TANMEI/EMB2757 Encodes a WD Repeat Protein Required for Embryo Development in Arabidopsis[1]

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We identified the Arabidopsis (*Arabidopsis thaliana*) *tanmei/emb2757* (*tan*) mutation that causes defects in both embryo and seedling development. *tan* mutant embryos share many characteristics with the *leafy cotyledon* (*lec*) class of mutants in that they accumulate anthocyanin, are intolerant of desiccation, form trichomes on cotyledons, and have reduced accumulation of storage proteins and lipids. Thus, *TAN* functions both in the early and late phases of embryo development. Moreover, the *TAN* and *LEC* genes interact synergistically, suggesting that they do not act in series in the same genetic pathway but, rather, that they have overlapping roles during embryogenesis. *tan* mutants die as embryos, but immature mutant seeds can be germinated in culture. However, *tan* mutant seedlings are defective in shoot and root development, their hypocotyls fail to elongate in the dark, and they die as seedlings. We isolated the *TAN* gene and showed that the predicted polypeptide has seven WD repeat motifs, suggesting that TAN forms complexes with other proteins. Together, these results suggest that TAN interacts with other proteins to control many aspects of embryo development.

Seed development in angiosperms begins with double fertilization of the egg cell and the central cell of the female gametophyte that leads to the formation of the diploid zygote and the triploid endosperm mother cell, respectively. The endosperm provides nutrients for the developing embryo and persists in the mature seed either as a layer of cells surrounding the embryo or as a storage tissue that serves a nutritional role for germinating seedlings (Lopes and Larkins, 1993; Olsen, 2004). Development of the zygote into the mature embryo can be divided conceptually into two overlapping phases (West and Harada, 1993; Goldberg et al., 1994). During the early morphogenesis phase, the basic body plan of the plant is established (Laux and Jurgens, 1997; Jurgens, 2001; Berleth and Chatfield, 2002). The initial cell divisions of the zygote generate the embryo proper and suspensor. Embryonic polarity is fixed with specification of the shoot-root axis. Along this axis, pattern elements, including the cotyledons, shoot apical meristem (SAM), hypocotyl, root, and root apical meristem, are established. Perpendicular to this axis, radial elements produce the embryonic tissue systems: protoderm, ground tissue, and procambium. During the late-maturation phase, the embryo undergoes unique developmental and physiological changes, including accumulation of nutrient reserves, acquisition of desiccation tolerance and dormancy, and developmental arrest and metabolic inactivation of the embryo as it desiccates (Bewley, 1997; Harada, 1997). Once the desiccated seed is exposed to an environment favorable for germination, the quiescent embryo becomes active metabolically and morphogenetically as postembryonic development is initiated. Although many aspects of embryogenesis have been characterized extensively, the molecular mechanisms underlying these events are poorly understood.

Genetic analyses have identified Arabidopsis (*Arabidopsis thaliana*) genes that play essential roles during embryogenesis. Several mutations that affect the early stages of the morphogenesis phase correspond to genes involved in morphogenetic processes that occur during vegetative development. For example, *SHOOTMERISTEMLESS* and *WUSCHEL* are involved

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in establishing the SAM, and *SHORT ROOT* and *SCARECROW* are involved in root apical meristem formation (Dilaurenzio et al., 1996; Long et al., 1996; Mayer et al., 1998; Helariutta et al., 2000). A different set of mutations has been shown to affect the maturation phase. *abi3*, *abi4*, and *abi5* are mutations that cause defects in storage protein accumulation and/or tolerance to desiccation (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). Furthermore, a large set of *embryo defective* (*emb*) mutations have been identified that disrupt embryo development and appear to be required for basic cellular functions (McElver et al., 2001; Tzafrir et al., 2004).

A distinct class of genes identified by the *leafy cotyledon* (*lec*) mutations of Arabidopsis play seed-specific roles in both the morphogenesis and maturation phases of embryogenesis (for review, see Harada, 2001). The *LEC1*, *LEC2*, and *FUS3* genes are required early in embryogenesis to maintain embryonic cell fate and to specify cotyledon identity (Meinke, 1992; Keith et al., 1994; Meinke et al., 1994; West et al., 1994; Lotan et al., 1998). Late in embryogenesis, these genes are required to initiate and/or maintain the maturation phase and to inhibit premature germination of developing embryos (Baumlein et al., 1994; Keith et al., 1994; Meinke et al., 1994; West et al., 1994; Nambara et al., 2000; Raz et al., 2001). Moreover, ectopic expression of *LEC1* or *LEC2* induces embryonic characteristics and somatic embryogenesis in vegetative cells (Lotan et al., 1998; Stone et al., 2001). The *LEC1* gene encodes a novel HAP3 subunit of the CCAAT binding factor, whereas *LEC2* and *FUS3* encode B3 domain transcription factors (Lotan et al., 1998; Stone et al., 2001; Kwong et al., 2003). We hypothesize that the LEC proteins are transcriptional regulators that integrate the early-morphogenesis and the late-maturation phase of embryogenesis by establishing cellular environments that promote embryo development.

In this article, we describe Arabidopsis mutants designated *tanmei* (*tan*) that have defects in embryogenesis. Analysis of *tan* mutants indicates that the gene plays roles in both the morphogenesis and maturation phases of embryogenesis, a hypothesis supported by genetic data suggesting that *TAN* has overlapping functions with the *LEC* genes. There are also indications that *TAN* is required for other aspects of plant development. We isolated the *TAN* gene and showed that it encodes a WD repeat protein predicted to function in protein-protein interaction. The potential role of *TAN* in plant development is discussed.

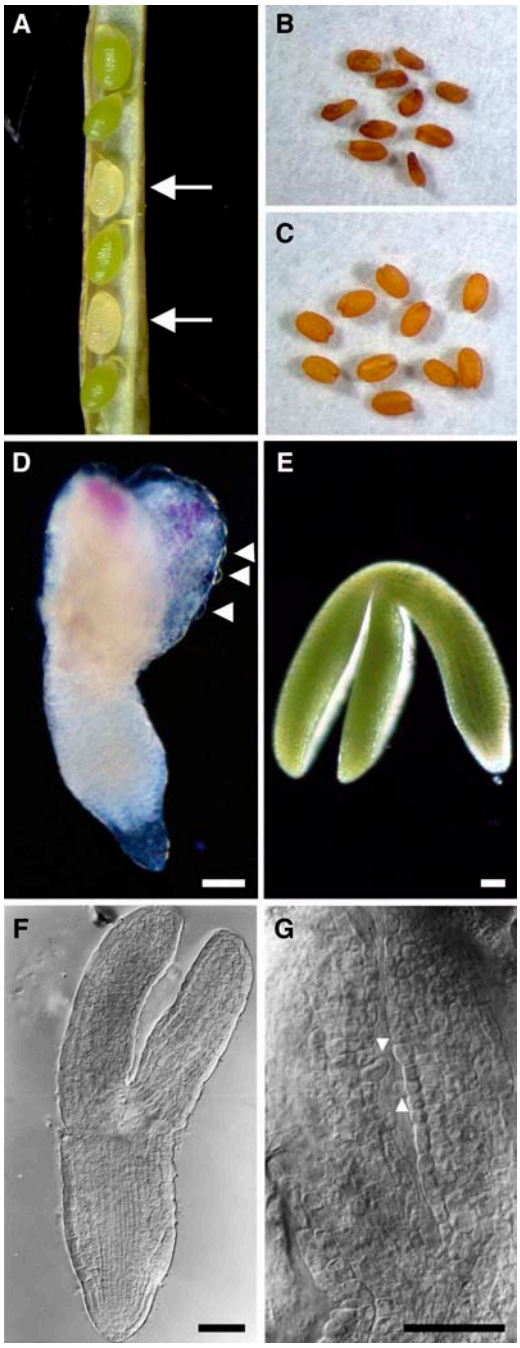

Figure 1. Embryonic defects of *tan* mutants. A, Developing seeds within a silique from a heterozygous *tan* mutant plant. Mutant seeds have a pale yellow color (arrows). B and C, Dried mutant and wild-type seeds, respectively. Mutant seeds are desiccation intolerant. D and E, Mature mutant and wild-type embryos, respectively. Mutant embryo accumulates anthocyanin and displays aberrant cell growth (arrowheads). F and G, Mutant embryos were cleared and photographed with Nomarski optics. Trichome initials and/or aberrant cell growth are observed at the protoderm of the adaxial cotyledon surface (arrow head). Bars = 50 μm.

RESULTS

tan Mutation Causes Defects in Embryogenesis

We screened a T3 population of Arabidopsis mutagenized with T-DNA and identified embryo-defective mutants (Yadegari et al., 1994). As shown in Figure 1A, one line segregated immature seeds that were pale

yellow as compared with their siblings that resembled green, wild-type seeds. After drying, the mutant seeds were shriveled and had a purple coloration as compared to wild-type seeds (Fig. 1, B and C), and they did not germinate, suggesting that the mutation caused embryo lethality. Furthermore, the mutation displayed monogenic, recessive inheritance. Twenty-five percent of progeny from plants heterozygous for the mutation had a mutant phenotype [68/286; $\chi^2(3:1) = 0.2$; $P > 0.5$]. Given that the mutant displayed embryonic lethality, we designated the corresponding gene *TANMEI* (*TAN*) because it is short lived.

Growth Defects of tan Mutant Embryos

The *tan-1* mutation caused defects in embryo morphology. As shown in Figure 1, D to F, mutant embryos differed from wild type in that their cotyledons did not curl within the seed at maturity because mutant embryos were shorter than wild type. Their embryonic axes were of comparable size to those of wild type, indicating that cotyledon growth was inhibited in the mutant. Mutant cotyledons also had regions of ectopic cell growth and patches of anthocyanin accumulation predominantly at their tips (Fig. 1D). We compared the SAMs of wild-type and *tan-1* mutant embryos. As shown in Figure 2, the SAM of *tan-1* mutant embryos (Fig. 2, A and B) did not appear to have the cell layer organization of wild-type sibling embryos at the bent-cotyledon stage (Fig. 2D) or of wild-type embryos at a similar morphological stage (Fig. 2C). In addition, root apices of *tan-1* mutant embryos were translucent, while those of the wild-type embryos were opaque (Fig. 1, D and E). Thus, the morphology of *tan-1* mutant embryos differed significantly from wild type.

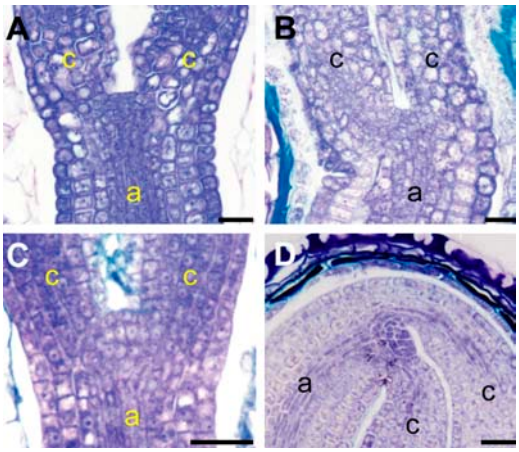

Figure 2. SAM organization in *tan* mutant embryos. A and B, Medial sections through the SAM of *tan-1* mutant embryos. *tan* mutant embryos were isolated from siliques at a stage when sibling embryos with a wild-type phenotype were at the bent-cotyledon stage. C, Wild-type embryo at the linear cotyledon stage. D, Wild-type embryo at the bent-cotyledon stage. a, Axis; c, cotyledon. Bars = 20 μm.

tan Mutation Affects the Maturation Program

We asked whether *tan-1* mutants died as embryos because they were not able to withstand desiccation that occurs at the end of seed development. *tan-1* mutant embryo cells from dried seeds cultured on callus-inducing medium did not grow or divide, whereas wild-type embryo cells proliferated to produce calli (data not shown). This result, along with the shriveled appearance of dried seeds, suggested that the cells in mutant embryos were dead because of their intolerance to desiccation. Because wild-type embryos normally acquire tolerance to desiccation during the maturation phase of embryogenesis (Harada, 1997), this result suggested that the *tan* mutation causes defects in maturation processes.

To determine whether the *tan* mutation affected other aspects of the maturation phase, we examined embryos at the ultrastructural level. As shown in Figure 3A, wild-type embryo cells in cotyledons and embryonic axes were packed with storage protein and lipid bodies. By contrast, accumulation of these organelles was defective in *tan* mutant embryos, particularly in apical regions. Storage protein and lipid bodies were much less prevalent in cotyledons (Fig. 3, B and C) versus axes (Fig. 3D) of *tan-1* mutants, although limited numbers of storage organelles were observed throughout the embryo. We conclude that the *TAN* gene is required for aspects of the maturation phase of embryogenesis.

tan Mutant Embryos Have Dormancy Defects

To obtain further insight into the role of the *TAN* gene, we asked whether mutant embryos could be rescued by germinating immature seeds in culture. Seventy to 90% of immature *tan-1* mutant seeds at 10 to 14 d after flowering germinated in the light without stratification and produced seedlings, whereas only approximately 10% of wild-type immature seeds germinated. Thus, the *tan* mutation appears to affect dormancy, although mutant seeds were not viviparous in planta. We note that immature *tan-1* mutant seeds did not germinate on medium containing 1 μM abscisic acid, suggesting that defects in dormancy did not result from abscisic acid insensitivity.

tan Mutants Display Defects in Seedling Morphology

Although immature *tan-1* mutant embryos could be germinated before desiccation, mutant seedlings did not continue to develop and eventually died. Thus, *tan-1* mutants exhibit both embryo lethality and seedling lethality. Analyses of mutant seedlings revealed striking defects in seedling morphology caused by the *tan* mutation. As shown in Figure 4A, *tan-1* mutant seedlings had a few trichomes either without branches or with one branch on the adaxial surfaces of their cotyledons that were initiated during embryogenesis (Fig. 1G). Trichomes are normally present only on

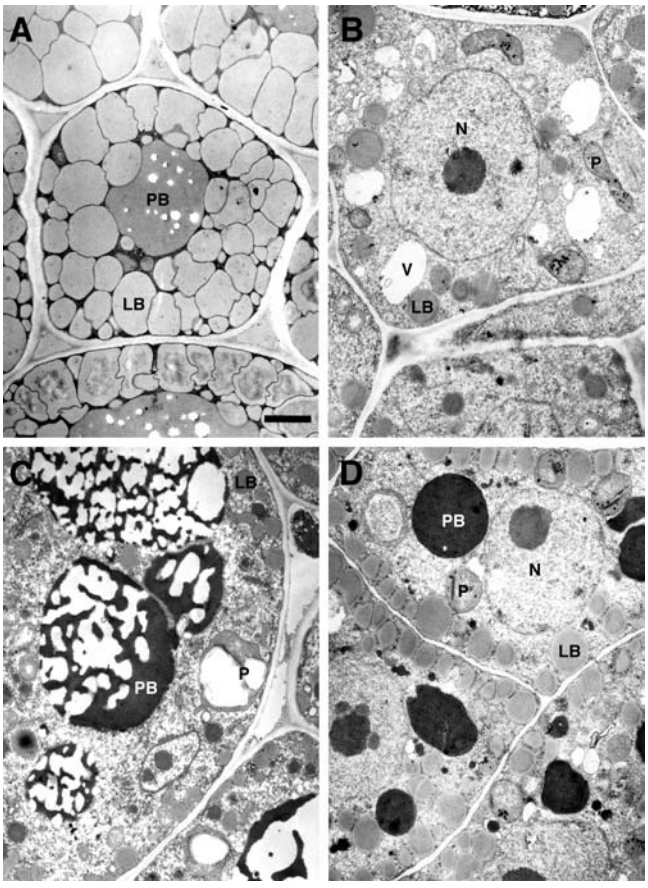

Figure 3. *tan* embryo cells contain fewer lipid and protein bodies. A, Cells from a wild-type embryo. B to D, Mutant embryonic cells in the tip and the middle of cotyledons and the embryonic axis, respectively. LB, Lipid body; PB, protein body; P, plastid; V, vacuole; N, nucleus. Bar = 1 μm; bar in A applies to B to D.

leaves, stems, and sepals and are rarely observed on cotyledons of wild-type Arabidopsis seedlings (Fig. 4C; Hulskamp et al., 1994; West et al., 1994). Furthermore, approximately 50% of *tan-1* seedlings had cotyledons that were fused along one side of their margins (Fig. 4B), another defect that was likely to be initiated during embryogenesis (Aida et al., 1997). It was also apparent that the *tan* mutation affected other aspects of seedling development. For example, mutant seedlings had stunted roots and hypocotyls that failed to elongate (Fig. 4, A and B). Moreover, true leaves rarely emerged, suggesting that the SAM is defective. To determine whether *tan-1* mutants were competent to form vegetative SAMs, we cultured immature *tan-1* embryos on callus-inducing medium. Unlike wild-type embryos that formed calli and adventitious shoots when cultured (Fig. 4H), *tan-1* mutant cells underwent limited proliferation and eventually stopped growing without producing shoots (Fig. 4G). We conclude that the *tan* mutation has pleiotropic effects on seedling development.

tan mutants share a subset of characteristics exhibited by the photomorphogenic *fusca/constitutive photomorphogenic/de-etiolated* mutants. This class of mutants accumulates anthocyanin in embryos, exhibits seedling lethality, and fails to etiolate when grown in the dark (Castle and Meinke, 1994; Chory et al., 1996; Wei and Deng, 1996). To determine whether *tan-1* mutants are defective in photomorphogenesis, we germinated immature *tan-1* seeds in the dark. As shown in Figure 4, D and E, dark-grown *tan* seedlings had short hypocotyls, and one-half the seedlings partially expanded their cotyledons. By contrast, wild-type seedlings had fully elongated hypocotyls and closed cotyledons (Fig. 4F). However, dark-grown *tan-1* seedlings did not exhibit other characteristics of photomorphogenic mutants such as development of leaves and expression of light-inducible genes encoding chlorophyll *a/b*-binding protein and D1 protein of PSII (data not shown), which are up-regulated in dark-grown photomorphogenic mutants. Consistent with these results, we used genetic complementation experiments to show that *TAN* is not allelic with the *FUS/COP/DET* genes, *COP1/FUS1*, *COP8/FUS8*, *COP9/FUS7*, *COP10/FUS9*, *COP11/FUS6*, *COP15/FUS5*, and *DET1/FUS2*. We conclude that *TAN* is not a typical *FUS/COP/DET* gene.

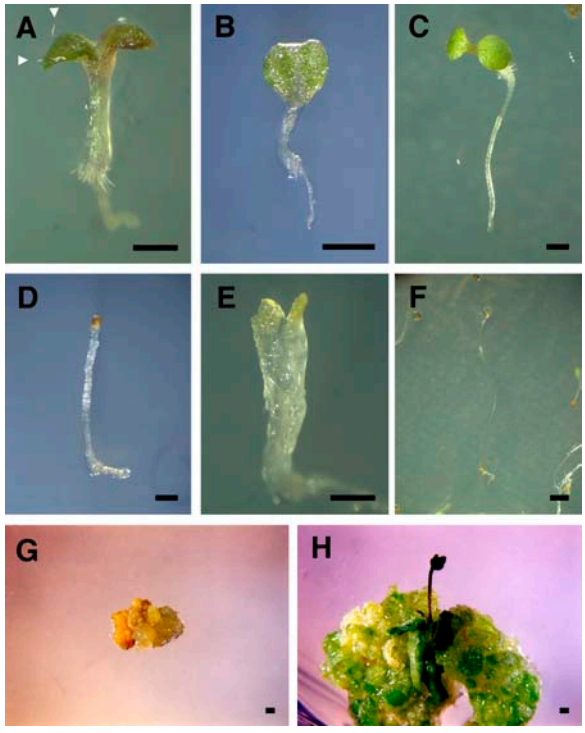

Figure 4. *tan* mutants are defective in vegetative development. A to F, Mutant and wild-type seedlings germinated from immature seeds on medium. A, Mutant seedlings grown in the light have trichomes on cotyledons (arrow heads). B, Many mutant seedlings have cotyledons fused along one margin. C, Wild-type seedling grown in the light. D and E, Mutant seedlings grown in the dark show etiolation defects. F, Wild-type seedling grown in the dark. G and H, Mutant and wild-type calli on shoot-inducing medium, respectively. Unlike wild-type callus, *tan* calli did not regenerate adventitious shoots. Bars = 500 μm.

Interaction between *TAN* and *LEC* Genes

tan-1 mutant embryos share many, but not all, characteristics of embryos with mutations in the *LEC* genes, *LEC1*, *LEC2*, and *FUS3.* For example, both *tan-1* and *lec* mutations cause desiccation intolerance, defects in storage protein and lipid accumulation, and trichome formation on cotyledons (Harada, 2001). However, the *lec* mutations do not cause seedling lethality. Genetic tests showed that *TAN* was not allelic with the *LEC* genes (data not shown). Therefore, we conducted digenic mutant analysis to investigate the relationship between *TAN* and the *LEC* genes.

TAN did not display epistasis with the *LEC* genes because, as shown in Figure 5, embryos with digenic mutations in *TAN* and any of the three *LEC* genes had characteristics that were not predicted by the single mutant phenotypes. For example, the vast majority of *tan-1 lec1-2* digenic mutant embryos arrested at the transition or heart stage, as shown in Figure 5, A and B, although a smaller number attained a shape similar to a torpedo-stage embryo (Fig. 5C). By contrast, each single mutant arrested at a late embryonic stage (Figs. 1D and 5D). Thus, *TAN* and *LEC1* did not display epistasis and did not interact additively in that the digenic mutant arrested at an earlier morphological stage than either monogenic mutant. *tan-1 lec1-2* mutant seeds were desiccation intolerant and germinated poorly even when cultured on agar plates. The morphology of *tan-1 lec2-1* digenic mutants differed from *lec2-1* mutant embryos (Fig. 5H) and resembled those with *tan-1 lec1-1* mutations in that most arrested primarily at the transition to heart stage (Fig. 5, E and F) and a few arrested at the torpedo stage (Fig. 5G). By contrast, we found that *tan-1 fus3-3* digenic mutants exhibited variable phenotypes. Approximately one-half of digenic mutants resembled transition- and heart-stage embryos (Fig. 5, I and J), whereas the remainder appeared to be intermediate forms of both parents (Figs. 1D and 5, K and L). Seeds with intermediate-form embryos germinated in culture and produced seedlings with trichomes on cotyledons, but they displayed lethality like *tan-1*. Together, these results indicate that the *TAN* and *LEC* genes, particularly *LEC1* and *LEC2*, interact synergistically. Thus, *TAN* and *LEC* genes may operate in closely related pathways during embryogenesis.

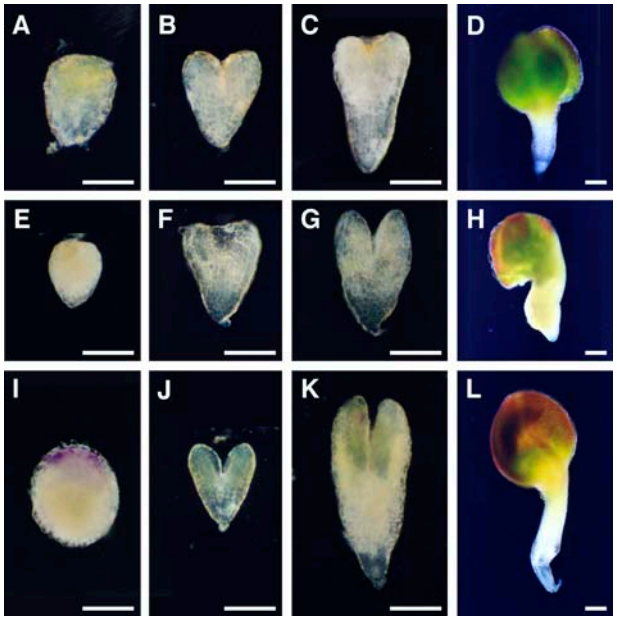

Figure 5. Lack of epistasis between *TAN* and *LEAFY COTYLEDON* genes. A to C, Terminal phenotypes of *tan lec1* digenic mutant embryos. Digenic mutant embryos are shaped like globular stage, heart-stage, and torpedo-stage embryos, respectively. D, Terminal phenotype of *lec1* monogenic mutant embryo. E to G, Terminal phenotype of *tan lec2* digenic mutant embryos. H, Terminal phenotype of *lec2* monogenic mutant embryo. I to K, Terminal phenotype of *tan fus3* digenic mutant embryos. L, Terminal phenotype of *fus3* embryo. Bars = 100 μm.

Identification of the *TAN* Gene

To determine its molecular function, we isolated the *TAN* gene. Genetic segregation and genomic DNA gel-blot analyses indicated that the *tan-1* mutation is linked with a single T-DNA locus (data not shown). Genomic clones containing the T-DNA were isolated from a library of *tan-1* DNA (see "Materials and Methods"). Plant DNA fragments flanking the T-DNA were used to isolate seven cDNA clones from a library of silique RNA sequences and the corresponding genomic clones containing wild-type genomic DNA. The cDNA clones represented two RNAs, 1.5 and 0.8 kb in size, that were encoded by different genes and present in wild-type siliques and seedlings. We used reverse transcription (RT)-PCR analysis to determine which cDNA clone corresponded to the *TAN* gene. As shown in Figure 6B, the 0.8-kb RNA was present in wild-type and *tan-1* mutant seedlings, whereas the 1.5-kb RNA was detected only in wild-type seedlings, suggesting that *TAN* encodes the 1.5-kb RNA. Consistent with this conclusion, a single recessive mutation [72/290, $\chi^2(3{:}1) = 0.004$; $P > 0.9$], *tan-2* (SALK_097510), was identified that is allelic to and that caused an identical mutant phenotype as *tan-1*. In *tan-2*, the left border of a T-DNA was present in the putative *TAN* gene, but we were unable to position the right border. Thus, the precise nature of the T-DNA insertion in *tan-2* is not known.

Because the longest *TAN* cDNA clone contained only a 759-bp insert, we performed RT-PCR to obtain 5′ RNA sequences and obtained two classes of cDNA sequences. One class, represented by the *RACE14* clone, contained the remaining 625 bp of the cDNA sequence and produced a long open reading frame (ORF) in the full-length RNA. Sequence alignment of the full-length RNA and the gene revealed that the *TAN* gene consists of 13 exons (Fig. 6A). This analysis also showed that the T-DNA in *tan-1* disrupts the

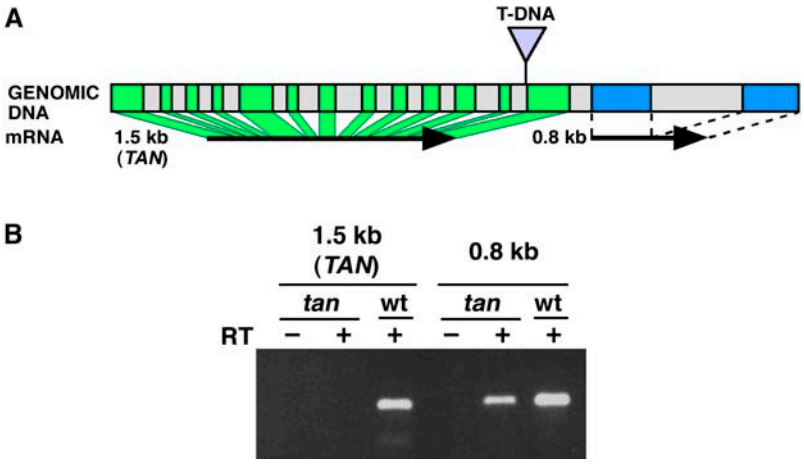

Figure 6. Identification of the *TAN* gene. A, Diagrammatic representation of the *TAN* locus. Green boxes represent exons of the *TAN* gene encoding a 1.5-kb RNA, gray boxes represent introns or intergenic regions, blue boxes represent the adjacent gene encoding an 0.8-kb RNA. Location of the T-DNA in *tan-1* is shown. B, RT-PCR analysis showed that the 0.8-kb RNA is present in both wild-type and *tan* mutant seedlings, whereas the 1.5-kb RNA is detected in wild-type, but not *tan*, mutants.

twelfth intron and that a deletion of 5 bp at the intron-exon junction occurred 7 bp downstream of the insertion (data not shown). To determine whether this cDNA clone represents the *TAN* gene, we constructed a full-length *TAN* cDNA clone, fused it with approximately 3 kb of 5′ flanking sequence from the *TAN* gene, and transferred the chimeric gene into plants heterozygous for the *tan-1* mutation. The transgene suppression experiments provided strong evidence that the *RACE14* clone represents a *TAN* cDNA clone. Embryos with mutant and wild-type phenotypes segregated at ratios suggesting that the transgene suppressed the *tan-1* mutation and was present at a single locus in one transgenic plant [20/258, $\chi^2(15:1) = 0.99$; $P > 0.3$] and at two loci in two transgenic plants, [2/261, $\chi^2(63:1) = 1.08$; $P > 0.3$] and [3/259, $\chi^2(63:1) = 0.28$; $P > 0.5$]. The segregation of progeny from one transgenic plant was distorted, suggesting transgene suppression of the mutation, but the ratio was not consistent with either one or two transgene loci [e.g. 8/254, $\chi^2(15:1) = 4.17$; $P > 0.01$]. We conclude that the cDNA clone represents the 1.5-kb RNA encoded by the *TAN* gene.

Other data suggest that alternative splicing of the *TAN* gene occurred, producing nonfunctional transcripts. We obtained another class of cDNA clone that had 5 bp missing at the beginning of the second exon of *TAN* that changed the reading frame and is expected to produce a truncated protein. We also found that two expressed sequence tags corresponding to *TAN* cDNA clones from the Columbia ecotype resulted from different splicing events as compared with the *RACE14* version of the RNA (accession nos. AA713092 and AK118989). Both alterations caused premature termination of the ORF, suggesting that RNAs corresponding to the expressed sequence tag clones are not functional. Subsequent sequencing of a bacterial artificial chromosome clone, F27B13 (accession no. AL050352), as part of the Arabidopsis Genome Initiative showed that the *TAN* gene is located on the bottom arm of chromosome IV (Arabidopsis Genome Initiative, 2000). Based on the locus designation, At4g29860, we learned that *TAN* corresponds to two recently reported *emb* mutations, *emb2757-1* and *emb2757-2*, described as part of a dataset of 250 *EMB* genes identified from T-DNA insertion lines (Tzafrir et al., 2004). Morphological mutant phenotypes attributed to these lines, such as seed and embryo color and embryo morphology (http://www.seedgenes.org), are consistent with those described for *tan-1* and *tan*-2. Thus, *TAN* appears to be allelic with *EMB2757*.

The *TAN* cDNA (accession no. AB191306) contains an ORF encoding a protein of 386 residues. As shown in Figure 7, the deduced amino acid sequence of the *TAN* gene contains seven WD repeats (Neer et al., 1994). WD repeats comprise 44 to 60 amino acid residues that often contain specific subsequence motifs, including the Trp-Asp (WD) dipeptide for which the repeat was named (Smith et al., 1999). The seven predicted WD repeats in TAN conform well with this motif with the possible exception of the third repeat.

RNA gel-blot (data not shown) and quantitative RT-PCR analyses, summarized in Figure 8A, showed that *TAN* RNA is present in floral buds, leaves, stems, roots, and four sequential stages of siliques. Quantitative RT-PCR experiments showed that *TAN* RNA was present at its highest levels in siliques that contain developing seeds. In situ hybridization analysis showed that *TAN* RNA accumulates throughout the embryo and in endosperm (Fig. 8B). We conclude that the *TAN* gene encodes a WD repeat protein that functions during embryogenesis and other phases of the life cycle.

DISCUSSION

TAN Is a WD Repeat Protein

We identified the *TAN/EMB2757* gene whose deduced amino acid sequence (Fig. 7) shows that it is one of the approximately 237 potential WD repeat proteins in Arabidopsis (van Nocker and Ludwig, 2003). This class of protein is involved in mediating interactions between proteins. WD repeat proteins have been

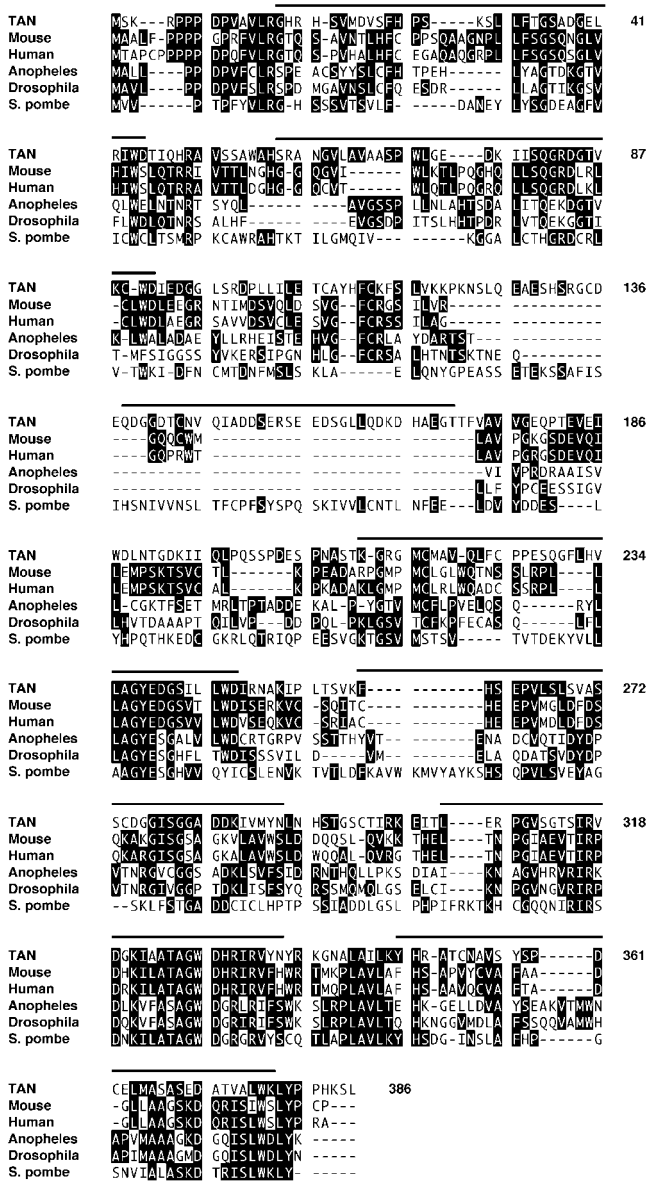

Figure 7. Amino acid sequence similarity between TAN and other WD repeat proteins. Comparison of the predicted amino acid sequence of TAN with WD protein homologs from mouse, human, Anopheles, Drosophila, and *Schizosaccharomyces pombe*. Identical amino acid residues are shown by black boxes. Overlined sequences show putative WD repeats. Numbers indicate amino acid positions in the TAN protein.

predicted to have between four and 16 copies of the repeat (Smith et al., 1999), although structural analyses of WD repeat proteins have shown that several proteins predicted to have fewer or more repeats actually possess seven (Voegtli et al., 2003; Madrona and Wilson, 2004). Similarly, TAN is predicted to possess seven WD repeats, suggesting that it is a functional WD repeat protein.

Identification of TAN as a likely WD repeat protein provides insight into its molecular function. WD repeat proteins participate in protein-protein interactions and have roles in a variety of physiological functions (Neer et al., 1994; Smith et al., 1999). Of proteins with a known function, TAN is similar to the β-subunit of G proteins (Jones, 2002). However, this similarity is based primarily on sequences within the WD repeat. Portions of sequential WD repeats interact to form propeller blades, and the propeller structure creates a surface with which other proteins can interact (Neer et al., 1994; Smith et al., 1999). Thus, regions between repeats determine the specificity of each WD repeat protein, and WD repeat proteins with common functions share sequence similarity in inter-repeat regions. No significant sequence similarity was observed between the inter-repeat regions of TAN with other proteins in the database (http://bmerc-www.bu.edu/wdrepeat).

TAN Functions Early and Late in Embryo Development

Phenotypic analyses suggest that the *TAN* gene plays roles during both the early-morphogenesis and

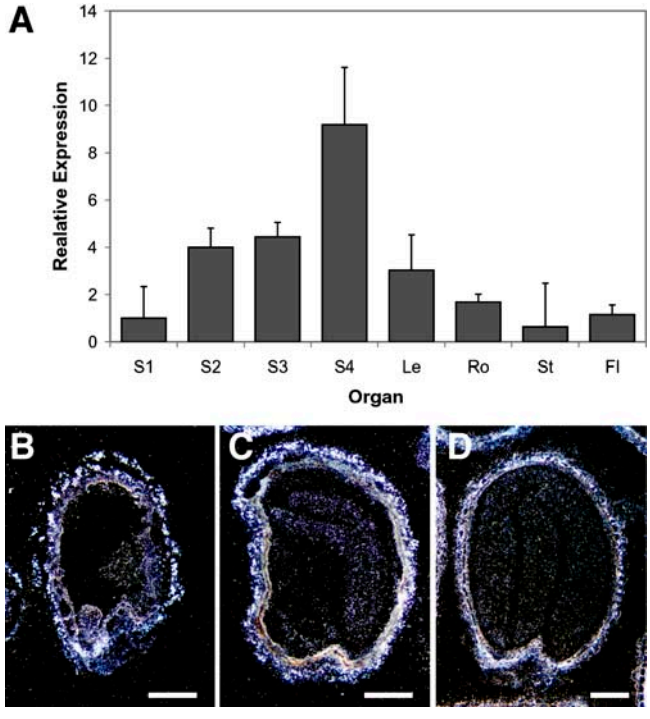

Figure 8. Detection of *TAN* RNA in organs and developing embryos. A, Detection of *TAN* RNA levels with quantitative RT-PCR analysis. *TAN* RNA was detected in total RNA from flower buds (Fl), rosette leaves (Le), stems (St), roots (Ro), siliques containing seeds with embryos at the zygote to early globular stage (S1), globular stage to heart stage (S2), torpedo stage to curled-cotyledon stage (S3), and mature green stage (S4). *TAN* RNA levels were normalized to that of the 60S ribosomal protein, L14. Bars indicate the average of two determinations. B to D, Detection of *TAN* RNA in developing embryos with in situ hybridization. Wild-type seed sections were hybridized with a *TAN* antisense probe. Control experiments with a sense probe indicated that the hybridization signals were specific to *TAN* RNA. B, Heart-stage embryo. C, Bent-cotyledon-stage embryo. D, Mature green-stage embryo. Bars = 100 μm.

late-maturation phases of embryogenesis. As summarized in Table I, several lines of evidence indicate that *TAN* is required for normal progression through the maturation phase. The acquisition of desiccation tolerance and the accumulation of storage reserves are hallmarks of this developmental phase. The *tan* mutation causes defects in both processes because mutant embryos are intolerant of desiccation and do not properly accumulate storage proteins and lipids (Fig. 3). Consistent with this observation, *tan* mutant embryos contain reduced levels of RNA encoding the small heat shock protein, HSP 17.4, a protein whose accumulation is correlated with an embryo's competence for desiccation tolerance (data not shown; Wehmeyer and Vierling, 2000). Another indication that *TAN* functions during the maturation phase is that immature *tan* mutant embryos germinate efficiently, indicating that dormancy is compromised by the *tan* mutation (Fig. 4). Together, these results suggest strongly that *TAN* is required for normal progression through the maturation phase.

Table I also summarizes results suggesting a role for *TAN* in the morphogenesis phase of embryogenesis. In support of this idea, *tan* mutant embryos do not appear to form functional embryonic SAMs, as indicated by defects in SAM organization in mutant embryos (Fig. 2), fusion of mutant cotyledons along one margin (Fig. 4), and the inability of seedlings to form stems or leaves (Fig. 4). Root apical meristem also appears to be defective in *tan* mutant embryos and seedlings (Figs. 1 and 4).

Although *tan* mutant seedlings have an abnormal morphology (Fig. 4), it is not clear whether the defects result from direct effects of the mutation on seedling development or indirect effects of the mutation that occurred originally during embryogenesis. For example, because SAM initiation occurs early in embryogenesis, abnormalities in the seedling SAM may be attributed to embryonic defects (Barton and Poethig, 1993). Furthermore, similar to *tan*, dark-grown *lec1* seedlings also have shorter hypocotyls as compared with dark-grown wild-type seedlings, yet *LEC1* gene expression is restricted to embryogenesis (Lotan et al., 1998; Brocard-Gifford et al., 2003; K. Yamagishi, J. Danao, and J. J. Harada, unpublished data). Thus, effects on seedling morphology could be explained by indirect effects of the *tan* mutation on embryo development. On the other hand, *tan* mutant calli failed to form shoots (Fig. 4), suggesting that the vegetative SAM is defective. Moreover, the *TAN* gene appears to be expressed throughout plant development (Fig. 8), suggesting a potential postembryonic role for the gene.

Relationship between *TAN* and the *LEC* Genes

Mutations in the *lec* genes induce many defects in embryo development (Harada, 2001) shared with those caused by the *tan* mutation, suggesting that the genes may have overlapping roles in embryogenesis. For example, a defining characteristic of *lec* mutants is the presence of trichomes on the adaxial surfaces of their cotyledons. Trichomes are present on true leaves, sepals, and stems and are not normally found on Arabidopsis cotyledons (Hulskamp et al., 1994; West et al., 1994). The presence of trichomes on the cotyledons of *lec* mutants has been attributed to heterochrony (West et al., 1994). That is, the shift from the maturation to the postgerminative phase of development induced by the *lec* mutations causes a heteroblastic change in which cotyledons adopt a partial leaf fate. Thus, one explanation for trichome initiation on the surfaces of *tan* mutant cotyledons (Figs. 1 and 4) is that defects in the maturation phase induce postgerminative development prematurely. However, we cannot eliminate the possibility that other factors are responsible for trichome formation on *tan* cotyledons. For example, *twin1* mutants with embryonic transformation of the suspensor (Vernon and Meinke, 1994) and transgenic plants overexpressing *GLABROUS1* and *AtMYB23*, genes involved in trichome initiation, develop cotyledonary trichomes after germination (Larkins et al., 1994; Kirik et al., 2001). Furthermore, trichomes have also been detected on cotyledons of

Table I. *Summary of defects in tan mutants*

Phenotypes and Defects	Role of *TAN* in Plant Development
tan Mutant Embryos	
Defects in protein and lipid body accumulation	Storage protein and storage lipid synthesis—maturation
Desiccation intolerance (embryo lethality)	Acquisition of desiccation tolerance—maturation
Immature seeds germinate efficiently	Dormancy—maturation
Defective elongation of cotyledons	Cell division and/or expansion—morphogenesis and/or maturation
tan Mutant Seedlings	
Trichomes on cotyledons	Specification of cotyledon identity during embryogenesis—morphogenesis
Cotyledons often fused along margins	Organ separation during embryogenesis—morphogenesis
Defective SAM	Meristem formation—morphogenesis
Stunted roots	Meristem formation and/or growth—morphogenesis

constitutive photomorphogenesis and dwarfism (*cpd*) mutants that have defects in brassinosteroid biosynthesis (Szekeres et al., 1996). In this context, we confirmed that *tan* mutant seedlings contained wild-type levels of major sterols and their defects were not rescued by the application of 24-epibrassinolide (K.Yamagishi and S.Fujioka, unpublished data).

Another indication that *tan* mutants prematurely enter the postgermination phase is the high germination frequency of cultured immature seeds, indicating competence to germinate. We have shown that isocitrate lyase and lipid transfer protein genes, normally expressed primarily during postgerminative growth, are activated prematurely in *tan* mutant embryos (data not shown). Thus, similar to the *lec* mutations, the *tan* mutation appears to induce at least some aspects of postgerminative development prematurely during seed maturation.

tan mutants are similar to *lec2* and *fus3* mutants in that they all display a gradient of defects in storage reserve accumulation, with defects in storage protein accumulation being severe in apical regions of cotyledons and mild or nonexistent in embryonic axes (Fig. 3; Meinke et al., 1994). However, *tan* and *lec2* mutants differ in that *lec2* mutant embryos exhibit a gradient of desiccation intolerance in apical regions (Meinke et al., 1994; Stone et al., 2001), whereas *tan* mutants do not. Rather, all cells of *tan* mutant embryos appear to be intolerant of desiccation because no cells in mature dried embryos are able to proliferate and form callus. One interpretation of these results is that storage reserve accumulation and desiccation tolerance are regulated independently in pathways downstream of *TAN*. Alternatively, *TAN* may directly regulate each pathway, but desiccation tolerance may be more sensitive to loss of *TAN* activity.

Genetic interactions between *TAN* and *LEC* genes provide compelling evidence for an overlap in their functions. *tan lec1*, *tan lec2*, and a subset of *tan fus3* digenic mutants display a synergistic phenotype similar to those observed for *lec1 lec2* digenic mutants (Fig. 5; Meinke et al., 1994; Lotan et al., 1998). *LEC1* and *LEC2* both act early in embryogenesis (Harada, 2001). The *lec1 lec2* digenic mutant arrests at an earlier morphological stage of embryogenesis than either monogenic mutant, and this result has been interpreted to indicate that the two genes have overlapping functions early in embryogenesis. By analogy, the striking similarities of *tan lec1, tan lec2,* and *lec1 lec2* digenic mutants suggest that *TAN* also functions early in embryogenesis and that it may have overlapping function with the *LEC* genes, particularly *LEC1* and *LEC2*. We note that both the *lec1-2* and *lec2-1* alleles used for the digenic mutant analysis are null alleles (Lotan et al., 1998; Stone et al., 2001), whereas it is not known whether *fus3-3* represents a null allele (Luerben et al., 1998).

In conclusion, we have identified the *TAN/EMB2757* gene that is required for several aspects of plant development. *TAN* is required both early and late in embryo development and potentially during vegetative development. Moreover, *TAN* interacts genetically with *LEC* genes, suggesting an overlap in functions. The demonstration that *TAN* is expressed throughout plant development and encodes a putative WD repeat protein suggests that it accomplishes its function by interacting with other proteins possibly to form regulatory complexes.

MATERIALS AND METHODS

Plant Material

The *tan-1* mutant (previously designated Line 24) was derived from a population of Arabidopsis (*Arabidopsis thaliana* ecotype Wassilewskija [Ws]) mutagenized with T-DNA insertions (Feldmann and Marks, 1987; Yadegari et al., 1994). *tan-2* (stock no. SALK_097510, ecotype Columbia), and *cop*, *det*, and *fus* mutants were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The *tan-1* mutant was used for experiments unless otherwise indicated. *fus3-3* mutants and the *lec2-1* mutant were provided by Peter McCourt (University of Toronto; Nambara et al., 1992; Keith et al., 1994) and by David Meinke (Oklahoma State University; Meinke et al., 1994), respectively. Plant growth, digenic mutant construction, and *lec1-2* and *lec2-1* mutants are as described previously (West et al., 1994; Lotan et al., 1998). Because *lec* mutants can be maintained as homozygotes, *tan-1 lec* digenic mutant embryos segregated at a 3:1 ratio. The genotype of each digenic mutant was verified by backcrosses with each single mutant.

Induction of Calli and Shoots

Embryos were dissected from imbibed or immature seeds and cultured for six weeks on callus-inducing medium and, subsequently, for five weeks on shoot-inducing medium according to Valvekens et al. (1988), except that the carbon source was 20 g/L Suc.

Isolation and Sequence Analysis of Genomic and cDNA Clones

A genomic DNA library from a heterozygous *tan-1* mutant was constructed in the λGEM 11 vector (Promega). A wild-type Ws-0 genomic library was provided by Ken Feldmann (University of Arizona). A cDNA library was prepared according to the manufacturer's specifications in the λZAPII bacteriophage vector (Stratagene) from wild-type siliques containing heart- to young-torpedo-stage embryos.

Clones were isolated from the *tan* genomic library using probes for the right and left T-DNA borders. *Hin*dIII fragments of 4.1 and 5.1 kb containing the plant DNA/T-DNA junctions were isolated and cloned into the pBluescript KS(+) phagemid (Stratagene) to create pRL3 and pL15, respectively. The DNA fragments were used to isolate genomic clones from a wild-type Ws-0 library. A 3.2-kb *Hin*dIII fragment present in a genomic clone that hybridized with both probes was inserted into the pBluescript KS(+) phagemid and used to identify *TAN* cDNA clones from wild-type silique cDNA libraries. Because the cDNA clones were smaller than the corresponding RNA, the 5′-end of the cDNA was obtained using the CapFinder PCR cDNA library construction kit (CLONTECH). cDNA was synthesized from poly(A)$^+$ RNA of 3-week-old plants according to the manufacturer's instructions and subjected to the nested PCR with a 5′ PCR primer (CLONTECH) and gene-specific primers. The amplified products were inserted into pCR2.1 plasmid (Invitrogen) and fused with the cDNA clone at an *Mfe*I site to make full-length cDNAs. Database searches were performed at the National Center for Biotechnology Information by using the BLAST network service. Alignment of protein sequences was done using DNASIS (Hitachi Software Engineering Co.). Analysis of WD repeat motifs was performed at the Bio-Molecular Engineering Research Center Web site (http://bmerc-www.bu.edu/index.html).

Generation of Transgenic Plants

For genetic suppression of the *tan* mutation, the full-length *TAN* cDNA was fused with a 3-kb fragment of *TAN* promoter and the octopine synthase

terminator in the plasmid, pART7, in the proper transcriptional orientation (Gleave, 1992). The fusion gene was transferred into the plant transformation vector, BJ49. Constructs were transferred into heterozygous *tan-1* mutant plants using in planta transformation procedures with *Agrobacterium tumefaciens* strain GV3101 (Bechtold et al., 1993). Genotypes of transgenic plants were verified in DNA amplification experiments.

RNA Analysis

RNA was isolated as described previously (Stone et al., 2001). For quantitative RT-PCR, RNAs were treated with DNase, *Mse*I, and *Dde*I to control for DNA contamination. RT reactions were carried out with 1 μg of total RNA in a 20-μL reaction volume. PCR reactions were done with the Bio-Rad iQ SYBR Green supermix (Bio-Rad Laboratories) according to specifications, except that 0.25 μM of each primer and 1 μL of a 20-fold dilution of RT products were used. The Bio-Rad iCycler iQ multicolor real-time detection system (Bio-Rad Laboratories) was used with the following reaction conditions: one repeat of 3 min at 95°C followed by 50 repeats of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, followed by a melt curve encompassing 80 steps of 0.5°C from 60°C to 100°C. Images were captured during the 72°C extension step and at each step in the melt curve using a FAM filter. The following primer sets were used: AT4G27090 Fwd 5′-GAGAGGATCCAGATGAACTTCAAGA-3′ and AT4G27090 Rev 5′-GCTCTTGCCTGACAACACCAGCTTT-3′; TanMei Fwd 5′-GACCTCCGCCTGATCCAGTG-3′ and TanMei Rev 5′-CCATTTGC-TCTACTATGAGCC-3′. Primary data analysis was performed with Bio-Rad iCycler iQ software. The Bio-Rad gene expression macro version 1.1 software (Bio-Rad Laboratories; Livak and Schmittgen, 2001; Pfaffl, 2001; Vandesompele et al., 2002) was used to calculate relative RNA levels normalized to an internal control (AT4G27090: 60S ribosomal protein L14; Thellin et al., 1999). Results are reported relative to RNA level in the S1 sample.

In situ hybridization experiments were performed as described previously (Dietrich et al., 1989).

Electron Microscopy

Transmission electron microscopy was performed according to Meinke et al. (1994).

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number AB191306.

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