Development 136, 955-964 (2009) doi:10.1242/dev.028613

The *more and smaller cells* mutants of *Arabidopsis thaliana* identify novel roles for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* genes in the control of heteroblasty

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Regulation of cell number and cell size is essential for controlling the shape and size of leaves. Here, we report a novel class of *Arabidopsis thaliana* mutants, *more and smaller cells 1* (*msc1*)-*msc3*, which have increased cell number and decreased cell size in leaves. *msc1* has a *miR156*-resistant mutation in the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15* (*SPL15*) gene. *msc2* and *msc3* are new alleles of *paused* and *squint* mutants, respectively. All *msc* mutants showed accelerated heteroblasty, a phenomenon in which several morphological traits of leaves change along with phase change. Consistent with this finding, in the wild type, leaves at higher nodes (adult leaves) also have increased cell number and reduced cell size compared with those at lower nodes (juvenile leaves). These facts indicate that precocious acquisition of adult leaf characteristics in the *msc* mutants may cause alterations in the number and size of cells, and that heteroblasty plays an important role in the regulation of cell number and size. In agreement with this suggestion, such heteroblasty-associated changes in cell number and size are severely inhibited by the constitutive overexpression of *miR156* and are promoted by the elevated expression of *miR156*-insensitive forms of *SPL* genes. By contrast, *rdr6*, *sgs3*, *zip*, *arf3* and *arf4* mutations, which affect progression of heteroblasty, had little or no effect on number or size of cells. These results suggest that cell number and size are mainly regulated by an *SPL*-dependent pathway rather than by a *tasiR-ARF*-dependent pathway.

KEY WORDS: Cell proliferation and expansion, Compensation syndrome, Heteroblasty, Leaf development, miR156

INTRODUCTION

Determination of leaf size and shape is a crucial process that influences the appearance of shoots. Plants have the ability to make leaves of amazingly diverse sizes and shapes, not only between different species but also within a species. The size and shape of leaves change dramatically in response to environmental conditions, such as light quality and quantity, daylength, nutrition and water availability (Ferjani et al., 2008). In addition to these external cues, internal signals that arise at certain developmental stages have a role in the regulation of leaf size and shape. In most species, some leaf traits change as a plant passes through developmental phases, such as embryogenic, juvenile vegetative, adult vegetative and reproductive phases (Tsukaya and Uchimiya, 1997; Kerstetter and Poethig, 1998; Tsukaya et al., 2000). This phenomenon is called phase change, or heteroblasty. In *Arabidopsis thaliana* (*Arabidopsis*, hereafter), lamina size, leaf length/width ratio, petiole length, serration number, and production of abaxial trichomes change in relation to heteroblasty. Cell number and cell size in leaves might also change with heteroblasty, as an increase in cell number and a decrease in cell size in higher node leaves is observed in various species (Ashby, 1948; Granier and Tardieu, 1998; Cnops et al., 2004; Cookson et al., 2007). However, reduced water availability caused by water deprivation to leaves at lower nodes, or diffusive inhibitory signals from lower leaves were previously thought to account for the reduction in cell size in leaves on higher nodes (Ashby, 1948); whether this phenomenon is physiological or genetically controlled remains to be answered.

Recent molecular genetic studies indicate that miRNAs and trans-acting siRNAs regulate heteroblasty. *miR172* in maize promotes the juvenile-to-adult phase change by repressing its target, *Glossy15*, an *APETALA2*-like gene required for juvenile leaf traits (Lauter et al., 2005). By contrast, *miR156* inhibits this phase change by repressing its target, SBP-box-containing genes, when overexpressed in a *Corngrass1* mutant (Chuck et al., 2007). Also, in *Arabidopsis*, constitutive overexpression of *miR156* severely inhibits the progression of heteroblasty. Moreover, overexpression of the *miR156*-insensitive form of the *Arabidopsis* SBP-box gene *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* (*SPL3*), *SPL4* or *SPL5* accelerates the juvenile-to-adult phase change (Wu and Poethig, 2006). In addition, involvement of *tasiR-ARF* and its target *ETTIN* (*ETT*)/*AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4* in the regulation of heteroblastic traits, such as abaxial trichome production, is also proposed (Peragine et al., 2004; Hunter et al., 2006). However, how these genes regulate leaf size and/or shape is still unknown.

Leaf size and shape regulation is dependent on the control of cell proliferation and cell expansion (Tsukaya, 2006), and spatial and temporal regulation of these two processes is crucial for leaf development. In eudicot species, cell proliferation first occurs throughout the leaf primordium and is gradually restricted to the proximal part (Donnelly et al., 1999). Arrest of cell cycle and subsequent post-mitotic cell expansion occur from the distal to the proximal part of a young leaf. The regulatory mechanisms of cell proliferation and cell expansion have been investigated in many studies. An interesting topic is how these two cellular processes are coordinated in the context of leaf development. The first example of such coordination was in barley leaves irradiated with γ rays, which show no cell division during development but develop significantly larger cells (Haber, 1962). Recent studies using various mutant or

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Accepted 15 January 2009

Table 1. Primers used in this study for quantitative RT-PCR

Template	Oligonucleotide sequence (5′-3′)	
	Forward primer	Reverse primer
SPL2	TGTAGCAGGTTCCACTGTCTCTC	TGTCCTTCCAGGATTTGGCTTG
SPL3	CTGAGTGAGGAAGAGGAAGAAGAAG	CGCGTGAAACCTGCTGCATTG
SPL4	AGGCATCTTCTGTCTTTCTCTCAGG	TGGTCGCTTGAATGATGAGTTTGG
SPL5	TCAGGCAACGCTTTTGTCAAC	GATCTTCCTCCTCCTCTCATTGTG
SPL6	GTGCAGCAGGTTTCATTTCCTCTC	TGCAGGTTTCCTTCTTCGTTCATTG
SPL9	GTCACTGTGGCTGGTATCGAAC	CCTGCGGCAACTCCTTTTCTC
SPL10	CACCCTCTCTTTCTCTGCGTTTC	GAGCATTCTCCCACACCTTTGTC
SPL11	CATGGCGAAGATGTGGGAGAATAC	CCGAAGAGGTTGACAGAAGAGAGAG
SPL13	TGGTAGGTGTATGGATTTGTGAGAG	ACCCACCAAACGAGATCGAAC
SPL15	TCGCTCCATCTCTTTACGGAAACC	GTTGACCACGCAGTAGGATCTC
UBQ10	CACACTCCACTTGGTCTTGCGT	TGGTCTTTCCGGTGAGAGTCTTCA

transgenic plants have provided several lines of evidence for the presence of organ-level coordination of cell proliferation and cell expansion (Tsukaya, 2002; Beemster et al., 2003; Tsukaya, 2003; Horiguchi et al., 2006a; Ferjani et al., 2007). When cell number is reduced by a mutation in a cell-proliferation-promoting gene, the size of individual cells often increases. This phenomenon, called compensation syndrome, is observed in several *Arabidopsis* mutants. However, overexpression of such a gene [for example, *ANGUSTIFOLIA 3* (*AN3*)/*GRF-INTERACTING FACTOR 1* (*GIF1*) or *AINTEGUMENTA* (*ANT*)] results in an increase in cell number, but cell size does not change (Mizukami and Fischer, 2000; Kim and Kende, 2004; Horiguchi et al., 2005). These observations indicate that cell number and cell size in a leaf might not be determined by a simple trade-off between cell proliferation and cell expansion. The precise mechanism coordinating these two processes is unknown.

To further investigate the regulation of cell proliferation and cell expansion, we isolated a number of mutants with altered cell number, size or both (Horiguchi et al., 2006a; Horiguchi et al., 2006b; Ferjani et al., 2007; Fujikura et al., 2007). Here, we report a new class of mutants named *more and smaller cells* (*msc*) that have increased cell number and decreased cell size, the opposite phenotype to compensation syndrome. These mutants also show accelerated heteroblasty. Analysis of leaves at various nodes demonstrated that adult leaves had an increased cell number and decreased cell size compared with those in juvenile leaves. This indicates that heteroblasty plays an important role in the regulation of cell number and size. Cloning of *MSC* genes and subsequent molecular and genetic analyses demonstrated that *miR156* and its target *SPL* genes are involved in the regulation of heteroblastic change of cell number and size, whereas another group of heteroblasty-related genes (*RDR6*, *SGS3*, *ZIP*, *ARF3* and *ARF4*) might not be involved.

MATERIALS AND METHODS

Plant materials and growth conditions

The wild-type accession used in this study was Columbia-0 (Col). The *msc1-D* (newly isolated), *msc2* (formerly line number 2025) and *msc3* (formerly 2058) mutants were isolated from a T-DNA mutagenized population (Horiguchi et al., 2006b). Before analyses, all of the mutants were backcrossed to the wild-type Col at least three times. We also used *an3-4* (Horiguchi et al., 2005), *ant-1* (Mizukami and Fischer, 2000), *fugu1*, *fugu2-1*, *fugu3-D*, *fugu4-D*, *fugu5-1* (Ferjani et al., 2007), *psd-1*, *psd-6* (Hunter et al., 2003a; Li and Chen, 2003), *rdr6-11*, *sgs3-11* (Peragine et al., 2004), *zip-1* (Hunter et al., 2003b) and *axr1-3* (Estelle and Somerville, 1987) mutants and the T-DNA insertion mutants *arf3-2* (CS24604) (Okushima et al., 2005), *arf4-2* (SALK_070506C) (Alonso et al., 2003) and *sqn-5* (SALK_033511) (Prunet et al., 2008). The *sqn-1* (Berardini et al., 2001) mutant and the transgenic plants that overexpress *miR156*, *SPL3m*, *SPL3Δ*, *SPL4Δ* or *SPL5Δ* were gifts from G. Wu and R. S. Poethig (Wu and Poethig, 2006). All of these mutants and transgenic plants were in the Col background. Plants were grown on rock wool at 22°C under a 16 hour light/8 hour dark photoperiod at a light intensity of approximately 40 μmol m^{-2} s^{-1}.

Phenotypic analyses

For histological analysis of cells, first, third or fifth leaves of 30-day-old seedlings were collected. Collected leaves were fixed in formalin/acetic acid/alcohol and cleared using chloral solution, as described by Tsuge et al. (Tsuge et al., 1996). Whole leaves and cells were observed as previously described (Fujikura et al., 2007). Because expansion of first, third and fifth leaves was already completed at that stage, cell area was uniform in all parts of the leaf (see Fig. S1 in the supplementary material). Areas of 20-30 cells were measured for each leaf and averaged. Mean ± s.d. of average cell areas from six individual plants are indicated in the figures. To calculate the total cell number in leaves, we measured cell density of observed images of cells, and multiplied the cell density by the area of the same leaf. For analysis of abaxial trichomes, plants were grown until the first few flowers opened.

Genetic mapping

The *msc* loci were genetically mapped using various genetic markers according to the sequence information available in The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/).

RNA analyses

Total RNA was extracted from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For quantitative RT-PCR, total RNA was treated with amplification grade DNase I (Invitrogen) before reverse transcription. Reverse transcription was performed with the oligo$(dT)_{20}$ primer using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed with *Power* SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The *UBIQUITIN10* (*UBQ10*) gene was used as an internal control. The primers used are listed in Table 1. At least three biologically independent samples were analyzed and triplicated reactions were performed with each sample. Reactions performed without reverse transcription did not result in any amplifications (data not shown). For 5′-RLM-RACE, total RNA was extracted from the first and second rosette leaves of 14-day-old plants and 5′-RLM-RACE was carried out using the GeneRacer Kit (Invitrogen). For small RNA blots, 5 μg of total RNA was separated using 8 M urea-denaturing polyacrylamide gels and electrically transferred to a Hybond-N^+ membrane (GE Healthcare, Buckinghamshire, UK). Blots were hybridized with a [γ-^{32}P]ATP labeled *miR156*-complementary oligonucleotide probe 5′-GTGCTCACTCTCTTCTGTCA-3′ at 40°C in ULTRAhyb-oligo hybridization buffer (Ambion, Austin, TX, USA). A *U6* snRNA-complementary oligonucleotide probe 5′-TCATCCTTGCGCAGGGGCCA-3′ was used as a loading control.

RESULTS

Isolation of *msc* mutants

From a collection of numerous *Arabidopsis* mutants with altered palisade mesophyll cell number or size, or both, in the first leaf (Horiguchi et al., 2006a; Horiguchi et al., 2006b), we selected three

mutants that had increased cell number and decreased cell size (Fig. 1C,D). We named these *more and smaller cells* (*msc*) mutants. All *msc* mutants had larger first leaves than the wild type (Fig. 1B). *msc1* had no obvious phenotype other than a larger leaf area and a slightly earlier flowering phenotype (Fig. 2E, reduced total rosette leaf number). *msc2* showed a delay in the initiation of the first pair of rosette leaves, as if the shoot apical meristem were inactive for a few days (Fig. 1E). *msc3* had a delay in leaf emergence and expansion, but the final leaf size was larger than in the wild type (Fig. 1F). *msc2* and *msc3* were inherited as recessive mutations and *msc1* was semi-dominant, and was thus designated as *msc1-D*.

msc2 and *msc3* are allelic to *paused* (*psd*) and *squint* (*sqn*) mutants, respectively

We cloned *MSC1-MSC3* genes using a map-based approach. *msc2* and *msc3* were found to be new alleles of previously reported mutants showing accelerated heteroblasty. These findings suggest that *msc1-D* might have a genetic lesion in the heteroblasty-related gene; therefore, we first described *MSC2* and *MSC3* genes and then considered *MSC1*.

The mutation in *MSC2* was mapped to the *PSD* gene (At1g72560) (Hunter et al., 2003a; Li and Chen, 2003). *msc2* has an 870 bp deletion spanning the seventh intron to the eleventh exon of *PSD* and an insertion of T-DNA in this region (Fig. 2A). *msc2* had a very similar phenotype to the *psd* mutant [meristem pause (Fig. 1E), pointed leaves (data not shown) and accelerated heteroblasty (Fig. 2E, see below)]. Therefore, we tested for the cell number and size of *psd-1* and *psd-6* alleles and found that they had increased cell number and decreased cell size, as found in *msc2* (see Fig. S2 in the supplementary material). F1 plants from a cross between *msc2* and *psd-6* failed to complement their phenotypes (see Fig. S2 in the supplementary material). We concluded that *msc2* was a new allele of *psd* (*psd-16*).

The mutation in *MSC3* was mapped to the *SQN* gene (At2g15790) (Berardini et al., 2001). *msc3* had a 127 bp deletion from the seventh intron through the eighth exon of *SQN* (Fig. 2A). *msc3* and *sqn* mutants also shared other phenotypes [leaf size and shape (Fig. 1F), disturbed flower phyllotaxis (data not shown) and accelerated heteroblasty (Fig. 2E, see below)]. We then examined the number and size of cells in *sqn-1* and *sqn-5* leaves and confirmed that they had similar phenotypes to that of *msc3* (see Fig. S2 in the supplementary material). F1 plants from the cross between *msc3* and *sqn-1* did not complement their phenotypes (see Fig. S2 in the supplementary material). Thus, we conclude that *msc3* is a new allele of *sqn* (*sqn-6*).

msc1-D has a mutation in the *miR156* target site of the *SPL15* gene

We mapped the *msc1-D* mutation at low resolution to the lower arm of chromosome 3, near the SNP marker SGCSNP7. Around the marker, the *SPL15* (At3g57920) gene was found, which has an *miR156* target site (Rhoades et al., 2002). Recently, *SPL15* and its closest homolog *SPL9* were reported to be involved in the regulation of heteroblasty (Schwarz et al., 2008). Based on this information, we sequenced the *SPL15* gene in the *msc1-D* background. We found a C-to-T nucleotide substitution in the *miR156* target site in the *msc1-D* mutant (Fig. 2A,C). Although this mutation does not cause amino acid substitution, it could lead to reduced efficiency of *miR156*-targeted mRNA cleavage. To test this, mRNA cleavage sites of *SPL15* transcripts were identified by 5′-RLM-RACE. A distinct band of amplified products of an expected size was observed in the wild type (Fig. 2B). We found that most cleavage events took place at the ninth and tenth residues from the miRNA 5′ end (Fig. 2C) as previously reported (Wu and Poethig, 2006). In *msc1-D*, however, the amount of amplified products was significantly reduced (Fig. 2B), and most of the cleavage events occurred outside of the *miR156* complementary site (Fig. 2C). We also tested expression levels of *SPL15* by quantitative RT-PCR. The expression levels in *msc1-D* were much higher than in the wild type (Fig. 2D). These results strongly suggest that the mutation in the *miR156* target site of the *SPL15* gene leads to

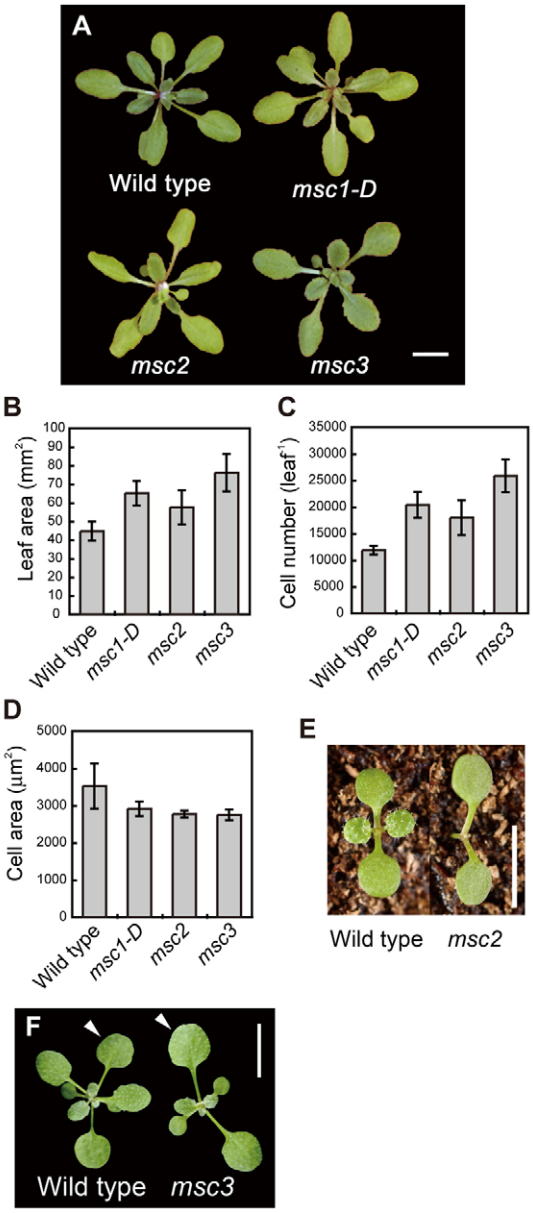

Fig. 1. Phenotypes of *Arabidopsis msc* mutants. (**A**) Morphology of the shoots of seedlings 25 days after sowing. (**B-D**) Leaf area (B), total cell number (C) and average cell area (D) of the first leaves of 30-day-old seedlings. Subepidermal palisade mesophyll cells were observed. The mean ±s.d. from six individual plants is indicated. (**E**) Seedling morphology of the wild type and *msc2* mutant 10 days after sowing. (**F**) Seedling morphology of the wild type and *msc3* mutant 18 days after sowing. Note that the wild type has seven visible true leaves, whereas the *msc3* mutant has five. Arrowheads indicate first leaves. Scale bars: 1 cm in A,F; 5 mm in E.

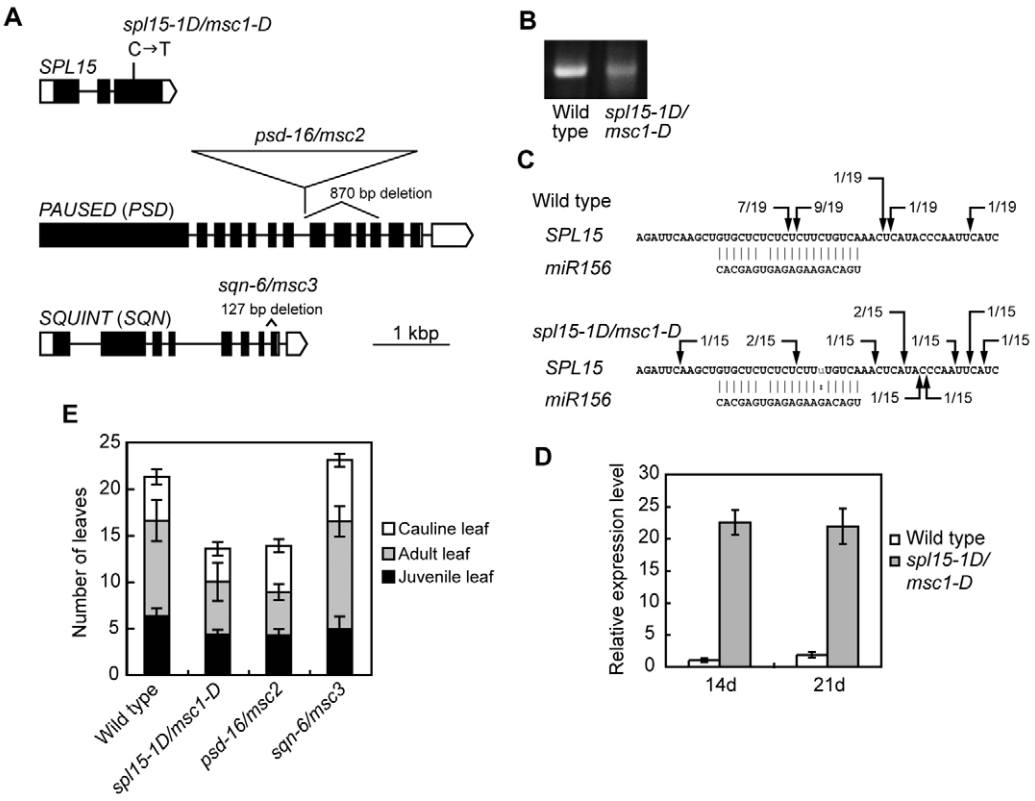

Fig. 2. *msc1-D*, *msc2* and *msc3* have genetic lesions in the heteroblasty-related genes *SPL15*, *PAUSED* and *SQUINT*, respectively. (**A**) Diagram of *Arabidopsis SPL15*, *PAUSED* (*PSD*) and *SQUINT* (*SQN*) genes. Coding and non-coding regions of exons are denoted by black and white boxes, respectively, and introns by lines between boxes. The C-to-T nucleotide substitution in the codon of Leu 226 (CTG to TTG) found in the *msc1-D* mutant does not cause an amino acid change. A T-DNA insertion and an 870 bp deletion found in *msc2*, and a 127 bp deletion in *msc3*, are indicated. (**B**,**C**) 5′RLM-RACE of *SPL15* indicates that the efficiency of *miR156*-directed cleavage of *SPL15* mRNA was reduced in *msc1-D*. Shown are the 5′RLM-RACE products of *SPL15* (B) and the cleavage sites identified by 5′RLM-RACE (C). (**D**) *SPL15* mRNA levels in the first and second leaves of 14-day-old seedlings (14d), and third and fourth leaves of 21-day-old seedlings (21d) of the wild type and *msc1-D* mutant as determined by quantitative RT-PCR. (**E**) Heteroblasty in *msc* mutants. The number of rosette leaves without abaxial trichomes (black) or with abaxial trichomes (gray) and cauline leaves (white) were counted. The mean ±s.d. (*n*=9-10) is indicated.

reduced cleavage and increased accumulation of *SPL15* mRNA in the mutant. In addition, *msc1-D* showed accelerated heteroblasty (Fig. 2E, see below). As *SPL15* promotes phase change (Schwarz et al., 2008), the mutated *SPL15* is highly likely to be responsible for the phenotypes of the *msc1-D* mutant. We hereafter designate the mutant *spl15-1D/msc1-D*.

spl15-1D/msc1-D, *psd-16/msc2* and *sqn-6/msc3* show accelerated heteroblasty

psd and *sqn* mutants were previously reported to exhibit accelerated heteroblasty (Telfer et al., 1997; Berardini et al., 2001). In *Arabidopsis*, juvenile leaves have trichomes only on the adaxial side and adult leaves have trichomes on both adaxial and abaxial sides; thus, trichome presence is a typical marker of heteroblasty. To investigate whether *spl15-1D/msc1-D* exhibits accelerated heteroblasty, we counted rosette leaves without abaxial trichomes (juvenile leaves) or with abaxial trichomes (adult leaves) and cauline leaves (reproductive leaves). The wild type produced 6.3 juvenile leaves on average, whereas the *spl15-1D/msc1-D* mutant had 4.3, indicating that it shows accelerated heteroblasty (Fig. 2E). *psd-16/msc2* and *sqn-6/msc3* produced 4.2 and 4.6 juvenile leaves, respectively, confirming the accelerated heteroblasty (Fig. 2E).

Leaves at higher nodes have increased cell number and decreased cell size compared with those at lower nodes

Because *msc1-msc3* mutants show accelerated heteroblasty, their leaves might have characteristics of those at higher nodes in the wild type. If this interpretation is correct, wild-type leaves should exhibit progressive changes in the number and size of cells during phase change; such changes have been observed mainly in epidermal cells in previous reports (Cnops et al., 2004; Cookson et al., 2007). To address this possibility, we determined the number and size of palisade mesophyll cells at various nodes in the wild type. Cell number increased and cell size decreased in leaves at higher nodes compared with those at lower nodes (Fig. 3A,B). In *spl15-1D/msc1-D* and *sqn-6/msc3* mutants, cell number and cell size resembled those in leaves at higher nodes than the corresponding nodes of the wild type (Fig. 3A,B). However, cell number in the third and fifth leaves of *psd-16/msc2* did not exceed that of the wild type (Fig. 3A,B). This might be because of a genetic lesion in the *PSD* gene, which encodes a tRNA export mediator exportin-t (Hunter et al., 2003a; Li and Chen, 2003) and could lead to reduced efficiency of protein synthesis, and thus to a decline in plant growth.

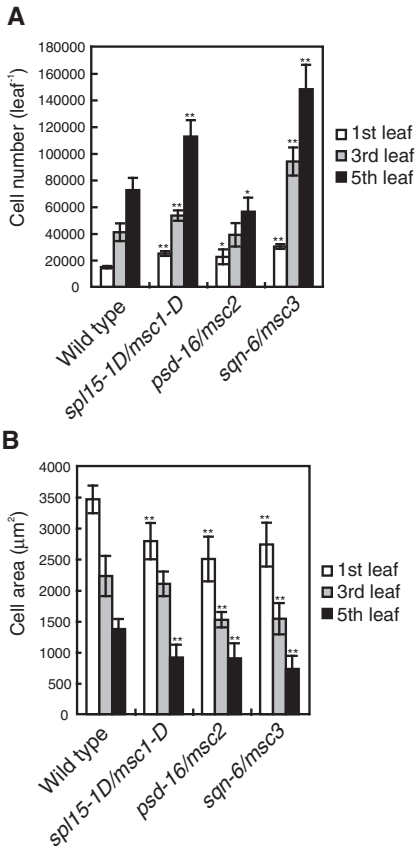

Fig. 3. Cell number increases and cell size decreases in leaves at higher nodes. Total cell number (**A**) and average cell area (**B**) of the first, third and fifth leaves of the wild type and *msc* mutants 30 days after sowing. The mean ±s.d. from six individual plants is indicated. **P*<0.05 and ***P*<0.01 versus wild type (Student's *t*-test).

Cell number and size in leaves are regulated by *miR156* and *SPLs*, not by *tasiR-ARF*

The progression of heteroblasty in *Arabidopsis*, in a broad sense, is inhibited by *tasiR-ARF*-mediated repression of *ETT/ARF3* and *ARF4*, and by *miR156*-mediated repression of *SPL* genes (Hunter et al., 2006; Wu and Poethig, 2006). We investigated these two pathways in relation to the heteroblastic regulation of cell number and size. Initially, we observed accelerated heteroblasty mutants *rdr6*, *sgs3* and *zip*, in which *tasiR-ARF* expression was not detected (Hunter et al., 2006). As shown in Fig. 4A,B, *rdr6* and *sgs3* had a slightly increased cell number, although cell size was not significantly affected, except for the fifth leaf of *sgs3*. The *zip* mutant, by contrast, showed no alteration in cell number and size (Fig. 4A,B). Next, we investigated the *arf3-2* and *arf4-2* mutants, in which heteroblasty is retarded (Hunter et al., 2006), and found that they showed no change in cell number or size in any leaves compared to the wild type (Fig. 4C,D). These results suggest that *tasiR-ARF*-mediated regulation of *ETT/ARF3* and *ARF4* might not be required for the heteroblastic change in cell number and size. In the *arf3-2 arf4-2* double mutant, although cell number was reduced in all leaves tested, an increase in cell number in the leaves at higher nodes occurred in a similar manner to the wild type (Fig. 4C,D). Moreover, cell size did not change compared to the wild type.

We then examined *miR156* constitutively overexpressing plants. In these plants, cell number and size in the first leaf were the same as in the wild type (Fig. 4E,F). However, the cell number increase and cell size reduction in leaves at higher nodes were considerably inhibited (Fig. 4E,F), indicating that heteroblastic change in cell number and size was severely inhibited. By contrast, transgenic plants constitutively overexpressing *SPL3* with a mutation in the *miR156* target site (*35S:SPL3m*) or *SPL3* or *4* with a deletion of the *miR156* target site (*35S:SPL3Δ* and *35S:SPL4Δ*) (Wu and Poethig, 2006) had a significantly increased cell number and decreased cell size, most remarkably in the first leaf (Fig. 4E,F). Those overexpressing *SPL5* with a deletion of the *miR156* target site (*35S:SPL5Δ*) showed no significant alteration in cell number but a considerable reduction in cell size (Fig. 4E,F). These results indicate that *miR156* and its target *SPL* genes (*SPL3* subclass) might be involved in the regulation of heteroblastic change in cell number and size.

We further investigated the expression levels of *miR156* or various *SPL* genes in *msc1-msc3* mutants. *miR156* levels were partially reduced in *psd-16/msc2* and *sqn-6/msc3* (Fig. 5A). By contrast, we found preferential upregulation of specific *SPL* genes among 10 *SPL* genes with the *miR156* target sequence: *SPL13* and *SPL15* were upregulated 2.6- and 3.6-fold, respectively, in *psd-16/msc2*, and *SPL3* and *SPL13* were upregulated 3.9- and 5.6-fold, respectively, in *sqn-6/msc3* (Fig. 5B). In *spl15-1D/msc1-D*, only *SPL15* was markedly upregulated, as expected (Fig. 5B). These results suggest that altered cell number and size in *msc* mutants might have arisen from upregulation of a few *SPL* genes, although the relative importance of individual *SPL* genes might differ in the respective *msc* mutants. To test whether the upregulation of these *SPL* genes accounts for the phenotypes of *psd-16/msc2* or *sqn-6/msc3*, we crossed them with *35S:miR156*. The phenotypes of *psd-16/msc2* and *sqn-6/msc3* were effectively suppressed by *miR156* overexpression (Fig. 6A,B), suggesting that their phenotypes are dependent on upregulated *SPL* genes.

Heteroblasty in other known mutants

Our finding that cell number and size change in relation to heteroblasty allowed us reconsider the phenotypes of two groups of other known mutants. The first group includes mutants exhibiting compensation syndrome and the second is an *auxin resistant 1* (*axr1*) mutant.

As three *msc* mutants show accelerated phase change, one might assume that mutants exhibiting compensation syndrome that have a decreased cell number and increased cell size opposite to the *msc* mutants should show delayed phase change. To test this possibility, we investigated phase change in various compensation-exhibiting mutants by counting the number of rosette leaves with or without abaxial trichomes and cauline leaves. Some mutants (*an3* and *fugu1*) clearly showed delayed juvenile-to-adult phase change, while others (*ant*, *fugu2*, *fugu3-D*, *fugu4-D* and *fugu5*) did not (Fig. 7A). However, note that the *an3* mutant showed rapid leaf production (shorter plastochron) and similar flowering time as the wild type (Horiguchi et al., 2005); therefore, the seemingly delayed phase change in *an3* might be an indirect consequence of the shorter plastochron. By contrast, *fugu1* showed markedly delayed flowering (Fig. 7A, increased rosette leaf number), suggesting that both the juvenile-to-adult vegetative phase change and vegetative-to-reproductive phase change were delayed. The modes of delay in phase change in these mutants differed from each other.

The other mutant, *axr1*, was originally reported as one whose leaves have fewer but normal-sized cells (Lincoln et al., 1990). Subsequently, we reported that the first leaves of *axr1* mutants have

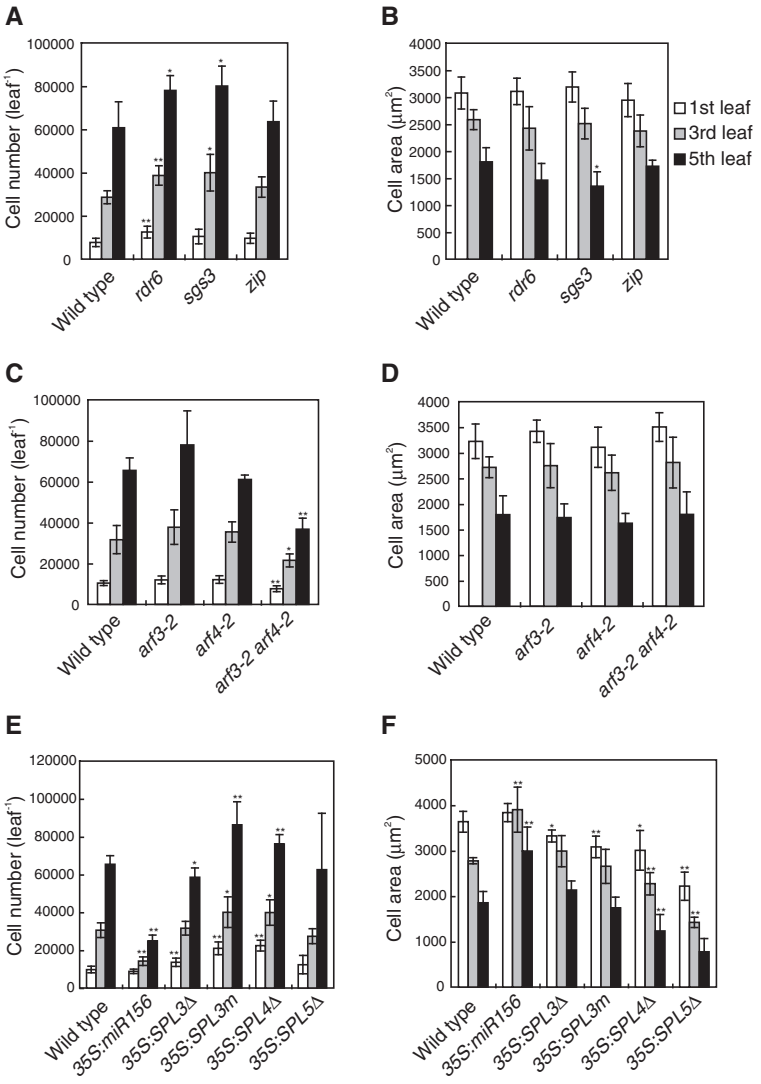

Fig. 4. Heteroblastic changes in cell number and cell size are controlled predominantly by *miR156*-dependent regulation of *SPL* genes. Total cell number (**A**) and average cell area (**B**) of *rdr6-11*, *sgs3-11* and *zip-1* mutants. Total cell number (**C**) and average cell area (**D**) of *arf3-2* and *arf4-2*, and the *arf3-2 arf4-2* double mutant. Total cell number (**E**) and average cell area (**F**) of the transgenic plants overexpressing *miR156* (*35S:miR156*), *SPL3* with a mutation in the *miR156* target site (*35S:SPL3m*), or *SPL3*, *SPL4* or *SPL5* with a deletion in the *miR156* target site (*35S:SPL3Δ*, *35S:SPL4Δ* and *35S:SPL5Δ*). All samples were observed 30 days after sowing. The mean ±s.d. from six individual plants is indicated. **P*<0.05 and ***P*<0.01 versus wild type (Student's *t*-test).

smaller cells compared with the wild type (Horiguchi et al., 2006b). To resolve this discrepancy, we determined the number and size of cells in the first, third and fifth leaves. In *axr1-3* mutants, cell number is significantly decreased in the third and fifth leaves (Fig. 7B). Moreover, cell size in the first, third and fifth leaves was almost the same in this mutant; as a consequence, cell size in the first or fifth leaves of *axr1-3* was smaller or larger, respectively, than the corresponding leaves of the wild type, whereas cell size in the third leaves of *axr1-3* and the wild type was similar (Fig. 7C). These results reasonably explain the paradoxical results reported previously and suggest that *AXR1* is required for the control of cell size during phase change. To investigate the relationship between *SPL*-regulated heteroblastic change in cell size and *AXR1*, we examined *axr1-3 msc1-D* double mutants. The double mutants showed increased cell number and slightly decreased cell size compared with the parental *axr1-3* in all leaves tested (Fig. 7B,C), indicating that *msc1-D* and *axr1-3* mutations have additive effects on cell number and size.

DISCUSSION

In this report, we described three *msc* mutants that have an increased cell number and decreased cell size. These mutants show acceleration in vegetative phase change. As adult leaves of the wild type have an increased cell number and a decreased cell size (Fig. 3A,B), precocious acquisition of the characteristics of adult leaves may be the cause of the phenotypes of these mutants. The results indicate that an unknown factor(s) underlying heteroblasty has an important role in the regulation of the cell number and cell size of leaves. Although an increase in cell number and a decrease in cell size in leaves at higher nodes have been observed in several species (Ashby, 1948; Granier and Tardieu, 1998; Cnops et al., 2004; Cookson et al., 2007), the cause of this phenomenon was unclear until the present study. In this report, we propose that *miR156* and *SPL* genes are involved in heteroblastic change of cell number and size (see below). Our findings highlight the novel roles of SPL transcription factors in the regulation of heteroblasty.

Regulation of cell number and size in relation to heteroblasty

A simple explanation for the increased cell number and decreased cell size in leaves at higher nodes is that a prolonged cell proliferation period in leaves at higher nodes may cause a shortening of the cell expansion period, resulting in the decreased final cell size. However, we cannot rule out another possibility: namely that a change in cell size is genetically separable from that in cell number. A notable example of this possibility is found in

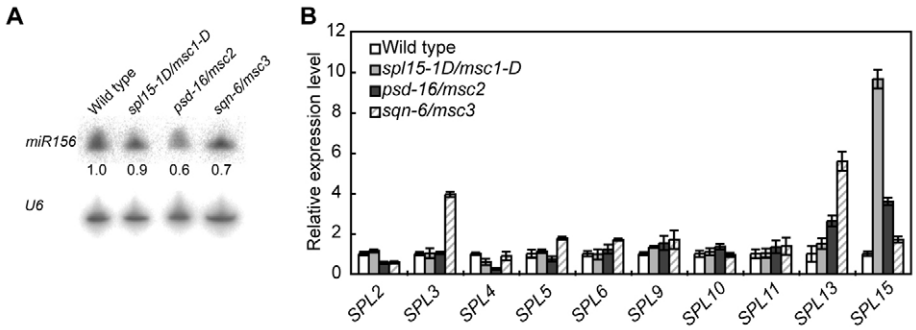

Fig. 5. Quantification of the expression levels of *miR156* and of ten *SPL* genes with the *miR156* target site. Total RNAs were extracted from the first and second leaves of 15-day-old wild-type and *msc1-msc3* mutant *Arabidopsis* seedlings. (**A**) Northern blot of *miR156*. Relative intensities of signals, normalized to *U6*, are indicated. (**B**) Quantitative RT-PCR of *SPL* genes. Relative expression levels in *msc1-msc3* as compared with the wild type are indicated.

the *rotunda2* (*ron2*) mutant, which is defective in the transcriptional co-repressor LEUNIG. In *ron2*, the cell size in the third leaf is as large as that in the first leaf of the wild type or *ron2*, whereas the cell number is almost the same as that of the third leaf of the wild type (Cnops et al., 2004). Another intriguing example is the *axr1-3* mutant, in which cell size is almost the same in any leaf tested (Fig. 7C), suggesting that auxin signal transduction is required for the heteroblastic regulation of cell size. Analysis on these types of mutants in relation to the *SPL* genes will help us to distinguish these possibilities.

With regard to heteroblasty-dependent cell size control, note that cell size within each leaf at different nodes is fairly uniform in *Arabidopsis* (see Fig. S1 in the supplementary material), as also seen in sunflower leaves (Granier and Tardieu, 1998). By contrast, many other heteroblastic traits are expressed heterogeneously within leaves produced during the transition from juvenile to adult phases. In these intermediate leaves, the distal part, which first arises from the shoot apical meristem, expresses more juvenile traits, whereas the proximal part, which arises later, expresses more adult traits (Kerstetter and Poethig, 1998). The highly homogeneous cell size within an intermediate leaf, such as the fifth leaf (see Fig. S1 in the supplementary material), suggests that the cell size is controlled at the organ level, whereas other traits are controlled in a cell-autonomous manner.

The *miR156-SPL*s pathway is involved in heteroblastic change of cell number and size

We propose that *miR156*-mediated regulation of *SPL* genes, including *SPL15* and *SPL3*, *-4* and *-5*, is involved in heteroblastic regulation of cell number and size for the following reasons. The *spl15-1D/msc1-D* mutant has a mutation in the *miR156* target site of the *SPL15* gene, and expression levels of *SPL15* are elevated in this mutant (Fig. 2A-D). In addition, *miR156*-overexpressing plants showed severe defects in heteroblastic change in cell number and size (Fig. 4E,F). Transgenic plants overexpressing the *miR156*-insensitive form of *SPL3* or *SPL4* have increased cell number and decreased cell size, particularly in the first leaf (Fig. 4E,F). Moreover, in the *psd-16/msc2* and *sqn-6/msc3* mutants, expression levels of a few *SPL* genes are significantly higher than in the wild type (Fig. 5B).

In the *rdr6*, *sgs3* and *zip* mutants, in which heteroblasty is accelerated but *miR156* levels are not changed (Peragine et al., 2004), some leaves have an increased cell number but do not show reduced cell size, except for the fifth leaf of *sgs3* (Fig. 4A,B). The results indicate that these genes have only minor effects on the heteroblastic regulation of cell number and size. Because *SPL3* expression levels were slightly (1.2- to 1.5-fold) elevated in these mutants (Peragine et al., 2004), such increases could affect cell number and size.

Because *tasiR-ARF* expression is not detected in *rdr6*, *sgs3* and *zip* mutants (Peragine et al., 2004), *tasiR-ARF*-mediated regulation of *ETT/ARF3* and *ARF4* is unlikely to be involved in heteroblastic change of cell number and size. This differs from the control of trichome production on the abaxial side of leaves, which is regulated by both *SPL* genes and by *ETT/ARF3* and *ARF4* (Hunter et al., 2006; Wu and Poethig, 2006). Under our experimental conditions, *rdr6* and *sgs3* clearly showed acceleration of abaxial trichome emergence, as previously reported (Peragine et al., 2004), although *zip* did not show this effect for unknown reasons (see Fig. S3 in the supplementary material). These facts suggest that heteroblastic regulation of abaxial trichome production and cell number and size are under the control of different pathways. This is further supported

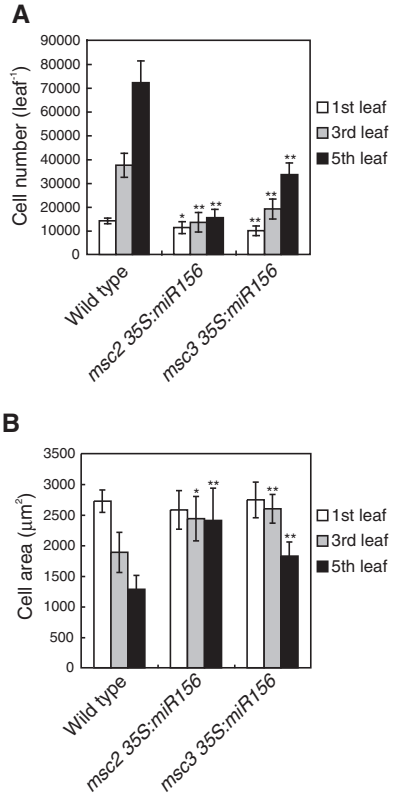

Fig. 6. The phenotypes of *msc2* and *msc3 Arabidopsis* mutants are suppressed by overexpression of *miR156*. Total cell number (**A**) and average cell area (**B**) of *msc2 35S:miR156* and *msc3 35S:miR156* double mutants 30 days after sowing. The mean ±s.d. from six individual plants is indicated. **P*<0.05 and ***P*<0.01 versus wild type (Student's *t*-test).

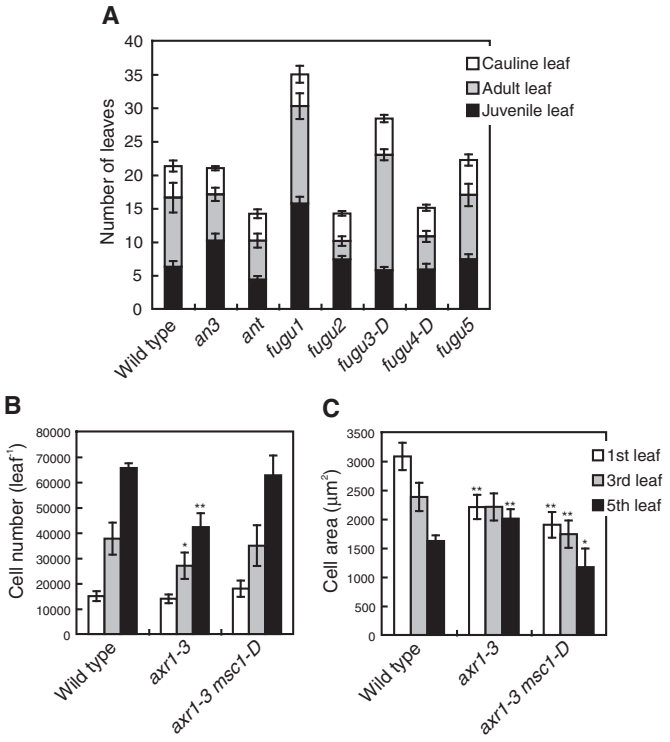

Fig. 7. Heteroblasty in compensation-exhibiting mutants and the *axr1* mutant. (**A**) The number of rosette leaves without abaxial trichomes (black), with abaxial trichomes (gray) and cauline leaves (white) were counted in *an3*, *ant* and *fugu1-fugu5* mutants. The mean ±s.d. (*n*=4-10) is indicated. Total cell number (**B**) and average cell area (**C**) of the wild type, *axr1-3* and *axr1-3 msc1-D* mutants 30 days after sowing. The mean ±s.d. from six individual plants is indicated. **P*<0.05 and ***P*<0.01 versus wild type (Student's *t*-test).

by the result that neither the *arf3* nor the *arf4* single mutant, which exhibit a delay in abaxial trichome emergence (Hunter et al., 2006), showed altered cell number or size compared to the wild type (Fig. 4C,D). The *arf3-2 arf4-2* double mutant also showed no change in cell size (Fig. 4D). Although cell number in this latter mutant was reduced, heteroblastic change in cell number occurred in a similar manner to that in the wild type (Fig. 4C,D). The reduced cell number could have been caused by disturbed leaf polarity, because the *arf3-2 arf4-2* double mutant shows defects in leaf adaxial/abaxial polarity and altered leaf morphology (Pekker et al., 2005).

spl15-1D/msc1-D is particularly intriguing, because a single synonymous nucleotide substitution occurred in the *miR156* target site of the *SPL15* gene (Fig. 2A). Transcriptional regulation of the gene should be the same in *spl15-1D/msc1-D* and in the wild type, suggesting that *miR156*-mediated cleavage of *SPL15* transcripts indeed plays a role in control of heteroblasty. This finding is consistent with a recent report that transgenic plants expressing the *miR156*-resistant *SPL9* gene under the control of its own promoter show significantly accelerated phase change (Wang et al., 2008). As only *SPL15* mRNA level was upregulated in *spl15-1D/msc1-D*, the mutated *SPL15* could have been the primary cause of the mutant phenotypes. In addition, because miRNAs, including *miR156*, also affect the translation of target genes (Gandikota et al., 2007; Brodersen et al., 2008), SPL15 protein level might be upregulated in *msc1-D*.

In *psd-16/msc2* and *sqn-6/msc3* mutants, the expression levels of *miR156* slightly decreased and those of a few *SPL* genes increased. However, the significance of such a slight reduction in *miR156* levels is not clear because only a subset of *SPL* genes was affected in their mRNA accumulation in these mutants (Fig. 5B). The increased expression of *SPL* genes should cause an alteration in cell number and size in leaves. This is further supported by the fact that increased cell number and decreased cell size in these two mutants were effectively suppressed by the overexpression of *miR156* (Fig. 6). The *PSD* gene encodes exportin-t, which regulates tRNA processing and nuclear export, but does not affect accumulation or export of miRNA (Park et al., 2005). *SQN* encodes cyclophilin 40, a protein that associates with the Hsp90 chaperone complex (Berardini et al., 2001), but its precise molecular function in plants is unknown. Although how these genes affect the expression levels of *miR156* or *SPL* genes is unknown, one possibility is that they regulate expression of a subset of *SPL* genes by unknown mechanisms. If this is correct, *psd* and *sqn* are useful mutants to clarify the mechanisms of heteroblasty operating upstream of the *SPL* genes.

Functions of *SPL* genes

We propose that the heteroblasty-promoting *SPL* genes, including *SPL3*, *-4*, *-5* and *-15*, increase cell number and reduce cell size in leaves. However, how these genes regulate cell number and size is still unknown. An intriguing possibility is that *SPL*s function through auxin signaling. Nevertheless, the additive phenotypes of the *axr1-3 msc1-D* double mutant indicate that *SPL15* and *AXR1* function in at least partially non-overlapping pathways.

SPLs comprise a family of transcription factors that share the SBP domain, a DNA-binding domain first identified in a protein that binds to a promoter of the *SQUAMOSA* gene in *Antirrhinum majus* (Klein et al., 1996; Cardon et al., 1997; Cardon et al., 1999). The amino acid sequence of the SBP domain is highly conserved among SPL proteins, but the sequence outside the SBP domain is diverse (Cardon et al., 1999). Although they are thought to bind to similar DNA sequences (Cardon et al., 1999), the molecular functions or target genes of SPLs remain unclear. Among the 10 *SPL* genes that have the *miR156* target site, *SPL3*, *-4*, *-5*, *-9* and *-15* are involved in the heteroblastic regulation of abaxial trichome production (Wu and Poethig, 2006; Schwarz et al., 2008). In this study, we found that heteroblastic change in cell number and size is also regulated by *SPL3*, *-4*, *-5* and *-15*, indicating that these genes might have overlapping functions. Among six *SPL* genes without the *miR156* target site, *SPL14* is particularly interesting because the loss-of-function mutant of this gene seems to have a truncated juvenile vegetative phase (Stone et al., 2005). SPL14 could have an antagonistic function to other SPL proteins that promote vegetative phase change. Identifying the targets of SPL transcription factors in further investigations will be necessary.

Regulation of cell number and cell size in leaves through distinct genetic pathways

Precise regulation of cell number and cell size is essential for leaf development. However, the genetic network controlling cell number and size in the context of leaf development is too complex to establish a unified view on its regulation. Instead, finding a particular pathway that confers unique effects on leaf development and comparing the relationship among individual pathways are needed. In the present study, we show that increased cell number and reduced cell size in *msc* mutants are caused by accelerated heteroblasty. In contrast to *msc* mutants, the compensation

syndrome, in which cell number is reduced and cell size is increased, is not necessarily caused by a genetic pathway associated with heteroblasty, because most compensation-exhibiting mutants do not show delayed phase change (Fig. 7A). Two compensation-exhibiting mutants (*an3* and *fugu1*) indeed show a delay in phase change. However, they had an increase in cell number and a decrease in cell size in leaves at higher nodes than the wild type (see Fig. S4 in the supplementary material), indicating that they are not deficient in the heteroblastic change in cell number and size. In addition, the *miR156*-overexpressing plant did not show a reduction in cell number and an increase in cell size in the first leaf (Fig. 4E,F). In other words, the first leaves might be in the ground state of the vegetative phase and cannot revert beyond it to a further immature phase. However, compensation syndrome can be seen in the first leaves (see Fig. S4 in the supplementary material). This indicates that cell number and cell size are controlled by at least two distinct genetic pathways, i.e. one associated with heteroblasty and one related to the compensation syndrome. Increasing numbers of genes identified from mutants with altered cell number/size will help us to understand the details of the genetic network and the mechanisms controlling cell number and size.

In summary, we conclude that heteroblasty has an important role in the regulation of cell number and cell size. Investigating the factor(s) that regulate cell number and size under the control of heteroblasty, particularly downstream of the *SPL* genes, is crucial for understanding the mechanisms that regulate cell number, cell size and organ size during leaf development.

We are grateful to Y. Mizukami, G. Wu, R. S. Poethig, and the *Arabidopsis* Biological Resource Center for providing seeds; and C. Yamaguchi, M. Nagura, and T. Kadowaki for technical assistance. This work was supported by Grants-in-Aid for Creative Scientific Research (to H.T.), Scientific Research (A; to H.T. and G.H.), and Exploratory Research (to G.H.) from the Japan Society for the Promotion of Science, and grants from the Toray Science Foundation (to H.T.).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/6/955/DC1

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