RESEARCH COMMUNICATION

Control of final seed and organ size by the *DA1* gene family in *Arabidopsis thaliana*

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Although the size of an organism is a defining feature, little is known about the mechanisms that set the final size of organs and whole organisms. Here we describe *Arabidopsis DA1*, encoding a predicted ubiquitin receptor, which sets final seed and organ size by restricting the period of cell proliferation. The mutant protein encoded by the *da1-1* allele has a negative activity toward DA1 and a DA1-related (DAR) protein, and overexpression of a *da1-1* cDNA dramatically increases seed and organ size of wild-type plants, identifying this small gene family as important regulators of seed and organ size in plants.

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Many experiments suggest that organs possess intrinsic information about their final size and grow until they reach a final predetermined mass (Conlon and Raff 1999; Day and Lawrence 2000), but the mechanisms setting the limits of growth are not well characterized despite their central importance. Recently, a key pathway suppressing cell proliferation during organogenesis has been identified (Dong et al. 2007) that is conserved in insects and mammals. However, many of the factors regulating organ size in animals have no obvious counterparts in plants, suggesting that the control of plant organ size involves novel mechanisms. Although external cues such as light, day length, and temperature influence plant growth and adapt sessile plants to their prevailing environment, the final size of plant seeds and determinate organs is reasonably constant within a given species, whereas interspecific seed and organ size variation is remarkably large, demonstrating that developing seeds and organs also possess intrinsic information about their final size (Tsukaya 2006). The mechanisms that establish the final size of seeds and organs and mediate environmental inputs into growth are poorly understood, de-

spite their fundamental importance and relevance to crop plant improvement.

Plant organ growth occurs by an initial proliferative phase in which cell numbers increase while their size remains fairly constant, followed by dramatic cell size increases that cease when the set size of the organ is reached. Increases in cell ploidy occur during later stages of organ growth that can be associated with the final size of cells (Sugimoto-Shirasu and Roberts 2003). In leaves, the transition from cell proliferation to cell expansion follows cell cycle arrest fronts that move from the tip to the base (Donnelly et al. 1999). Modulation of the time and location of cell proliferation arrest (Nath et al. 2003; Dinneny et al. 2004; Disch et al. 2006; White 2006) have been established as key regulatory points during leaf and petal formation that set final organ size and establish its shape. Interaction between organs also influences seed size. Reduced maternal integument size reduces final seed size (Garcia et al. 2005), and reduced endosperm proliferation arrests cell elongation in the integument (Garcia et al. 2003).

The growth regulator auxin promotes growth through ARGOS (Hu et al. 2003), which mediates expression of *AINTEGUMENTA* (*ANT*), a transcription factor that regulates expression of cell cycle genes (Mizukami and Fischer 2000). Elevated *ANT* levels increase cell numbers and increases the size of all shoot organs. Conversely, low *ANT* levels reduce the final size of organs (Krizek 1999; Mizukami and Fischer 2000). The transcription factors *ANGUSTIFOLIA* (*AN3*) and *AtGRF5* function together to promote cell proliferation in leaves (Horiguchi et al. 2005), and their overexpression increases leaf growth. Several factors that limit cell proliferation during organ formation have been described. Mutations in *ARF2*, an auxin-responsive protein, cause extra cell divisions, resulting in larger organ and integument size, which leads to larger seeds (Schruff et al. 2006) as well as partial sterility and other phenotypes. *ARF2* has also been shown to mediate auxin-responsive gene expression and negatively regulates cell expansion (Li et al. 2004). Several organ-specific growth regulators have been identified. The *BIGPETALp* gene limits petal size by restricting cell size (Szecsi et al. 2006). Deletion of the *PEAPOD* (*PPD*) genes increases leaf area and curvature due to prolonged division of dispersed meristematic cells in the leaf blade (White 2006). Loss-of-function mutations in *AP2*, encoding a transcription factor, lead to a range of floral defects that correlate with increased seed mass (Jofuku et al. 2005; Ohto et al. 2005).

One of the mechanisms setting final seed and organ size may potentially affect the time taken for an organ to reach its set final size, given that the period of proliferation is a key control point. The E3 ligase *BIG BROTHER* (*BB*) negatively regulates the duration of cell proliferation in leaves and petals, possibly by targeting growth stimulators for degradation, and is proposed as a bona fide organ size regulator (Disch et al. 2006). Despite being expressed in all plant tissues, loss-of-function *bb* mutations seem to influence primarily petal and stem growth. Here we describe the *DA1* gene, which defines a novel growth control pathway that sets the final size of seeds and determinate organs in *Arabidopsis* by controlling the period of cell proliferation. A modifier screen

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Figure 1. Seed size in *da1-1*. (A,B) Dry seeds of Col-0 (A) and *da1-1* (B). (C,D) Mature embryos of Col-0 (C) and *da1-1* (D). (E,F) Ovules of Col-0 (E) and *da1-1* (F). (G) Prewashed batches of wild-type Col-0, *da1-1*, complementation line (*DA1COM*), and *35S::DA1^{R358K}* #5 seeds from individual plants were passed through a series of wire sieves of decreasing mesh size (in microns) as described in the Supplemental Material. Seeds retained by each sieve were weighed, and the mass of each fraction was expressed as a percentage of the total mass of the seed sample analyzed. (H) Average seed weights of Col-0, *da1-1*, *DA1COM*, *35S::DA1^{R358K}* #5, *da1-ko1*, *da1-1*, and *da1-ko1da1-1* double mutants are shown as milligrams per 100 seeds. Standard deviations are shown ($n = 5$). (I) Average seed weight per plant. Standard deviation values were given ($n = 5$). Plants were grown under identical conditions. Bars: A,B, 200 μm; C,D, 100 μm; E,F, 50 μm.

identified an enhancer of *da1-1* (*EOD1*), which is allelic to *BB* (Disch et al. 2006). Mutations in *EOD1/BB* synergistically enhance both seed and organ size phenotypes of *da1-1*, indicating that *DA1* acts in parallel with *EOD1/BB* to control the final size of seeds and organs in *Arabidopsis*. *DA1* expression is induced by the growth regulator abscisic acid (ABA) and organ growth has reduced sensitivity to ABA in *da1-1*, suggesting ABA may be an important factor in establishing final organ size.

Results and Discussion

We identified the *da1-1* (DA means “large” in Chinese) mutant in genetic screens in *Arabidopsis thaliana* that increased both seed and organ size (Figs. 1, 2A–L) and has normal fertility compared with wild type (data not shown). The seed mass of *da1-1* was increased to 132% of wild type (Figs. 1H, 5D [below]); ovule and seed volume, embryo size, and cotyledon areas were substantially increased (Figs. 1A–G, 2A,B; Supplemental Fig. 1); and total seed yield per plant was increased (Fig. 1I). Seeds produced by a *da1-1* mother, regardless of the genotype of the pollen donor, were consistently heavier and larger than those produced by maternal wild-type plants, and *da1-1* mutant pollen in a wild-type mother produced seeds with wild-type mass (Supplemental Table 1), suggesting that *DA1* can act maternally to control seed mass. The integuments surrounding the ovule are derived from maternal tissues and form the seed coat. Increased integument size such as that seen in *da1-1* ovules (Fig. 1E,F) is known to contribute to increased seed size (Garcia et al. 2005; Schruoff et al. 2006).

da1-1 plants have large flowers with extra petals and carpels (Fig. 2D,E; Supplemental Fig. 2), enlarged and somewhat flattened siliques (Fig. 2F,G), rounder and large leaves (Figs. 2C, 3C), and thick stems as well as increased biomass compared with wild type (Fig. 2H,I,K,L). In all organs of *da1-1* plants, the size of cells was comparable with that measured in wild type (Fig. 2M), whereas the number of cells was increased—for example, in the stem epidermal layer, up to 180% of wild-type numbers (Fig. 2I). Kinematic analysis of petal and leaf growth (Fig. 3A,C) illustrated that *da1-1* plants undergo a longer phase of proliferative growth and have a longer life span than wild-type plants (Fig. 5C [below]; Supplemental Fig. 9D,E). Measurement of the mitotic index using a *pCYCB1:1::GUS* reporter fusion that is specifically expressed in mitosis during petal and leaf growth demonstrated that cell cycle activity was arrested later in growth (Fig. 3B,D,E). These analyses show that *DA1* functions to limit the period of proliferative growth during early stages of organ development and controls the time taken for most organs to reach their final size.

The *DA1* gene was identified by map-based cloning (Supplemental Fig. 3). DNA sequencing revealed that the *da1-1* allele has a single-nucleotide G-to-A transition in gene At1g19270 predicted to cause an arginine-to-lysine change in a conserved amino acid at position 358 (Fig. 4A; Supplemental Figs. 3, 4A, 5). All *da1-1* phenotypes were complemented by the wild-type *DA1* gene and by wild-type *DA1* full-length cDNA expressed from a 35S

Figure 2. Organ size in *da1-1*. (A,B) (A) Nine-day-old seedlings of Col-0 (A) and *da1-1* (B). *da1-1* has larger cotyledons than wild type. (C) The fifth leaves of Col-0 (left) and *da1-1* (right). *da1-1* has larger and rounder leaves compared with wild-type Col-0. (D,E) Flowers of Col-0 (D) and *da1-1* (E). (F,G) Siliques of Col-0 (F) and *da1-1* (G). (H–J) Stem diameter (H), epidermal cell number in stem cross-sections (I), and petal area (J) of Col-0, *da1-1*, *DA1COM*, and *35S::DA1^{R358K}* #5. (K,L) Mass of five fresh flowers (stage 14) (K) and leaves (first through seventh) of 35-d-old plants (L). (M) Cell area of embryos (E), petals (P), and leaves (L) in Col-0 and *da1-1*. Values are given as mean $\pm$ SD relative to the respective wild-type value, set at 100%. (N) Relative expression levels of *DA1* in Col-0 and *35S::DA1^{R358K}* #5 seedlings were measured by quantitative real-time RT-PCR. Bars: A,B, 1 mm; C, 0.5 cm; D–G, 1 mm.

Figure 3. Kinematic analysis of petal and leaf growth. (A) Growth of Col-0 and *da1-1* mutant petals. The largest petals of each series are from opened flowers (stage 14). (B) Mitotic index in wild-type and *da1-1* mutant petals. The time axis in B corresponds to that in A. (C) Fifth leaf area of Col-0, *da1-1*, *DA1COM*#2, and *35S::DA1^{R358K}*#5 measured over 25 d. (DAE) Days after emergence. (D,E) *pCyclinB1;1::GUS* activity in the first leaves (9 d after germination) of wild-type (D) and *da1-1* (E) seedlings grown on MS medium containing 1% glucose. Bar, 1 mm.

promoter, indicating that At1g19270 is indeed the *DA1* gene (Figs. 1G,H, 2H–L, 3C). The *DA1* gene is predicted to encode a 532-amino-acid protein containing two ubiquitin interaction motifs (UIM) typical of ubiquitin receptors (Hicke et al. 2005) and a single zinc-binding LIM domain defined by its conservation with the canonical Lin-11, Isl-1, and Mec-3 domains (Fig. 4B; Supplemental Fig. 4A–C; Frey et al. 1990; Hiyama et al. 1999). To test whether mutation in *da1-1* affects the predicted ubiquitin-binding activity of DA1, we expressed full-length GST-DA1 and GST-DA1^{R358K} fusion proteins in *Escherichia coli*, incubated protein extracts with ubiquitin-agarose beads, and identified bound proteins using DA1 antibody. No significant difference in ubiquitin-binding activity between GST-DA1 and GST-DA1^{R358K} was detected (Fig. 4C), demonstrating that the *da1-1* mutation does not affect DA1 ubiquitin binding. The ubiquitin receptor function of the UIMs was demonstrated using a GST fusion protein in *E. coli* to bind ubiquitin in pull-down assays (Supplemental Fig. 6). Seven other predicted proteins in *Arabidopsis* share extensive amino acid similarity with DA1 and have been named DA1-related (DAR) proteins (Supplemental Fig. 7A), of which four are found in a tandem cluster on chromosome 5. Proteins that share significant homology with DA1 outside the UIM and LIM domains are found in crop plants including rice and maize (Supplemental Fig. 7B), but not animals, suggesting the family has evolved to control growth in plants.

A screen for modifiers of *da1-1* function was conducted to identify other components in the *DA1* pathway. We identified two suppressors of large seed and organ phenotypes of *da1-1* (*sod*) alleles that mapped to the original *DA1* locus. Sequencing revealed that these *sod1* alleles harbored second site mutations in *DA1* that were

predicted to reduce gene function (Fig. 4A; Supplemental Fig. 4A), indicating that the R358K mutation in *DA1* is responsible for increased seed and organ size. Consistent with this, disruption of the *DA1* gene with T-DNA insertions in the predicted coding region (*da1-ko1*, *da1-ko2*, and *da1-ko3*) (Fig. 4A) did not cause an obvious growth phenotype, nor did T-DNA insertion alleles in *DAR1*, the most closely related family member (Figs. 1H, 4K). Lines heterozygous for the original *da1-1* mutation and wild-type Col-0 had a seed and organ size nearly similar to the wild type, whereas all the F₁ plants from crosses of *da1-1* with *da1-ko* lines displayed a similar phenotype to *da1-1* (Supplemental Fig. 8A,B). The *da1-1* phenotype was also observed in wild-type Col-0 plants expressing a *35S::DA1^{R358K}* transgene (Figs. 1G,H, 2H–L,N, 3C; Supplemental Fig. 8C). These data suggested that the protein encoded by the *da1-1* allele

Figure 4. Identification, expression, function, and genetic interactions of the *DA1* gene. (A) *DA1* gene structure showing the mutated sites of *da1-1*, *sod1-1*, and *sod1-2* alleles. The start codon (ATG) and the stop codon (TAA) are indicated. Closed boxes indicate the coding sequence, and lines between boxes indicate introns. T-DNA insertion sites (*da1-ko1*, *da1-ko2*, and *da1-ko3*) in *DA1* gene are shown. (B) The predicted DA1 protein contains two UIM and one LIM domains. (C) In vitro ubiquitin-binding assay. GST-DA1 and GST-DA1^{R358K} fusion proteins expressed in *E. coli* were incubated with ubiquitin-agarose beads. Bound GST-DA1 and GST-DA1^{R358K} fusion proteins (top panel) and 0.3% input proteins (bottom panel) were detected by immunoblotting (IB) with an antibody against DA1 (anti-DA1). (D–H) *DA1* expression activity was monitored by *pDA1::GUS* transgene expression. GUS staining during early leaf development (D), developing (E) and mature (F) petals, a developing ovule (G), and early-stage embryo (H). (I,J) Flowers of Col-0 (I) and *da1-ko1da1-1* double mutant (J). (K) Petal area of Col-0, *da1-ko1*, *da1-1*, and *da1-ko1da1-1* double mutants. The *da1-ko1da1-1* double mutant displays a *da1-1* phenotype including large flowers and petals. (L) Quantitative RT-PCR analysis revealed that expression of *DA1* is slowly induced by ABA. Seven-day-old wild-type seedlings were treated with 10 μ M ABA for 2, 4, 6, 18, and 30 h. (M) Seedlings of Col-0 (bottom) and *da1-1* (top) were established on MS medium for 4 d and transferred to MS + 1% glucose medium without ABA and with 5 μ M ABA, 10 μ M ABA, and 30 μ M ABA (from left to right) for 2 wk, respectively. The *da1-1* mutant seedlings exhibit ABA-insensitive organ growth, compared with Col-0. (N) The shoot fresh weight of Col-0 and *da1-1* in M. Values are given as mean $\pm$ SE relative to the respective wild-type value, set at 100%. Bars: D,M, 0.5 mm; E,F, 0.1 mm; G,H, 50 μ m; I,J, 1 mm; O, 2 mm.

exerts a negative activity toward DA1 in a dose-dependent manner. This result also indicated that this negative activity of the *da1-1* allele is weak because *da1-1/Col-0* F₁ plants display very similar phenotypes to Col-0 (Supplemental Fig. 8A,B). Because the loss-of-function alleles of *DA1* display no obvious phenotype, we postulated that DA1 may act redundantly with DAR proteins and that the *da1-1* allele may also exert a negative activity on the function of DAR proteins. Consistent with this hypothesis, double *da1-ko1dar1-1* knockout mutants exhibited the original *da1-1* phenotype (Figs. 1G,H, 4I–K). These analyses demonstrate that the R358K mutation in DA1 is necessary and sufficient to cause the *da1-1* phenotype and the DA1^{R358K} mutant protein has a negative effect on the function of DA1 and DAR1 proteins.

We also identified an enhancer of *da1-1* (*eod1-1*) in the screen for modifiers, and an *eod1-1da1-1* double mutant exhibited substantially larger seed and organ size and longer life span than *da1-1* (Fig. 5C; Supplemental Fig. 9). The *EOD1* gene was mapped to the *Big Brother* (*BB*) locus (At3g63530), encoding an E3 ubiquitin ligase that also represses organ (but not seed) growth in *Arabidopsis* (Disch et al. 2006). Sequencing revealed that the *eod1-1* allele is a single-nucleotide G-to-A transition resulting in an alanine-to-threonine change in BB protein (Fig. 5A). Both a T-DNA insertion in the 3' untranslated region (UTR) (*eod1-2*) and the original *bb-1* mutation also enhanced the *da1-1* phenotypes (Fig. 5B–E; Supplemental

Fig. 9C–E), and transformation of *eod1-1* with a binary vector expressing the At3g63530 cDNA from a 35S promoter rescued the *eod1* phenotype (data not shown), demonstrating that *EOD1* is the *BB* gene. Seed and petal measurements in *eod1-2da1-1* and *bb-1da1-1*^{L_{er}} double mutants demonstrated a synergistic enhancement of seed and organ size of *da1-1* by mutations in *EOD1/BB* (Fig. 5B–E; Supplemental Fig. 9C–E), suggesting that the two genes act in parallel pathways to control the final size of seeds and organs in *Arabidopsis*. Double mutants of *da1-1* with *ant-5*, *axr1-12*, *arf2-7*, and *ap2-7* mutants that also exhibit altered seed or organ size were assessed (Lincoln et al. 1990; Krizek 1999; Mizukami and Fischer 2000; Jofuku et al. 2005; Ohto et al. 2005; Schruoff et al. 2006). The genetic interactions between *da1-1* and these mutants were essentially additive for petal or seed size, compared with their parental lines (Supplemental Fig. 10), suggesting that *da1-1* acts independently of *ANT*, *AXR1*, *ARF2*, and *AP2*.

A *DA1* promoter::GUS fusion (*pDA1::GUS*) was expressed most strongly during the early stages of leaf, petal, integument, and embryo formation (Fig. 4D–H) and at much reduced levels later in petal and leaf development, consistent with its role in controlling the period of cell proliferation. Microarray data showed that *DA1* is expressed at higher levels in ABA-treated seedlings (Li et al. 2006), and further experiments established that *DA1* is slowly induced by ABA (Fig. 4L) and not by other growth regulators such as jasmonic acid, auxin, cytokinin, brassinosteroids, gibberellins, or glucose (data not shown). Interestingly, seedling and root growth, seed germination, and seedling establishment in the *da1-1* mutant are relatively insensitive to inhibition by ABA (Fig. 4M–N; Supplemental Fig. 11), suggesting ABA may be an endogenous or environmental cue that limits the period of proliferative growth during organogenesis through regulation of *DA1* expression. To gain a better understanding of ABA function in size control, we generated double mutants of *da1-1* with *abi4-1* and *abi5-1* mutants that control ABA growth and developmental responses (Soderman et al. 2000; Lopez-Molina et al. 2001). Genetic analysis revealed that the seed and petal size phenotypes of *abi4-1da1-1* and *abi5-1da1-1* double mutants were essentially additive (data not shown), compared with their parental lines, suggesting that *DA1* is independent of *ABI4* and *ABI5*, two key regulators of ABA responses in *Arabidopsis*.

Our genetic, physiological, biochemical, and kinematic data established that the novel growth regulator gene *DA1* plays a major role in setting final sizes of both seeds and organs by restricting the duration of proliferative growth. The mutant DA1^{R358K} protein has a negative activity toward DA1 function in a dose-dependent manner, and it also has a negative activity toward the activity of the closely related DAR1 protein. This type of effect has also been reported for the *wol* and *sleepy1* mutants (Mahonen et al. 2000, 2006; Strader et al. 2004). The R358K change affects an invariant amino acid in an extensive region conserved in all members of the DA1 family, two of which can function redundantly. It is possible that the R358K mutation in an invariant amino acid in this conserved region interferes with the interaction of DA1 family members with a common target—for example, by forming an unproductive complex that normally restricts growth.

Analysis of double *eod1-2da1-1* mutants shows that

Figure 5. Mutations in *EOD1/BB* synergistically enhance the phenotypes of *da1-1*. (A) *EOD1/BB* gene structure, showing the mutated sites of the two *eod1* alleles. The start codon (ATG) and the stop codon (TGA) are indicated. Closed boxes indicate the coding sequence, and lines between boxes indicate introns. The mutated site in *eod1-1* and T-DNA insertion site in *eod1-2* also are shown. (B) Flowers of Col-0, *da1-1*, *eod1-2*, and *eod1-2da1-1* double mutants. (C) Soil-grown plants of Col-0, *da1-1*, *eod1-2da1-1* double mutant, and *eod1-2* are shown. (D) Average seed weights of Col-0, *da1-1*, *eod1-2*, and *eod1-2da1-1* double mutants are shown as milligrams per 100 seeds. Standard deviations are shown ($n = 5$). Plants were grown under identical conditions. (E) Petal areas of Col-0, *da1-1*, *eod1-2*, and *eod1-2da1-1* double mutant. Standard deviation values are shown ($n > 50$). (F) A model of *DA1* and *EOD1/BB* controlling seed and organ size. Bars: B, 2 mm; C, 50 mm.

DA1 and EOD1/BB, another negative regulator of the proliferative phase of organogenesis (Disch et al. 2006), function in parallel pathways and may modulate the activity of a common target (Fig. 5F). However, the DA1^{R358K} mutant protein appears to have a stronger negative influence on growth inhibition than EOD1/BB loss-of-function mutants, and affects the size of most determinate organs, including seeds (Fig. 5D). The ubiquitin-binding activity of DA1 is consistent with a potential signaling function. UIMs bind molecules modified by monoubiquitin or ubiquitin chains (Hicke et al. 2005) and promote coupled monoubiquitination, leading to conformational changes that regulate diverse cellular processes, including signaling and endocytosis (Hurley et al. 2006; Penengo et al. 2006). LIM domains, which serve as protein interaction interfaces, are involved in a variety of fundamental biological processes (Dawid et al. 1998; Kadrmaz and Beckerle 2004). The close involvement of E3 ligase activity from EOD1/BB, the ubiquitin receptor activity, and potential coupled monoubiquitination activities from DA1 suggests direct involvement of ubiquitin-based cell signaling mechanisms in plant organ size control.

The induction of DA1 expression in response to ABA and the ABA-insensitive growth seen in the *da1-1* mutant suggests a mechanism for limiting proliferative growth during organogenesis by ABA. ABA signaling has the potential to link environmental inputs, such as stress, to final organ size (Finkelstein et al. 2002), and ABA is required for normal plant development (Phillips et al. 1997; Cheng et al. 2002). The large effects of DA1 on the duration of cell proliferation during seed and organ formation, the absence of pleiotropic effects, and DA1's genetic interactions with EOD1/BB, emphasize the central role of the period of cell proliferation in setting final seed and organ size in plants and define a major role for DA1 in coordinating size control at the whole-plant level. Our current knowledge of DA1 function and the identification of conserved DA1-like genes suggest that the *da1-1* allele can be used to engineer large seed size and increased biomass in crop plants.

Materials and methods

Plant materials and growth conditions

A. thaliana Columbia (Col-0) was the wild-type line used. All mutants were in the Col-0 background, except for *da1-1^{Ler}*, *bb-1*, and *ant-5*, which were in *Landsberg erecta* (Ler). Plant materials and growth conditions are available in the Supplemental Material.

Genetic screening

da1-1 was identified as a novel seed and organ size mutant from an ethyl-methanesulphonate (EMS)-treated M₂ population of Col-0 accession. *sod1-1*, *sod1-2*, and *eod1-1* were identified as suppressors and enhancers, respectively, of *da1-1* from EMS-treated M₂ populations of *da1-1*.

Morphological and cellular analysis

Detailed protocols, sample preparation, measurement, microscopy, and histochemical staining for β -glucuronidase activity are described in the Supplemental Material.

Map-based cloning and plant transformation

F₂ mapping populations were generated from a single cross of *Ler/da1-1*, *Ler/sod1-3da1-1*, and *da1-1^{Ler}/eod1-1da1-1* plants. The DA1, SOD1, and EOD1 genes were isolated by using a map-based cloning method. Detailed information for map-based cloning, constructs, and plant transformation is described in the Supplemental Material.

Protein expression and ubiquitin-binding assays

GST-DA1 and GST-DA1^{R358K} fusion proteins were cloned in pGEX-4T-2 (Amersham-Pharmacia), expressed in *E. coli* BL21(DE3), and incubated with ubiquitin-agarose beads (Santa Cruz Biotechnologies). Bound GST-DA1 and GST-DA1^{R358K} fusion proteins were detected by immunoblotting (IB) with an antibody against DA1 (anti-DA1). The UIM1 + 2 domain from DA1 (residues 63–127) was cloned into pGEX-4T-2 and expressed in *E. coli* BL21 as a fusion with N-terminal GST tag. Detailed methods for protein expression and ubiquitin-binding assays are described in the Supplemental Material.

RT-PCR, quantitative real-time RT-PCR, and Northern blot analysis

Total RNA was extracted from *Arabidopsis* seedlings using an RNeasy Plant Mini kit (Qiagen). Detailed protocols for RT-PCR, quantitative real-time RT-PCR, and Northern blot analysis are described in the Supplemental Material.

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