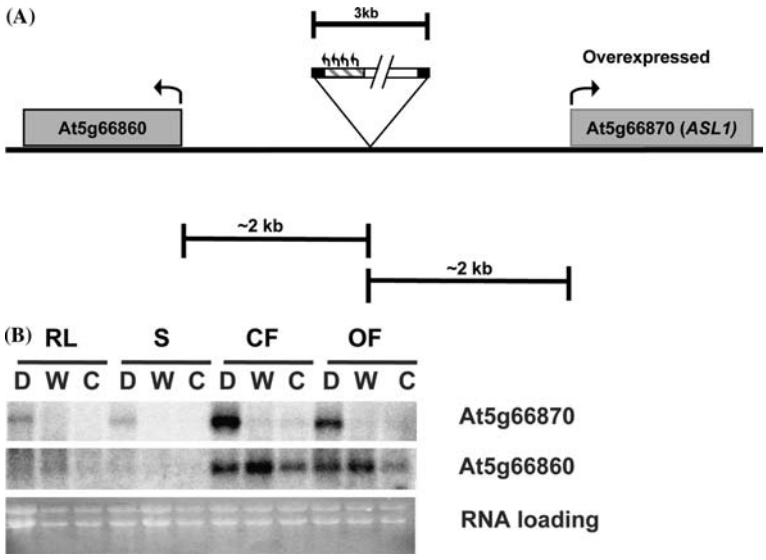

Figure 2. (A) Schematic representation of the insertion position of the activation I element (AIE) in the *dsl1-D* genome. The insertion was located between two genes at chromosome 5. The distances from the transcription start sites of the genes to the 4 × *35S* enhancer elements in the AIE are indicated in kilobasepairs (kb). Gray boxes depict the two genes and the arrows represent the direction of transcription. Numbers beneath the gray boxes show where the ATG start codon is positioned and the number beneath the AIE represent the insertion point in the genome. The AIE element is about 3.0 kb in length and is composed of the 4 × *35S* enhancers (dashed box) and the *BAR* resistant gene (filled gray box). The black boxes are the left and the right junctions of the AIE element.(B) Northern blots showing the expression of the two genes that flank the AIE. The upper blot was hybridised with gene At5g66860 and lower blot with gene At5g66870. Samples were collected from RL, stems (S), closed flower buds (top of inflorescence) (CF) and OF from *dsl1-D* (D) plants and wild type WS-3 (W) and Col-0 (C) plants. An image of the gel prior to blotting and stained with ethidium bromide is presented as a control for RNA loading.

overexpression of the nearest gene to the 4 × *35S* enhancers (Hayashi *et al*., 1992; Kardailsky *et al*., 1999; Weigel, 2000).

The affected gene, which was overexpressed in the *dsl1-D* mutant was previously annotated as the *ASYMMETRIC LEAVES2-LIKE1* (*ASL1*) / *LATERAL ORGAN BOUNDARIES DOMAIN36* (*LBD36*) gene. We will refer to *ASL1* in this report. It encodes a novel cysteine rich protein with a so-called AS2/LOB domain (Iwakawa *et al*., 2002; Shuai *et al*., 2002). The AS2/LOB domain is a conserved domain of ~100 amino acids residues, which contains four conserved Cys residues ($CX_2CX_6CX_3C$) present in members of the *AS2* family. The *ASL1* gene is very similar in amino acid sequence to *AS2* (also known as *LBD6*) (82% sequence similarity). A phylogenetic tree of the AS2 family, including the close homologues AS2 and *ASL1* is presented by Iwakawa *et al*. (2002). Using a T-DNA activation tagging approach, Nakazawa *et al*. (2003) described very recently a similar *downwards siliques* mutant phenotype obtained by overexpressing the *ASL1* gene.

Confirmation of the phenotype

To confirm that the observed *downwards siliques1* phenotype was caused by the activation of the *ASL1* gene, the predicted *ASL1* cDNA was cloned behind the CaMV *35S* promoter. This *35S::ASL1* construct was introduced in *Arabidopsis* wild type plants ecotype Col-0. From 10 Col-0 transformants, 9 exhibited the downward flower/silique phenotype, similar to *dsl1-D* (Figure 1H and I). Among those 9 lines, one line showed increased severity in hyponastic rosette leaves compared to *dsl1-D*. Two plants were smaller than the *dsl1-D* mutant and another had a severe reduction in pedicel length (Figure 1J). In conclusion, the overall phenotype of the *35S::ASL1* lines resembles the phenotype of the *dsl1-D* mutant obtained by activation tagging, although some lines were more severe, most likely due to a higher *ASL1* expression.

Expression analysis of ASL1

To analyse *ASL1* expression, a construct harbouring a 1.8 kb putative *ASL1* promoter fragment fused to the *β*-glucuronidase (*GUS*) gene, *pASL1::GUS*, was introduced in *Arabidopsis* Col-0 plants. The GUS expression patterns observed were consistently in all GUS expressing lines (17 out of 22 lines) and are shown in Figure 3. GUS expression was detected in many parts of the plant at various stages of development. Expression was observed in trichomes of mature rosette leaves and in cauline leaves. In young floral buds, the GUS activity was very strong in the distal part of the pistil at stages when style and stigma start to develop (Figure 3B and C). Most interesting, GUS activity was strongest at the base of many lateral organs, including branching points of the inflorescence and floral organs (Figure 3C, D and F). This expression pattern observed in many organ boundaries is similar to the result reported by Shuai *et al*. (2002) with an enhancer trap line showing expression of the *LOB* gene. GUS expression was also detected in pedicels, particularly those from older flowers and siliques, with a gradient of staining coming downwards from the abscission zone to the lateral axil (Figure 3E). In mature flowers, specific staining is visible at the base of petals and sepals, and progressively decreases towards the distal part of these floral organs (Figure 3F and G). Furthermore, GUS expression is detectable in ovules (Figure 3H), although this was only seen in high-expressing lines.

Knockout mutant of ASL1

To get further insight into the function of the *ASL1* gene, a loss-of-function mutant was identified by screening the Wisconsin population (Sussman *et al*., 2000). Homozygous mutant lines were generated and the insertion was confirmed by PCR analysis (data not shown). Northern blot analysis revealed the absence of *ASL1* mRNA suggesting that the *asl1* mutant is a null allele (data not shown). No visible mutant phenotype was observed in this *asl1* loss-of-function mutant, which suggests that *ASL1* is redundant with other closely related genes from the *AS2* family. The most likely candidate for redundancy is *AS2*, therefore, double mutants between *asl1* and *as2* were generated. A T-DNA insertion line affecting the *AS2* gene was obtained from the GABI population (Li *et al*., 2003). Confirmation of the T-DNA insertion was done by sequencing the flanking DNA sequences. Plants homozygous for the single T-DNA insertion in the *AS2* gene, as confirmed by PCR and Southern blot analysis

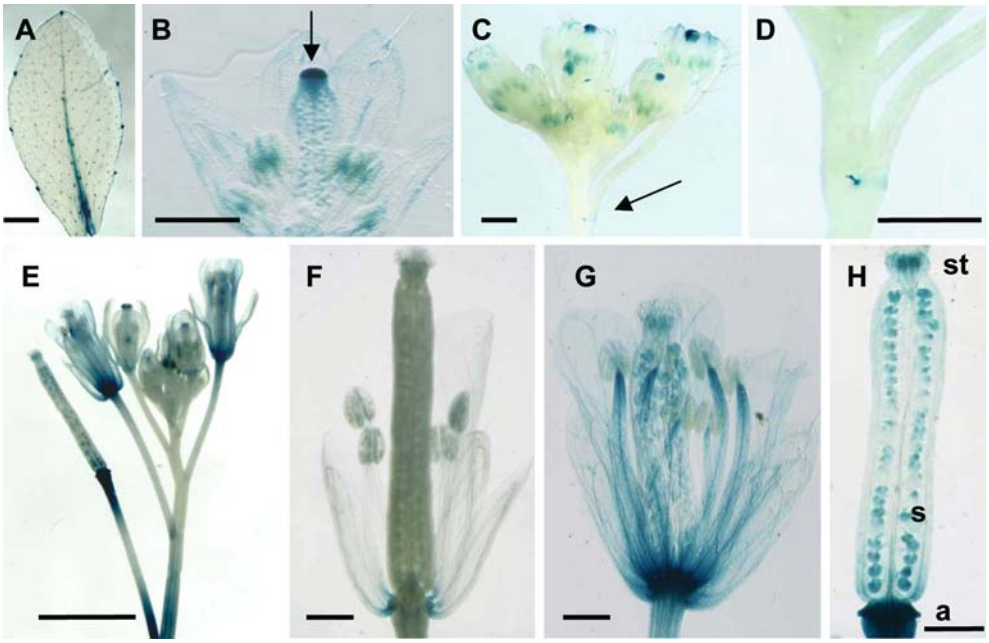

Figure 3. GUS expression analysis of *pASL1::GUS* plants. (A) Rosette leaf showing GUS expression in trichomes and the main vein. (B) Young floral bud with high GUS expression at the distal part of the pistil (arrow). (C) Inflorescence of a *pASL1::GUS* transgenic line with GUS staining in the style and at the base of lateral organs and branch points (arrow) (D) Close-up of (C) at the position of inflorescence branching. (E) Older inflorescence showing GUS expression in pedicels and floral organs. (F) Weak *pASL1::GUS* expressing line with expression at the basis of petals and sepals. (G) Mature flower of a strong *pASL1::GUS* expressing line. GUS activity is present at the basis of petals and sepals, in the anther filaments and ovules. (H) GUS expression in a young silique of the same line as shown in (G). st = stigma, s = developing seed, a = abscission zone. Bar = 5 mm in (A, E); = 0.5 mm in (B), = 1 mm in (C, D, F, G, H).

(results not shown), exhibit aberrations in RL, CL and sepals. The RL are lobed and curled downwards and have shorter petioles. Both the cauline leaves and sepals show serrations at the margins. These phenotypic aberrations for the *as2* mutant have been described previously (Semiarti *et al*., 2001; Iwakawa *et al*., 2002). The *as2* mutant plants were used to generate a double mutant with *asl1*. Reciprocal crossings were made between *asl1* and *as2*. F2 segregating plants were analysed molecularly and phenotypically. In the two F2 populations, three classes of phenotypes were segregating: plants with a wild type phenotype (69 plants out of 96), mutants resembling the single *as2* mutant (19 out of 96) and lines with a stronger floral mutant phenotype (8 out of 96). The genotypes of all plants were determined by PCR (results not shown) and revealed that the lines with the most dramatic phenotypic changes were all double mutants. Because the *as2* and *asl1* mutants were in different genetic backgrounds, Col-1 and Ws, respectively, we studied the single mutant phenotypes also in a Col-1 × Ws background. Although the overall architecture of the offspring plants differed slightly and segregate for the different ecotype backgrounds, the aberrations in the flower were hardly influenced by the genetic background.

When comparing the *as2* single and *asl1 as2* double mutants, differences were observed in the floral buds (Figures 4 and 5). Wild type floral buds at around stage 6 are enclosed by sepals (Bowman, 1994). The buds remain closed until stage 13, prior to anthesis (Figures 4A and D, 5A and B). Petals become apparent just before that stage, when the sepals cannot cover the expanding petals completely (Figures 4D and 5B). At stage 6, when sepals should enclose the wild type buds completely, the *asl1 as2* double mutant plant, has narrower sepals resulting in exposed inner floral organs (Figures 4C and F; 5E and F). Another striking phenomenon, in addition to the exposed inner organs, is the outwards curling of the sepals and petals (Figures 4H and 5E). In contrast, in

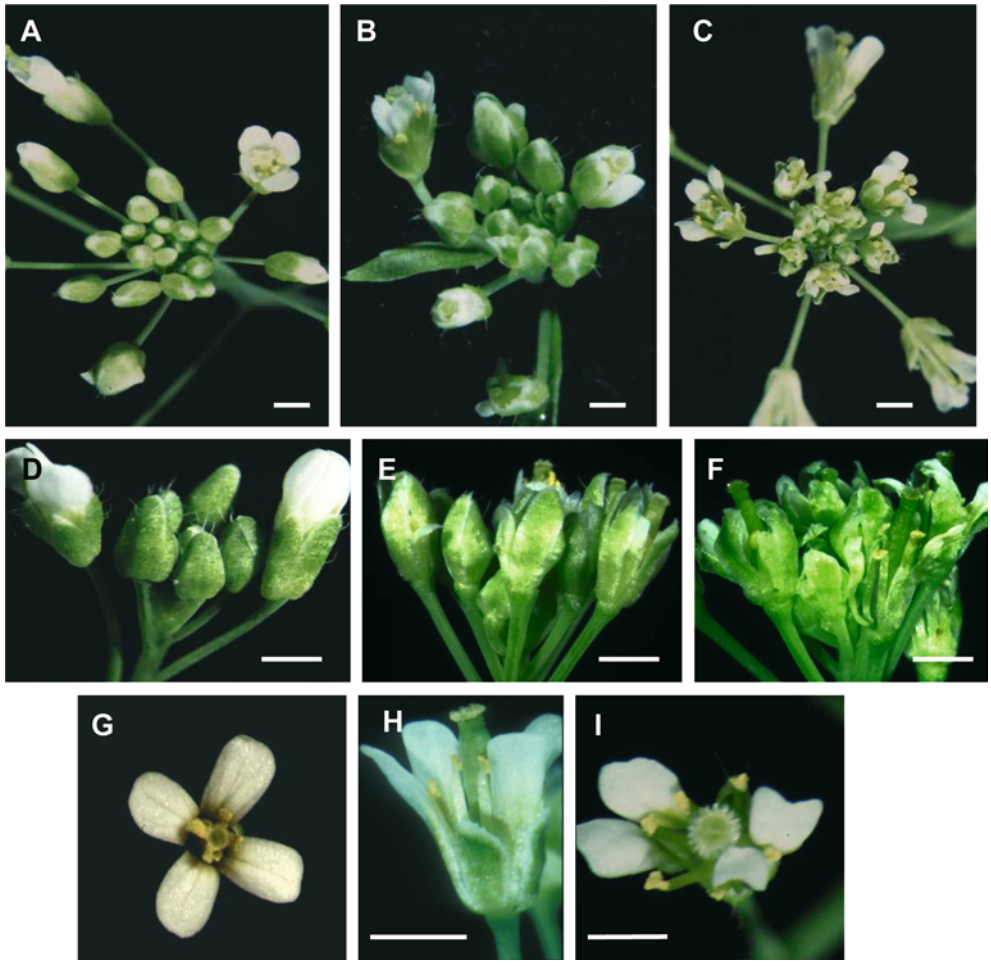

Figure 4. Phenotypes of wild type, *as2* mutant and *asl1 as2* double mutant plants. (A) Top view of a wild type inflorescence. (B) Top view of an *as2* inflorescence. (C) Top view of a *asl1 as2* double mutant inflorescence. (D) Side view of wild type inflorescence. (E) Side view of the *as2* mutant plant. (F) Side view of the *asl1 as2* double mutant plant, showing that the flower buds are opened earlier. (G) Top view of a wild type flower. (H) Detailed few of a *as2* single mutant flower. (I) Top view a *asl1 as2* mutant flower depicting petals curling outwards and loss of symmetry. Bar = 1 mm.

wild type and single mutant flowers, the perianth organs are folded inwards and thereby, covering the reproductive organs perfectly (Figures 4A, B, D and E, 5A and C). The phenotype of premature flower bud opening has been described previously for *as2* (Ori *et al*., 2000; Byrne *et al*., 2002), although *as2* mutants grown under the conditions used in this experiment showed only mild defects in the flower (Figures 4B and E, 5C and D).

The wild type sepals overlap each other as can be seen in the SEM illustrations (Figure 5A), while the overlap is absent in the double mutant flower buds from approximately stage 6 onwards (Figure 5E and F). The incomplete overlap of the sepals in the double mutant leaves a space between the sepals, allowing the inner organs to be exposed. The SEM and macroscopic analyses also revealed a dramatic reduction in number of trichomes at the abaxial site of sepals of the double mutant (Figure 5E and F). Because the vegetative part of the *as2* mutant is indistinguishable from the double mutant, *asl1* does not contribute to the aberrations in the rosette and cauline leaves. The RL are lobed and they are also curled downwards. CL are serrated and form lobes at the base (data not shown).

To get more insight into the morphological abnormalities of the double mutant, histology of wild type and mutant flowers was examined (Figure 6). In a wild type flower at stage 10, the

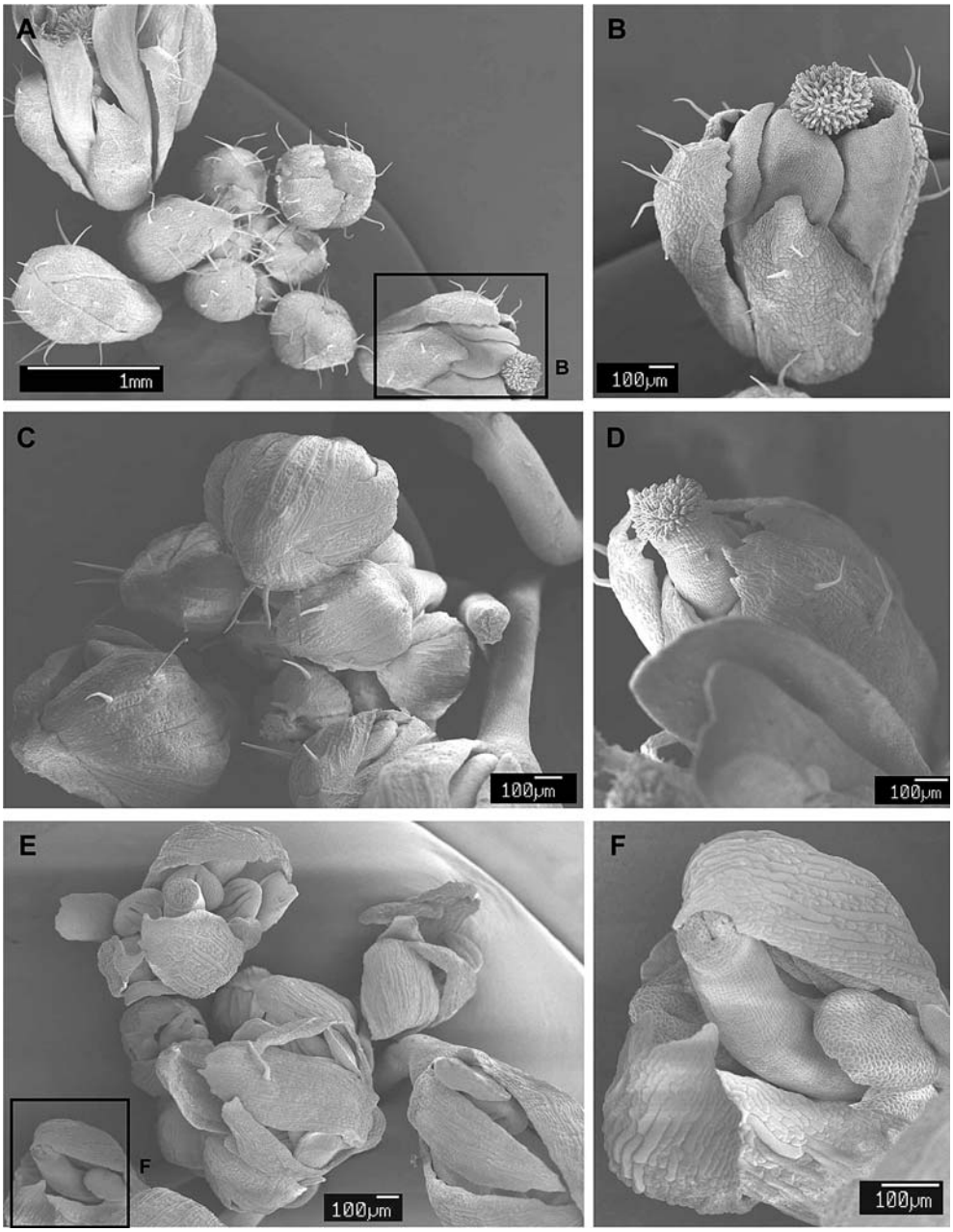

Figure 5. Scanning Electron Microscopy analysis. (A) Wild type inflorescence showing that flower buds are completely closed. (B) A wild type flower just before anthesis, when petals become apparent. The sepals are covered with trichomes (C) *as2* mutant inflorescence. (D) An *as2* mutant flower. (E) *asl1 as2* double mutant inflorescence showing that flower buds open earlier than in wild type lacking trichomes in the sepals (F) Close-up of a *asl1 as2* flower at floral stage 10, which is already opened and the small petals are exposed.

organs in each of the four floral whorls are symmetrically organised. The petals have the adaxial surface facing the inner organs, with a perfect concave shape (Figure 6A). In the *asl1 as2* double mutant, this concave adaxial petal shape is highly distorted: the form of the petal is irregular and spaces are present between the perianth organs (Figure 6B and C). It appears that the position of the four petals is not changed in the mutant. They are still alternating with the four sepals, thereby

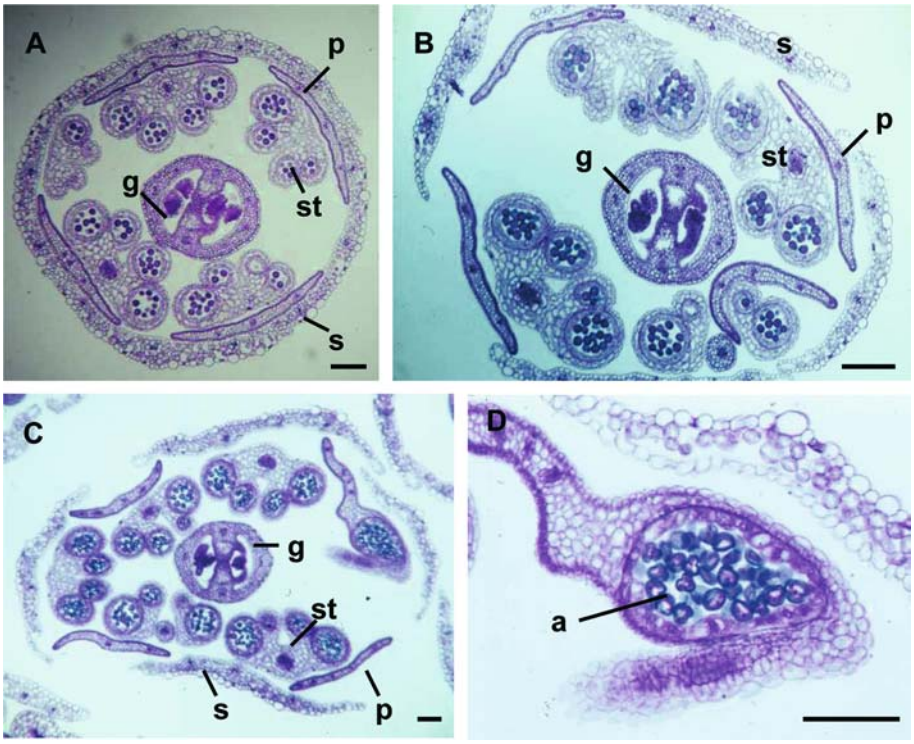

Figure 6. Light microscopic analysis of wild type and *asl1 as2* mutant flowers. The flower is composed of 4 sepals (s), 4 petals (p), 6 stamens (st) and a gynoecium (g). (A) Cross-section through a wild type flower. The flower bud is still fully enclosed by symmetrically organised sepals and petals. One stamen is missing in this section. (B and C) *asl1 as2* mutant flowers, showing that sepals are not enclosing the other inner organs. The petals are irregular in shape and position and are facing outwards. (D) Occasionally petals are fused to antheroid tissue containing pollen (a). Bars = 100 μm.

positioned in between the outer perianth organs. Occasionally, anther locule-like structures containing pollen are fused with a petal (Figure 6C and D).

Morphology of sepal and petal epidermal cells

Because changes in abaxial/adaxial symmetric has been reported for leaves of the *as2* mutant (Lin *et al*., 2003), the identities of the epidermal cells on both sites of the sepals and petals were monitored by SEM analysis (Figure 7). In the blade part of the wild type abaxial petal, epidermal cells are round shaped with irregular epicuticular ridges, whereas cells at the adaxial site are conical (Figure 7C and M) (Bowman, 1994). The basal part of the wild type petal has long elongated epidermal cells at both sides. The domain of these elongated cells spans about one third of the total size of a wild type and *as2* single mutant petal (Figure 7). In the double mutant, conical cells are present at the adaxial site (Figure 7S) and irregular serrated cells cover the distal part of the petal at the abaxial side (Figure 7J), indicating that the abaxial/adaxial symmetry is not changed. However, in contrast, the proximal–distal organisation is affected. The elongated petiole-like cells are also present in the central part of the petal and even appear at the tip of the petal between normal round-shaped cells (Figure 7I, J and S). Due to this expansion of petiole-like cells into the distal portion of the petal, the boundary of the two cell types is shifted to approximately two-third of the petal size in the double mutant.

These changes in proximal–distal organisation may result in the outwards folding of the *asl1 as2* double mutant petal, which could be explained by unequal expansion of the abaxial and adaxial sides.

Analysis of the epidermal cells of the sepals revealed no pronounced differences between wild type and double mutant (results not shown). The

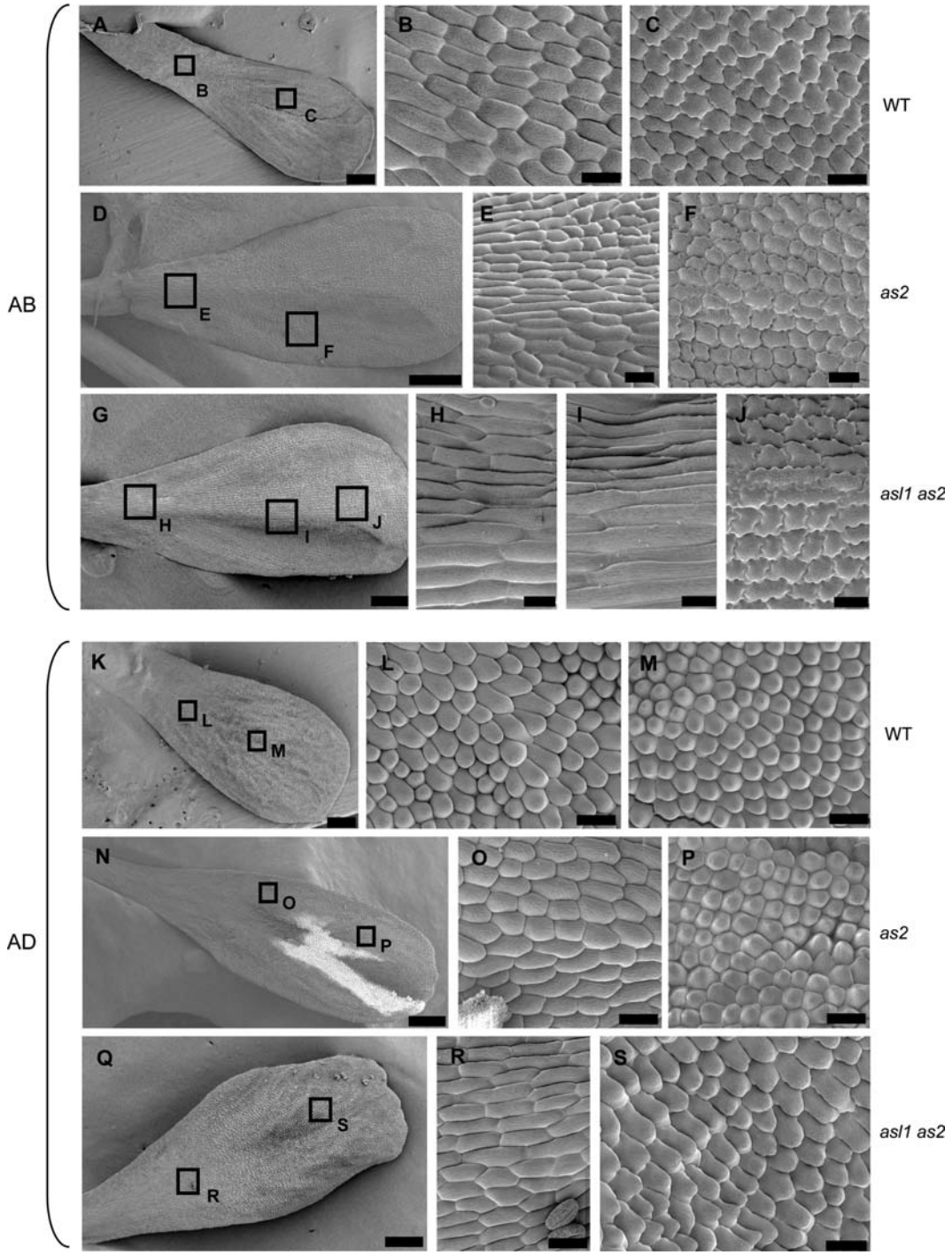

Figure 7. Scanning Electron Microscopy analysis of petal epidermal cells. The boxes in (A), (D), (G), (K), (N) and (Q) indicate the position of the close-ups depicted in the other SEM figures. (A–C) Abaxial epidermal cells of a wild type petal. (D and E) Abaxial epidermal cells of an *as2* petal. (G–J) Abaxial epidermal cells of a *asl1 as2* petal. (K–M) Adaxial epidermal cells of a wild type petal. (N–P) Adaxial epidermal cells of an *as2* petal. (Q–S) Abaxial epidermal cells of a *asl1 as2* petal. Bars in (A, D, G, K, N, Q) = 200 μm; in (B, C, E, F, H–J, L, M, O, P, R and S) = 20 μm.

epidermal cells are very similar at both sides of the sepal and a clear boundary between petiole-like and blade cells is missing. Obvious is the reduced number of trichomes at the abaxial side of mutant sepals (compare Figure 5A and E).

Genetic interaction between ASL1 *and BREVIPEDICELLUS (BP)*

The *dsl1-D* activation tagging mutant resembles the loss-of-function mutant *bp*, also known as *knat1* (Douglas *et al*., 2002; Venglat *et al*., 2002), suggesting that overexpression of the *ASL1* gene suppresses the expression of the homeobox gene *BP*. To provide evidence for this hypothesis, northern blot hybridisations were performed (Figure 8). Tissue samples from roots (R), rossette leaves (RL), cauline leaves (CL), young closed flower buds (CF), OF, stems (ST) and siliques (S) were collected from *dsl1-D* and *bp* mutants. The northern blots containing *dsl1-D* and *bp* material were hybridised with *BP* and *ASL1* specific probes, respectively. Based on the reciprocal expression patterns it can be concluded that there is an antagonistic relationship between these two genes. In the *dsl1-D* mutant transcript levels of *BP* are dramatically decreased compared to *BP* expression levels in wild type Col-0 tissues. In the young floral buds of *dsl1-D* mutant, *BP* transcripts could not be detected. In the same blot low levels of *BP* expression were detected in mature open flowers and stems of wild type samples (Figure 8), which is in agreement with previously reported *BP* expression patterns (Lincoln *et al*., 1994). Furthermore, *ASL1* expression is highly upregulated in the inflorescence of the loss-of-function *bp* mutant demonstrating the antagonistic relationship between the two genes.

Discussion

ASL1 and the AS2/LOB gene family

ASL1 is a member of the AS2/LOB family, which consists of 42 members in the *Arabidopsis* genome (Iwakawa *et al*., 2002; Shuai *et al*., 2002). They all share the AS2/LOB domain. The *LOB* (corresponding to *ASL4*) gene is expressed at the base of the lateral organs in shoots and roots and is excluded from the floral organs (Shuai *et al*., 2002). In contrast, *ASL1* appears to have a broader expression pattern, besides the expression at the axils, it exhibits expression in floral organs, the receptacle and style, suggesting that no redundancy exists between *LOB* and *ASL1* in the flower. These two *ASL/LBD* genes overlap in expression mainly at the boundaries of the lateral organs, such as leaves and pedicels. *AS2*, the closest homologue of *ASL1*, is expressed in almost

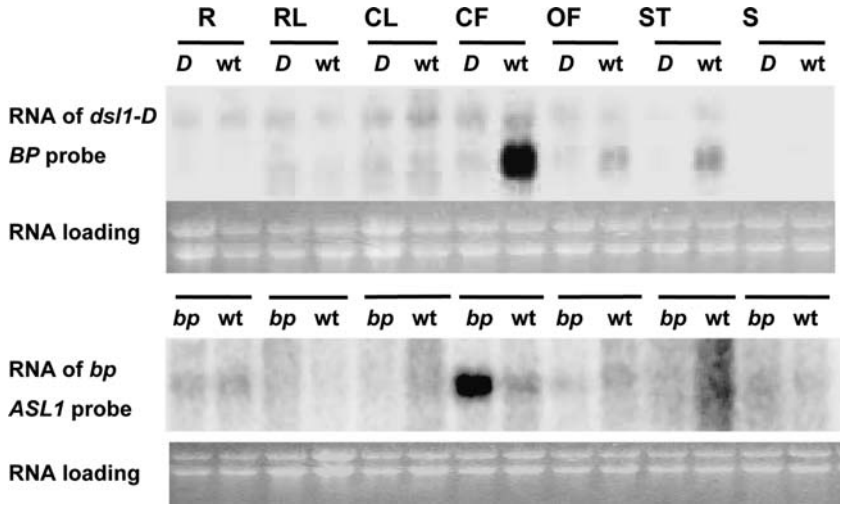

Figure 8. Expression analysis of *BP* and *ASL1* in wild type and mutant plants. Expression patterns of *ASL1* and *BP* were examined in *bp* mutant and *dsl1-D* tissues, respectively. Samples were collected from roots (R), RL, closed flower buds (top of inflorescence) (CF), OF, stems (ST) and siliques (S) from *dsl1-D* (D), wild type (wt, Ws in upper blot, Col-0 in lower blot) and *bp* plants. An image of each gel stained with ethidium bromide is presented as a control for RNA loading.

all of the above ground parts of the plant except internodes and pedicels (Iwakawa *et al.*, 2002; Lin *et al.*, 2003; Xu *et al.*, 2003), demonstrating only a partial overlap in expression pattern between *ASL1* and *AS2*. This is in line with ideas explaining how members of a large gene family may have evolved specific functions: duplication of the ancestral gene, followed by the modification of their expression patterns leads to diversification in gene functions (Ferrario *et al.*, 2004).

The function of the conserved AS2/LOB domain is not known, although it has been suggested that this domain with a putative coiled-coil motif is involved in protein–protein interactions (Shuai *et al.*, 2002). Recently, it has been reported that AS2 interacts with the MYB-domain containing protein AS1 in yeast two-hybrid experiments (Xu *et al.*, 2003). This does not exclude interactions between AS2/LOB family members, which is even very likely, because proteins with a similar amphipathic coiled-coil structure often interact with each other (Newman *et al.*, 2000; Immink *et al.*, 2002).

ASL1 *suppresses* BP *expression*

In the *dsl1-D* mutant internodes are shorter and lateral organs such as flowers are pointing downwards. A very similar effect on inflorescence architecture was observed when the closely related gene *AS2* is overexpressed (Iwakawa *et al.*, 2002; Lin *et al.*, 2003; Nakazawa *et al.*, 2003; Xu *et al.*, 2003). This phenotype is reminiscent with knockout mutants of class 1 *KNOX* (*KNOTTED-LIKE* homeobox) genes, such as *BP* (Ori *et al.*, 2000). The *bp* mutants are affected in internodes and pedicel development and show downwards pointing flowers and siliques (Douglas *et al.*, 2002; Venglat *et al.*, 2002). This is in line with its expression pattern being localised in the peripheral zone of the shoot apical meristem (Lincoln *et al.*, 1994) and also in the cortical cell layers of the inflorescence stem and pedicel, but excluded from leaves and perianth organs (Douglas *et al.*, 2002; Venglat *et al.*, 2002). These phenotypes could be assigned to defects in differentiation of abaxial cells in the inflorescence axils, which demonstrates that *BP* is an important regulator of inflorescence architecture. We have shown that *ASL1* down-regulates *BP* and, *vice versa*, *ASL1* is upregulated in the *bp* mutant at the transcriptional level. Therefore, it is likely that the phenotype of the *ASL1* gain-of-function mutant is due, at least partly, to the down-regulation of *BP*. A similar antagonistic relationship was also reported between *AS2* and several members of the *KNOX* family (Lin *et al.*, 2003). This demonstrates that the closely related members of the *AS2*/*LOB* family, *AS2* and *ASL1*, share the same role in down-regulating *KNOX* genes, although the differences in their spatial and temporal expression patterns determine where and when the *KNOX* genes are suppressed.

Ectopic expression of the *BP* gene gave rise to lobed leaves and the formation of ectopic shoot meristems in the leaf blade, indicating that this *KNOX* gene mainly regulates adaxial/abaxial and proximal/distal polarity and differentiation zones (Chuck *et al.*, 1996; Xu *et al.*, 2003). The *as2* loss-of-function mutant phenocopies the *BP* misexpression mutant, demonstrating again the antagonistic relationship between these genes (Ori *et al.*, 2000). In addition, overexpression of *AS2* by the CaMV *35S* promoter resulted in changes in abaxial and adaxial features of leaf epidermal cells, demonstrating that *AS2* is required for proper polarity determination.

In contrast, the *asl1* mutant does not show these changes in cell fate in the leaves, which is in line with its lack of expression in the leaves. In flower organs, where *ASL1* is expressed, it may play a role in cell fate determination and suppression of *KNOX* genes. However, this role is not apparent in the *asl1* single mutant, because most likely it is masked by the action of a functionally redundant gene.

ASL1 *and* AS2 *acting together in proximal–distal symmetry determination*

The *AS2* gene is the closest homologue of *ASL1* and plays an important role in the establishment of normal leaf formation (Serrano-Cartagena *et al.*, 1999; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Lin *et al.*, 2003). *35S::AS2* plants display adaxial/abaxial changes in the leaves (Xu *et al.*, 2003) and the formation of abaxial epidermal cells with a adaxial identity (Lin *et al.*, 2003). In *as2* knockout mutants the polarity in petioles is affected, particularly obvious when the *as2* mutant is analysed in the L*er* genetic background (Xu *et al.*, 2003). In flowers, the *as2* mutation causes mild effects in the sepals: they are slightly curved outwards and are

shorter with a partly serrated appearance (Ori *et al.*, 2000). In addition, mutations in the *AS2* gene causes proximal–distal changes in leaves, which is also observed in mutants of the MYB-domain containing AS1 protein (Serrano-Cartagena *et al.*, 1999; Sun *et al.*, 2002). In particular in the *rough sheath2* (*rs2*) mutant, which is the maize equivalent of *as1*, the role of *RS2* in establishing proximal–distal polarity in leaves is apparent. In these mutants sheath cells expand in the distal region of the maize leaf blade without effects on dorso–ventral symmetry (Schneeberger *et al.*, 1998; Tsiantis *et al.*, 1999).

Whereas *dsl1* loss-of-function mutants lack a visible phenotype, more pronounced aberrations were observed in the flower of the double *asl1 as2* mutants when compared to either of the single mutants. The flower buds opened precociously, which causes an exposure of the inner organs at early bud stages. These effects on the perianth organs could be attributed to an expansion of petiole-like cells towards the blade area, which causes a slight increase in length of the petals and outwards growth of sepals and petals. The phenotype observed in the *asl1 as2* double mutants indicates that there is partial redundancy between the two members of the AS2/LOB family and that their roles in establishing proximal–distal polarity in the perianth overlap. The fact that the identity transformation of blade to petiole-like cells is subtle and not complete suggests that other closely related members of the AS2/LOB family, e.g. *ASL2*/*LBD10*, may play a role in this process as well.

LBD *and* KNOX *genes in lateral organ development*

Leaves of higher plants are produced through the differentiation of cells derived from the shoot apical meristem. Similarly, cells that form the floral organs undergo a comparable transition from floral meristem identity to differentiated state. Important factors in this transition are members of the *KNOTTED1-LIKE (KNOX)* homeobox family, such as *STM*, *BP*, *KNAT2*, and *KNAT6*, which are predominantly expressed in the meristematic cells and are down-regulated prior to lateral organ initiation. Recent studies in *Arabidopsis* revealed a central role of a novel class of regulators, belonging to the AS2/LOB family, in the spatial control of *KNOX* gene expression. It has been shown that genes such as *LOB* and *AS2* are mainly active in leaves to control polarity, while in this report we have shown that *ASL1* plays a role in differentiation of the perianth organs. Although it has been demonstrated here that *BP* and *ASL1* can act antagonistically, it does not mean that *ASL1* is the one that excludes *BP* expression from sepals and petals in wild type *Arabidopsis* flowers. However, Ori *et al.* (2000) showed that *BP* is ectopically expressed in the floral organs in an *as2* mutant background. Based on this and the functional redundancy between *AS2* and *ASL1* reported here, *ASL1* might do the same.

The role of the MYB-gene *AS1* in this regulatory pathway has been postulated by Xu *et al.* (2003). The physical interaction between AS1 and AS2 proteins, forming a potential heterodimeric transcription factor may directly or indirectly control *KNOX* expression and promote cell differentiation in leaves. Also ASL1 interacts with AS1 in yeast two-hybrid studies, which corroborates our finding that ASL1 and AS2 are partly redundant (A. Chalfun-Junior; R. Immink; G.C. Angenent, unpublished data).

How adaxial–abaxial symmetry is promoted in sepals and petals remains to be elucidated, although it is very likely that AS2/LOB family members are essential in that process as well. Functional characterization of more *ASL*/*LBD* genes and defining their partial overlapping roles in lateral organ development will provide new insights into floral organ differentiation and underlying regulatory networks.

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