Arabidopsis TSO1 Regulates Directional Processes in Cells During Floral Organogenesis

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ABSTRACT

Flowers of the previously described Arabidopsis tso1-1 mutant had aberrant, highly reduced organs in place of petals, stamens, and carpels. Cells of tso1-1 flowers had division defects, including failure in cytokinesis, partial cell wall formation, and elevated nuclear DNA content. We describe here two new tso1 alleles (tso1-3 and tso1-4), which caused defects in ovule development, but had little effect on gross floral morphology. Early ovule development occurred normally in tso1-3 and tso1-4, but the shapes and alignments of integument cells became increasingly more disordered as development progressed. tso1-3 ovules usually lacked embryo sacs due to a failure to form megaspore mother cells. The cell division defects described for the strong tso1-1 mutant were rarely observed in tso1-3 ovules. The aberrations in tso1-3 mutants primarily resulted from a failure in directional expansion of cells and/or coordination of this process among adjacent cells. Effects of tso1-3 appeared to be independent of effects of other ovule development mutations, with the exception of leunig, which exhibited a synergistic interaction. The data are consistent with TSO1 acting in processes governing directional movement of cellular components, indicating a likely role for TSO1 in cytoskeletal function.

The relative importance of the orientation of cell divisions, rate of cell divisions, and the directional expansion of cells in plant morphogenesis is still debated (Jacobs 1997). In one view, morphogenesis is regulated primarily by expansion processes acting at the level of whole organs, with cell divisions being a necessary consequence for maintenance of practical cell size (Kaplan and Cooke 1997). A contrasting view is that the orientation of cell divisions predicts the shape of plant organs (Fosket 1990). Despite the differences between these views, both are consistent with the idea that cells in meristematic regions respond to positional signals that induce and control morphogenesis.

Recently, a number of mutants that provide new insight into the relative roles of directional expansion and division in morphogenesis have been isolated. The tangled-1 mutant of maize exhibited significant misorientation of the planes of cell division, but its morphology was like that of wild type (Smith et al. 1996). The orientation of cell divisions in the Arabidopsis fass mutants was essentially random (Torres-Ruiz and Jürgens 1994), the plants were dwarf, but the overall morphology of these mutants was similar to wild type. In addition, an increase in the rate of cell division in roots, induced by ectopic cyclin expression, did not alter root morphology (Doerner et al. 1996), leading to speculation that root form is not determined by cell division rates (Jacobs 1997). Thus, examples of morphology appearing to be regulated at a supercellular level exist.

Several mutations, however, could directly link morphogenesis and cell division. For example, cell division defects have been hypothesized to be the underlying cause of the abnormal development observed in superman (sup) and tousled (tsl) mutants (Sakai et al. 1995; Roe et al. 1997) and have been offered as one possible explanation for the effects of the tso1 mutation (Liu et al. 1997).

In the case of the tso1-1 mutant, the phenotype was not only associated with altered planes of cell division, but also included the following: (1) abnormal cell plate formation; (2) disorganized architecture of the floral meristem; (3) large nuclei that were aberrant in shape and had high DNA content; and (4) formation of rudimentary, misshapen petals, stamens, and carpels (Liu et al. 1997). Herein we describe two apparently weaker alleles of tso1 (tso1-3 and tso1-4) that have novel phenotypes. While flower formation was essentially complete in these mutants, ovule development was aberrant, and the mutants enabled an examination of the role of tso1 in this later developmental process. The greatest effects of these weak alleles was on directional expansion of cells and organs, rather than on cytokinesis. These alleles showed that tso1 mutations affected morphogenesis independent of failure in cell division. These findings indicate that TSO1 is involved in general spatial organization of a variety of cellular processes, rather than having a specific role in cell division.
MATERIALS AND METHODS

Plant growth: Arabidopsis plants were grown under continuous illumination with fluorescent light (\(-50 \mu\text{E/m}^2\text{sec}\)) in a mixture of equal parts vermiculite, perlite, and peat moss. Plants were fertilized weekly with complete nutrient solution (Kranz and Kirschheim 1987), and insects were controlled using weekly treatments of Gnatrol (Abbott Laboratories, Chicago, IL) and biweekly treatments of orthene and malathion (both from Chevron Chemical Co., San Ramon, CA), as suggested by the manufacturers. All genetic crosses were done as previously described (Kranz and Kirschheim 1987).

Mutant isolation, allelism test, and mapping: The tso1-3 allele was isolated from a population derived from ethyl methanesulfonate-mutagenized Arabidopsis Landsberg erecta (Ler) seed (Lehle Seeds, Tucson, AZ) as previously described (Robinson-Beer et al. 1992). tso1-4 was identified as a contaminant in a pot of other mutants in the Ler background. Because tso1-4 was identified after tso1-3, it is possible that it represents a reisolation of this same mutant. The significant phenotypic differences between the two isolates (see results), however, indicate that this mutant is likely to be a different allele and will be referred to as such throughout the text. The tso1-1 mutant was a gift of Zhonghui Liu (University of Maryland).

Segregation of genetic markers was analyzed in the progeny from a cross between wild-type Columbia (Col-3) plants and tso1-3. DNA was isolated (Edwards et al. 1991) from F2 progeny, and the map position of this locus was determined by linkage analysis with cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993) and simple sequence length polymorphisms (Bell and Ecke 1994). Map distances were determined using Mapmaker v. 2 for Macintosh computers (Lander et al. 1987).

Microscopy: Ovules and flowers were prepared for scanning electron microscopy (SEM) as previously described (Robinson-Beer et al. 1992), except that samples were fixed in 25 mm sodium cacodylate pH 7.0, 5% glutaraldehyde, and 0.1% Tween 20. Samples were examined using an ISI DS130 microscope (Topcon Technologies, Paramus, NJ) at an accelerating voltage of 10 kV. Images were recorded from this microscope using the Semicaps digital capture system (Semicaps, Santa Clara, CA). Images were cropped and contrast-adjusted using Adobe Photoshop (v. 4.0 for Macintosh, Adobe Systems, Inc., San Jose, CA).

Samples were prepared for light microscopy as described by Baum and Rost (1996), except that they were infiltrated for 1 wk each with two changes of Historesin (Cambridge Instruments, Deerfield, IL). Plastic-embedded pistils were cut into 2- to 3-µm sections using a Reichert Jung 2050 Supercut microtome (Cambridge Instruments, Buffalo, NY). Sections were mounted on gelatin-coated slides, stained with a periodic acid-Schiff reaction (O’Brien and McCully 1981), and counterstained with toluidine blue. Stained sections were photographed with a Zeiss Axioplan fluorescence microscope (Oberkochen, Germany) using bright field illumination and Kodak Ectachrome 160T film (Eastman Kodak, Rochester, NY). Images were digitized using a Nikon LS-1000 slide scanner (Nikon Corp., Melville, NY) and edited as for SEM images.

Pollen tube and seed staining: Pollen tubes were stained as previously described (Kandasamy et al. 1994). The pollen tubes from pistil squashes were visualized with a Zeiss Axioplan fluorescence microscope using a G365 filter for excitation and an LP420 filter for observation. Pollen tubes were photographed using Kodak Ectachrome 100 film. Slides were digitized and edited as described above.

To examine mucilage, seeds were hydrated, stained with either 0.05% toluidine blue or 0.1% ruthenium red, and rinsed with water. Stained seeds were examined using a dissecting microscope.

Double-mutant analyses: Crosses were done to look for interactions of tso1-3 and other ovule mutants. Pistils from heterozygous tso1-3/+ plants were pollinated with stamens from plants homozygous for bd-1 (Robinson-Beer et al. 1992), ino-1 (Baker et al. 1997), ap2-1 (Jofuku et al. 1994), ant5 (Baker et al. 1997), lug1 (Liu and Meyerowitz 1995), flo-10 (Schultz et al. 1991) [also designated sup-2 (Bowman et al. 1992)], or sin-12 (Ray et al. 1996). As expected for these recessive mutations, all F1 progeny were phenotypically wild-type, and F2 seeds were collected from individual F1 plants. Double mutants were identified through phenotypic screening of segregating F2 progeny using a dissecting microscope. Double-mutant identification was facilitated by the floral phenotypes, which are diagnostic of ap2, ant5, lug1, sup-2, and tso1-3 mutants, and the vegetative phenotype of sin-12. The tight genetic linkage between tso1 and sup necessitated a screen for a crossover event between these two loci to enable generation of a tso1 sup double mutant line. Because the sup-2 allele is in the Columbia ecotype and tso1-3 is in the Ler ecotype, we were able to use a CAPS marker at the sup locus (H. Sakai, personal communication) to differentiate the wild-type and mutant alleles. The tso1-3 sup-2 double mutant was identified from among the progeny of an F2 plant that exhibited the tso1-3+ phenotype and that was heterozygous at the sup locus (indicating a single crossover event linking mutant alleles of the two loci).

RESULTS

Isolation of novel tso1 alleles: Two ovule mutants with similar phenotypes were identified in screens for mutants with reduced female fertility. The mutants were initially designated pretty few seeds-1 (pfs-1) and pfs-2. Because of the extreme reduction in female fertility of both mutants, known pfs-1/+ heterozygotes were used as female parents in crosses with pollen from homozygous pfs-2 plants to test for allelism. F1 progeny from these crosses segregated 15 wild:18 mutant, consistent with the 1:1 ratio expected if the two mutants were allelic ($\chi^2 = 0.27$, $P = 0.60$). The pfs locus was mapped to the middle of chromosome 3 (see materials and methods), 16.6 cm south of nga162 and 4.5 cm north of g4711.

Complementation tests were done with other ovule mutants and with floral mutants mapping near pfs. In the case of the tso1 mutation, which mapped to the same region of chromosome 3 as pfs ([Liu et al. 1997] and this work), tso1-1/+ heterozygotes were crossed with pollen from pfs-1 homozygotes. F1 progeny from three independent crosses consisted of 16 mutant and 13 wild-type plants. This failure to complement fit the 1:1 segregation ratio ($\chi^2 = 0.31$, $P = 0.58$) expected for allelic mutations. To confirm this result, a putative heteroallelic tso1-1/pfs-1 plant in the F1 generation was crossed to a wild-type Ler plant to produce the F1′ generation. On the basis of the segregation of the F2′ plants, each of the five F1′ plants was found to be heterozygous for either pfs or tso1-1, confirming that the parental heteroallelic plant contained both pfs-1 and tso1-1. Thus, complementation tests and map position indicated that the pfs mutants are alleles of tso1 (but see also Discussion).
**tsO1-Regulated Ovule Development**

**Figure 1.**—tsO1 alleles differentially affect floral structure. All parts show representative scanning electron micrographs of flowers at anthesis. (A) Wild-type flowers. Bar, 500 μm. (B) Flowers from a heteroallelic tsO1-1/tsO1-3 plant exhibited aberrant sepal margins, serrated petal margins, and twisted pistils, which were only partially fused along the replum. Bar, 500 μm. (C) Homozygous tsO1-3 flowers were similar to wild type, except for defects in sepal tips (which are not obvious at this magnification, but are apparent in Figure 2B). Bar, 500 μm. (D) tsO1-1 mutant flowers had highly reduced, aberrant petals, stamens, and pistils. Bar, 150 μm (note: magnification is over threefold greater than other panels). pe, petal; pi, pistil; se, sepal; st, stamen.

Gross floral morphology of tsO1 mutants: Liu et al. (1997) observed that petals, stamens, and carpels failed to develop in the strong tsO1-1 allele. While we observed somewhat lesser effects of this allele (due to differences in growth conditions or genetic background), these organs were still highly reduced and abnormally shaped (Figure 1D). In striking contrast, petals, stamens, and carpels appeared normal in tsO1-3 mutants (Figure 1C). The only effect of tsO1-3 on gross floral morphology was in the sepals. In wild-type sepals, the tips are made up of small rectangular cells that form a smooth outline (Figure 2A). Other cells on the abaxial surface are irregular in shape (Figure 2A). While the overall size and shape of tsO1-3 sepals were normal, the sepal tips appeared ragged due to the presence of two to four projections (Figure 2B). In addition, cells at the tips of tsO1-3 sepals appeared similar to those on the rest of the abaxial sepal surface (Figure 2B). Sepals of tsO1-1 mutants also lacked the small cells near the sepal margin and exhibited similar projections at the tips (Figure 2D). Effects of tsO1-4 on gross floral morphology were indistinguishable from those of tsO1-3 (data not shown).

Flowers of the tsO1-1/tsO1-3 heteroallelic plants had an intermediate phenotype between the strong tsO1-1 allele and the weak tsO1-3 allele (Figure 1B). tsO1-1/tsO1-3 flowers had sepal margins with elongated protrusions (Figure 2C), petals with serrated margins, stamens that sometimes failed to dehisce (almost always with the short stamens), and bifurcated pistils due to incomplete fusion along the replum (Figure 1B). The mutant phenotype increased acropetally—later-forming flowers exhibited more severe petal serration, male sterility, and incomplete carpel fusion (data not shown).

Effects of tsO1 mutations on ovule development: The rudimentary gynoecium of tsO1-1 flowers described by Liu et al. (1997) did not produce ovules. While we observed somewhat lesser effects of this allele (Figure 1D), ovules were still only rarely observed. In contrast, ovules were present in the complete gynoecium of tsO1-3 and tsO1-4 mutants. Wild-type and tsO1-3 ovules were compared at different stages of development to determine the nature and timing of deviations in ovule development resulting from the mutation. Developing wild-type integuments consisted of regular cuboidal cells, which subsequently elongated in the direction of integument growth (Figure 3A). Elongation was coordinated such that a smooth surface of regular files of cells and a uniform leading margin were observed throughout development (Figure 3, C, E, and G). Development of tsO1-3 integuments initially appeared normal (Figure 3B), but became progressively more aberrant as the integuments grew (Figure 3, D, F, and H). Individual cells had wrinkled walls and were highly variable in shape (Figure 3H). Files of cells were usually discernible, but were not straight. The overall surface of tsO1-3 integuments was markedly rough, and the integument margins were ragged (Figure 3, F and H). Effects of tsO1-3
were most pronounced near the micropyle, where deviations from normal organization commonly produced gaps between adjacent files of cells (Figure 3H). Morphology of tso1-4 ovules was indistinguishable from that of tso1-3 ovules, while ovule morphology was more severely disrupted in tso1-1/tso1-3 heteroallelic plants (data not shown). The small number of ovules we observed in tso1-1 mutants appeared to be even more aberrant than those of tso1-3 and tso1-4. The phenotypes observed in this limited sample varied markedly, preventing firm conclusions concerning tso1-1 effects on ovule development.

The internal anatomy of tso1-3 ovules was examined in sections of plastic-embedded pistils. The most apparent difference from wild-type ovules (Figure 4A) was the absence of an embryo sac in the majority of tso1-3 ovules (Figure 4B). This absence appeared to be due to a block in megaspore genesis in tso1-3 mutants at the stage of megaspore mother cell formation. The megaspore mother cell, which has a large nucleus and densely staining cytoplasm (Figure 4C), was rarely observed in tso1-3 ovules (Figure 4, B and D, and data not shown).

Consistent with our SEM observations, the sections showed reduced elongation of the integument cells of tso1-3 ovules, especially in the region proximal to the micropyle (Figure 4B). Cells of this portion of the wild-type outer integument exhibit the greatest degree of directional expansion (Figure 4A). In addition, there was a 20% decrease in the number of cells in each file of the outer cell layer on the abaxial side of tso1-3 ovules, relative to wild type. This layer consisted of 26.8 ± 3.3 cells in wild-type ovules and 21.3 ± 2.8 cells in tso1-3 ovules (sample size of 10 in both cases). This difference was statistically significant (P = 0.008). However, the failure of tso1-3 ovules to obtain the correct morphology primarily resulted from defects in directional expansion of cells and a failure to coordinate expansion among adjacent cells. Despite these aberrations, the total quantity of integument tissue in tso1-3 ovules appeared similar to that of wild-type ovules at anthesis (Figure 4).

Liu et al. (1997) reported specific cell division defects in flowers of tso1-1 mutants. During division, cell plates often failed to form completely, leaving “cell wall stubs,” which only partially separated two daughter cells. In addition, the nuclei of cells were commonly misshapen. In contrast, in our examination of tso1-3 flowers using light microscopy, incomplete cell walls were rarely observed. The vast majority of cells in tso1-3 ovules formed complete cell plates, but the cells were still misshapen (Figure 4 and data not shown). tso1-3 nuclei appeared normal in these observations. Thus, incomplete cell division is a feature of stronger tso1 mutations and was rarely manifest in weaker alleles.

Effects of tso1 mutations on fertility: Both tso1-3 and tso1-4 exhibited significant reductions in female fertility, with tso1-3 showing the greatest reduction (Table 1). Seeds from both mutants showed normal viability (data not shown). We observed a direct correlation between the number of embryo sacs and the number of seeds produced in the two mutants (Table 1). Thus, the failure in embryo sac formation could completely explain the reduced fertility in these two mutants.

Examination of tso1-3 and tso1-4 pistils stained with decolorized aniline blue, a fluorescent stain for callose (Vishnyakova 1991), showed normal pollen germination on the stigma and growth of pollen tubes through the style and transmitting tract (data not shown). Under these staining conditions, ovules lacking embryo sacs exhibited an accumulation in the nucellus of brightly fluorescent material (Figure 5A), and wild-type ovules showed only weak fluorescence (Figure 5B). These observations were consistent with previous reports showing accumulation of callose in the nucelli of sterile ovules (Vishnyakova 1991). In both tso1-3 and tso1-4, pollen tubes tracked to the microplies of those ovules containing embryo sacs, but failed to do so for ovules lacking callose.
Figure 3.—Morphogenesis of wild-type and tso1-3 mutant ovules observed in scanning electron micrographs. Wild-type ovules are shown in A, C, E, and G, while Tso1-3- ovules are shown in B, D, F, and H. Each part is oriented such that the stigma of the pistil containing the ovules is toward the right. (A) In wild-type ovules at stage 2-III (stages according to Schneitz et al. (1995)), integument primordia have emerged from the chalazal region of the ovule. (B) Tso1-3- ovules at stage 2-III. (C) The integuments in wild-type ovules at stage 3-I expand to enclose the nucellus. (D) Tso1-3- ovules at stage 3-I. Disruptions in the organization of files of cells are visible (arrows). (E) In wild-type ovules at stage 4-I (anthesis), the outer integument has covered the inner integument, and the micropyle is adjacent to the funiculus. (F) Tso1-3- ovules at stage 4-I. Anomalously shaped cells are visible in the integuments. (G) Wild-type ovules at stage 4-I (after pollination). (H) Tso1-3- ovules at stage 4-I (after pollination) had gaps (arrows) among the files of integument cells. Bars in A–D are 20 μm and E–H are 25 μm. f, funiculus; ii, inner integument; m, micropyle; oi, outer integument.

Embryo sacs (Figure 5, Table 1, and data not shown). Thus, fertilization appeared to occur normally in tso1-3 and tso1-4 ovules containing embryo sacs. This finding is consistent with the hypothesis that a viable embryo sac is necessary for pollen tube guidance (Hülskamp et al. 1995; Ray et al. 1997).

tso1 mutant seed morphology: In contrast to the elliptical shape of wild-type seeds (Figure 6A), mature tso1-3 and tso1-4 seeds were roughly spherical (Figure 6, B and C). During seed maturation, the integument cells in fertilized wild-type ovules expand four-to-sixfold, with almost no cell divisions. Much of this expansion occurs prior to embryo expansion and is, therefore, an independent process of the seed coat, rather than a simple passive stretching to accommodate the growing embryo (Robinson-Beers et al. 1992). On the basis of the size and overall morphology of tso1-3 and tso1-4 seeds, this process appears to occur relatively normally in both alleles. Thus, directional cellular expansion is largely normal during seed development from fertile tso1-3 and tso1-4 ovules.

Cells on the outer surface of wild-type seeds are desiccated, and the anticlinal walls of these cells are visible as raised polygonal structures (Figure 6A). Central elevations in each cell, the columellae (Figure 6A), are sites of mucilage accumulation (Koornneef 1981).
While the surface features of tso1-4 seeds (Figure 6B) were like those of wild type (Figure 6A), in tso1-3 seeds the columellae were absent and the raised border of each cell was less defined (Figure 6C).

Staining of hydrated seeds with toluidine blue or ruthenium red revealed a large halo of mucilage around wild-type and tso1-4 seeds (data not shown). No mucilage was detected in tso1-3 seeds (data not shown), indicating that the absence of columellae resulted from an absence of mucilage, rather than from a failure of the mucilage to concentrate at the center of each cell.

**Interactions of tso1-3 with other ovule mutants:** To learn more about the nature of T501 and its interactions with other genes regulating ovule development, double mutant lines were generated and characterized. tso1-3 was used for all such experiments because it was the stronger of the two alleles producing significant numbers of ovules. For each of the double mutants, the observed segregation ratios were similar to expected segregation ratios (Table 2).

ant-5 tso1-3: Integument development ceases in strong anteguments (ant) mutants just after the formation of a rudimentary ridge of cells near the chalaza (Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997). ant-5 tso1-3 double mutants were identified in populations segregating for both mutations as those plants with both the characteristic narrow Ant petals (Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997) and Tso1-3-3 sepals. Ovules of ant-5 tso1-3 mutants (Figure 7B) were indistinguishable from those of ant-5 mutants (Figure 7A). Thus, with respect to ovule development, ant-5 was epistatic to tso1-3.

bel1-1 tso1-3: In place of integuments, ovules of bel1 (bel1) mutants form a single, relatively amorphous collar of tissue (the “integument-like structure,” or ILS), which is made up of small, relatively isodiametric cells (Figure

**TABLE 1**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Seeds per silique</th>
<th>Embryo sacs per silique</th>
<th>Pollen tubes per silique</th>
<th>Ovules per silique</th>
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<tr>
<td>tso1-3</td>
<td>0.36 ± 0.48 (n = 72)</td>
<td>0.4 ± 0.5 (n = 5)</td>
<td>0.3 ± 0.5 (n = 10)</td>
<td>36.4 ± 3.0 (n = 10)</td>
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<tr>
<td>tso1-4</td>
<td>2.7 ± 1.4 (n = 30)</td>
<td>3.0 ± 0.7 (n = 5)</td>
<td>2.1 ± 0.6 (n = 10)</td>
<td>38.1 ± 3.1 (n = 10)</td>
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<tr>
<td>Wild type</td>
<td>35.1 ± 4.1 (n = 30)</td>
<td>44.8 ± 3.7 (n = 5)</td>
<td>40.4 ± 3.8 (n = 5)</td>
<td>42.2 ± 4.5 (n = 10)</td>
</tr>
</tbody>
</table>

* Pollen tubes that entered the micropyles of ovules.
Figure 5.—Pollen tubes in wild-type plants and tso1-3 mutants. Pollen tubes were visualized by epifluorescence in pistil squashes stained with decolorized aniline blue. (A) In Tso1-3 mutant pistils, the pollen tubes were present throughout the transmitting tissue, but did not track to sterile ovules. (B) In wild-type pistils, pollen tubes emerged from the transmitting tissue and directly tracked along the funiculi to enter the micropyles of the ovules. aes, aborted embryo sac; es, embryo sac; m, micropyle; pt, pollen tube; tt, transmitting tissue; vt, vascular trace.

7C; Robinson-Beers et al. 1992; Modrusan et al. 1994). An ILS was formed in bel1-1 tso1-3 double mutants, but it consisted of cells that were more variable in shape than those of the bel1-1 single mutant ILS (Figure 7D), indicating an additive effect of these two mutations.

sin1-2 tso1-3: Integuments of short integument1 (sin1) mutants are short because cells of these structures fail to undergo normal directional elongation parallel to the nucellar axis (Figure 7E; Robinson-Beers et al. 1992; Lang et al. 1994). Despite the lack of expansion, integument cells in sin1 mutants were regular in shape and were arranged in neat files (Figure 7E). In contrast, corresponding cells in sin1-2 tso1-3 double mutants were irregular in shape and the organization of the files of cells was highly disrupted (Figure 7F). The integument cells in the sin1 tso1-3 double mutant did expand, but expansion was not constrained to a single direction. sin1 plants are short stunted in the erecta background (Robinson-Beers et al. 1992; Lang et al. 1994). The vegetative phenotype of the double mutants was like that of a sin1 plant. Thus, the effects of sin1-2 and tso1-3 were largely additive.

ino-1 tso1-3: In inner no outer (ino) mutants, the outer integument primordium initiates in an anomalous location on the adaxial side of the chalaza (Baker et al. 1997) and development of this structure ceases prematurely (Figure 7G; Baker et al. 1997; Schneitz et al. 1997).

Figure 6.—Scanning electron micrographs of wild-type and Tso1-4 seeds. (A) Wild-type seeds were elliptical and had a raised ridge, visible at the top. Polygonal surface features are anticlinal walls of desiccated testa cells. The central elevation in each cell is the columella. (B) Tso1-4 seeds were round and had well-defined testa cell walls and columnellae. The seed is viewed from above the raised ridge (rotated 90° relative to the seed in (A)). (C) Tso1-3 seeds were round, lacked columnellae, and had less pronounced testa cell walls. This seed is orientated like the seed in (A). Bar corresponds to 100 μm in all parts. c, columella.
The inner integuments of ino mutants appeared to be similar to those of wild-type ovules, except for differences resulting from absence of outer integuments (Baker et al. 1997). Outer integument primordia of ino-1 tso1-3 double mutants exhibited the same misorientation observed in ino single mutants, but underwent less subsequent development (Figure 7H). Inner integuments of the double mutants exhibited one of two different developmental aberrations. The majority of double-mutant ovules had inner integuments that consisted of abnormally shaped cells and failed to completely sheathe the nucellus (Figure 7H). This appeared to be primarily due to reduced expansion of inner integument cells in the direction parallel to the nucellar axis (Figure 7H). Approximately 30% of ovules exhibited hyperproliferation of the inner integument in addition to aberrant cellular expansion in this structure (Figure 7H). The frequency of this second phenotype was higher among stage 4-II ovules than among stage 4-I ovules (stages from Schneitz et al. 1995), indicating that some of the ovules with reduced inner integuments would later undergo hypertrophy. The ino-1 tso1-3 double mutant shows that tso1-3 affects directional cell expansion in the inner integument, independent of the presence of an outer integument. An apparently stochastic process determines whether this aberration will manifest as reduced development or excessive proliferation.

Sup-2 tso1-3: Flowers of sup plants have supernumerary stamens and a corresponding reduction in the amount of carpel tissue (Schultz et al. 1991; Bowman et al. 1992). In addition, the outer integument grows equally on all sides of a sup ovule, resulting in a long tubular structure with a well-defined micropyle (Figure 7I; Gaiser et al. 1995). Ovules of sup-2 tso1-3 plants also had tubular integuments, but the cells around the micropyle were misshapen, causing the edges surrounding this opening to appear ragged (Figure 7J). The portion of the integument distal to the micropyle consisted of well-organized files of cells elongated in the direction of the nucellar axis and was thus similar to this same region in sup-2 single mutants (Figure 7I and J). One possible explanation for this pattern of effects is that the sup-2 mutation leads to specific enlargement of the basal region of the outer integument—the part of the integument least affected by the tso1-3 mutation. The effects of these mutations can be seen as being additive.

Ap2-1 tso1-3: Seeds of apetala2-1 (ap2-1) mutant plants differ in shape from wild-type seeds, have little or no mucilage, and fail to form the characteristic central elevations (columellae) on their testa cells (Jofuku et al. 1994; Leon-Kloosterziel et al. 1994). In the ap2-1 mutant these differences appeared to be primarily due to developmental alterations following fertilization, because ap2-1 ovules exhibit only minor aberrations in cell shape near the micropyle (Figure 7K). In ap2-1 tso1-3 double mutants, however, the cells surrounding the micropyle were more disorganized than in tso1-3 single mutants, and large invaginations formed between cell files in this region (Figure 7L). This demonstrated that ap2-1 affects ovule development prior to fertilization and that this mutation can exacerbate the effects of tso1-3 on cell shape and organization near the micropyle.

Lug1 tso1-3: leunig (lug) mutations were identified by their ability to enhance the effects of ap2 mutations on

### TABLE 2

Double-mutant analysis with tso1-3

<table>
<thead>
<tr>
<th>Mutant</th>
<th>F2 segregation</th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
<th>P</th>
<th>Double-mutant description</th>
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<td>ant-5</td>
<td></td>
<td>119:41:36:6</td>
<td>9:3:3:1</td>
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<td>0.25</td>
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<td>ap2-1</td>
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<td>11:4:3:1</td>
<td>9:3:3:1</td>
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<td>0.98</td>
<td>Tso1- Ap2- ovule Ap2- flower</td>
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<tr>
<td>bd-1</td>
<td></td>
<td>47:47:16:3</td>
<td>9:3:3:1</td>
<td>1.1</td>
<td>0.78</td>
<td>Tso1- Bel1- ovule Tso1- sepal</td>
</tr>
<tr>
<td>ino-1</td>
<td></td>
<td>88:33:30:10</td>
<td>9:3:3:1</td>
<td>0.34</td>
<td>0.95</td>
<td>Tso1- Ino1- ovule Tso1- sepal</td>
</tr>
<tr>
<td>lug-1</td>
<td></td>
<td>37:8:11:2</td>
<td>9:3:3:1</td>
<td>1.4</td>
<td>0.70</td>
<td>Tso1- Lug1- ovule Tso1- sepal</td>
</tr>
<tr>
<td>sin-2</td>
<td></td>
<td>40:10:9:3</td>
<td>12:4:3:1</td>
<td>1.7</td>
<td>0.63</td>
<td>Tso1- Sin1- ovule Sin1- vegetative organs Tso1- sepal</td>
</tr>
<tr>
<td>sup-2</td>
<td>(fl110-1)</td>
<td>22:7</td>
<td>3:1</td>
<td>0.11</td>
<td>0.91</td>
<td>Tso1- Sup-1 ovule Sup- floral phenotype Tso1- sepal</td>
</tr>
</tbody>
</table>

*a* Wild type.

*b* sin1 mutants segregate $\sim 4:1$ (Robinson-Beers et al. 1992).
Figure 7.—Double-mutant analysis of tso1-3 with other ovule mutations. Scanning electron micrographs of single and double mutants are shown from flowers at anthesis. (A) Ant5- ovule. Integument development ceased after a single integumentary ridge formed. (B) Ant5- Tso1-3- ovules were indistinguishable from of ant5 single mutants. (C) Bel1-1- ovules had ILS in place of integuments. (D) Bel1-1- Tso1-3- ovules. Cells of the ILS are more variable in shape than those of bel1-1 single mutants. (E) The integuments in Sin1-2- ovules did not cover the nucellus as a result of reduced cell expansion along the nucellar axis. (F) Sin1-2- Tso1-3- integuments were short and were made up of cells of variable shape and size. (G) Ino-1- ovules have only rudimentary outer integuments. (H) Ino-1- Tso1-3- ovules exhibited an even greater reduction in outer integument development than Ino-1- ovules and usually also had reduced inner integument length. A subset of the Ino-1- Tso1-3- ovules had extensive hypertrophy of the inner integument (arrows). (I) Sup2- ovules. The outer integuments of Sup- ovules grew equally on the abaxial and adaxial sides of the ovule, resulting in a long tubular structure. (J) Sup2- Tso1-3- ovules exhibited an overall shape similar to that of Sup- ovules, but large gaps formed between the files of aberrantly shaped cells near the micropyle. (K) Ap2-1- ovules exhibited altered cell shapes near the micropyle. (L) Ap2-1- Tso1-3- ovules. Cells surrounding the micropyle were highly aberrant. (M) Lug1-1- ovules have protruding inner and recessed outer integuments. (N) Lug1-1- Tso1-3- ovules had highly reduced integumentary structures. Cells of these structures were variable in shape and size and were often organized into multiple separate lobes. f, funiculus; ii, inner integument; ils, integument-like structure; ir, integumentary ridge; m, micropyle; n, nucellus; oi, outer integument.
flower development (Liu and Meyerowitz 1995). lug-1 ovules have protruding inner integuments and slightly recessed outer integuments (Figure 7M; Roe et al. 1997). lug-1 tsol-3 double mutants formed highly reduced integuments with altered morphology. The integuments consisted of fewer cells than in either single mutant, and the inner integuments were commonly replaced by several separate projections around the nucellus (Figure 7N). In addition, the onset of the tsol-3 cellular phenotype (anomalous shape and expansion of ovule cells) occurs just after integument initiation in the lug-1 tsol-3 double mutant, as opposed to just before micropyle formation in the tsol-3 single mutant (data not shown). Thus, the lug-1 tsol-3 double mutant revealed a synergistic interaction between these two mutations and showed that the tsol-3 mutation can affect ovule development earlier than indicated by the phenotype of the single mutant.

**DISCUSSION**

**Allelism to tsol:** The two new mutations described in this work produced similar phenotypes and were shown to be allelic. These mutations also mapped to the same location as tsol and failed to complement tsol-1. These observations satisfy the criteria most commonly used to conclude that these mutations are allelic. Because of the significant differences in phenotypes produced by tsol-1 and the new mutations described here, we have also considered the possibility that the failure to complement could represent an example of nonallelic noncomplementation. While examples of this have been documented [e.g., clavata1 and clavata3 (Clark et al. 1995)], there is no precedent in plants for two fully recessive loci to produce a strong phenotype in a doubly heterozygous plant. In addition, a floral phenotype intermediate between that of the strong tsol-1 mutants and the weak tsol-3 mutant was observed. This result is precisely what would be expected for a heteroallelic state. While we are unable to prove that these mutations are not in two different loci, it is difficult to reconcile the intermediate phenotype with models for nonallelic noncomplementation in doubly heterozygous plants. Thus, it seems most likely that these mutations are allelic, and for this reason allelism has been assumed throughout this report.

**Effects of tsol mutations:** While the previously described tsol-1 mutant exhibited severe distortion of all floral organs (Liu et al. 1997), effects of homozygous tsol-3 or tsol-4 mutations were restricted to ovules and the tips of sepals (Figures 1 and 2). Aberrations in all floral whorls were, however, observed in tsol-1/tsol-3 heteroallelic plants (Figure 1B). These results demonstrate that effects of the tsol-3 mutation were not confined to ovules and sepals, but effects on other floral organs were visible only in combination with tsol-1. A likely explanation for these results is that the tsol-3 mutation leads to a partial loss of TSOL1 activity and that this loss of activity differentially affects floral organs. Sepal margins and ovules appeared to be most sensitive to reductions in TSOL1 activity, followed by petal margins and pistils, while sepal blades and stamens were least sensitive. TSOL1 activity would be reduced in all floral organs in tsol-3 mutants, but would fall below the threshold necessary for normal development only in the tips of sepals and in ovules. Because tsol-1 is a more severe mutation than tsol-3, there would be a lower level of TSOL1 activity in tsol-1/tsol-3 plants, which would fall slightly below this threshold in all floral organs. The level in tsol-1 homozygotes would be well below the threshold throughout flowers. The differential sensitivity to tsol mutations seen in distinct zones within flowers could be due either to differential basal expression of TSOL1 or to different levels of activity being required for normal development in the different structures.

The tsol-1 mutation caused severe effects on cytokinesis and karyokinesis in floral organs, resulting in increased cell size, decreased cell number, incomplete cell plate formation, elevated DNA content, and misorientation of the spindle apparatus (Liu et al. 1997). On the basis of these observations, Liu et al. (1997) hypothesized that TSOL1 could function in cytoskeletal structure, regulation of cytoskeletal function, cell wall formation, or in cell cycle control. The effects of tsol-3 were much less severe. The numbers of cells in tsol-3 ovules were only slightly reduced when compared to wild-type plants, and incomplete cell wall formation was rarely observed. The primary effects of tsol-3 were to disrupt directional elongation of ovule cells and coordination of cell enlargement among adjacent cells (Figure 3). Thus, tsol-3 mutants have significant morphological aberrations even though they do not exhibit incomplete wall formation or failure in cell division. This indicates that defects in cell division processes cannot be the primary effects of tsol mutations. Of the previously proposed mechanisms for the nature of TSOL1, only those relating to a role for TSOL1 in mediating a variety of positional or directional events within cells can explain the phenotypes of all tsol mutants. The cytoskeleton is a structure involved in all such events in plant cells (Kropf et al. 1998). Defects in the assembly, structure, or function of cytoskeletal elements could lead to inappropriate cell expansion and misshapen cells [due to misplacement of cell wall components or factors mediating cell wall extension (Cosgrove 1997)] and, in more extreme manifestations, could also lead to incomplete wall formation and a failure in karyokinesis. Thus, we hypothesize that TSOL1 is a critical component of cytoskeletal function in flowers.

While effects of known tsol mutations were confined to flowers, the activities proposed for TSOL1 would be necessary components of morphogenesis in all parts of plants. There are several possible explanations for the absence of effects of these mutations outside of flowers.
Liu et al. (1997) hypothesized that there maybe a redundant activity to perform this function in vegetative parts of plants or that TSO1 actually mediates a floral-specific process. Our results are consistent with these hypotheses, but our observation that effects of different tso1 alleles can be spatially confined also suggests another possibility. We hypothesized that tso1-3 and tso1-4 affected only ovules and sepals because they cause only a partial loss of function and that this loss is sufficient only to affect these most sensitive structures. It may be that tso1-1 is also a partial loss-of-function mutation and that flowers are generally more sensitive to a decrease in TSO1 activity than are vegetative parts of plants. If this hypothesis is correct, then complete loss-of-function alleles could be lethal and would not have been detected in the screens used to identify current alleles. Discrimination among these hypotheses may be possible when sequencing of tso1-1 allows determination of the nature of the lesion in this allele.

The size and overall morphology of rare tso1-3 and tso1-4 seeds indicate that the integuments in these seeds expanded relatively normally following fertilization (Figure 6). The mutant seeds must have derived from ovules with viable embryo sacs, and it is possible that integument development may be more similar to wild type in this subset of ovules. Alternatively, it may be that cell expansion after fertilization is less affected by reductions in TSO1 activity than are earlier stages of ovule development.

Columellae are largely absent from tso1-3 seeds, but were present on tso1-4 seeds. This indicates that TSO1 activity is still required for some aspects of postfertilization seed development. That columellae formation is not affected in the weakest (tso1-4) allele indicates that this process is less sensitive to a decrease in TSO1 activity than is integument development. Our inability to detect mucilage on tso1-3 seeds indicates that this mutation causes a failure of mucilage accumulation, rather than a failure of mucilage localization to the centers of the cells. While this could indicate a separate effect of tso1 mutations on a particular aspect of cellular metabolism, it is also possible that the failure to accumulate mucilage is a secondary effect of defects in directional movement of substances to the cell wall.

Double-mutant analyses: Analysis of genetic interactions of the tso1 mutation with previously characterized ovule mutants has allowed us to further define the role of TSO1 in ovule development. While it is usually best to use a null mutant in such studies, the common absence of ovules in the strongest tso1 allele precluded its use in genetic interaction studies on ovule development. For this reason, all such studies were performed with tso1-3.

In several cases the phenotypic effects of the double mutants on ovule development were consistent with what would be predicted from the effects of the two single mutants. With respect to ovule development, ant-5 was epistatic to tso1-3, apparently as a result of the ant mutation preventing formation of integuments and embryo sacs—the primary sites of visible tso1-3 effects. There was apparent simple additivity between the effects of bel-1 and tso1-3. ap2-1 showed additive effects with tso1-3, resulting in a micropylar region that was even more irregular than those in tso1-3 single mutants. sup-2 and tso1-3 were also simply additive, but the double mutant provided some additional information about both mutants. The fact that tso1-3 effects were confined to the region of the double-mutant ovules near the micropyle shows that sup primarily acts to expand the part of the outer integument that is proximal to the funiculus and emphasizes the fact that tso1-3 effects were most severe in the parts of the integment proximal to the micropyle. The region proximal to the micropyle is also where integument cells exhibit the greatest asymmetric expansion.

The majority of ovules of ino1 tso1-3 double mutants exhibited a simple additive phenotype where the outer integument was absent and the cells of the exposed inner integument were irregular. The observation that a subset of such ovules exhibited a novel phenotype where significant hypertrophy of the inner integument occurred is more surprising. The capacity of the inner integment to undergo hypertrophy was previously shown by the phenotype of the sin1 mutant in an ERECTA background, where this effect was also seen in only a subset of ovules (Lang et al. 1994). The hypertrophy in the ino1 tso1-3 double mutant indicates that a combination of the presence of an outer integument and some directional processes normally under control of TSO1 may be necessary for cessation of inner integument growth.

The number and arrangement of integument cells in the sin1 mutant is the same as those in wild type, and the phenotype is derived solely from a failure of cells to elongate to enclose the nucellus (Robinson-Beers et al. 1992). In contrast, the sin1-2 tso1-3 double mutant exhibited decreased and less organized cell expansion, and also a reduction in cell number. Thus, while the two mutations were largely additive, the phenotypic effects of tso1-3 were exacerbated, leading to a reduction in cell division.

The lug mutation produces a complex phenotype, which includes narrow leaves and floral organs as a result of a reduction in longitudinal expansion or cell divisions (Liu and Meyerowitz 1995). The allocation of tissue into the inner and outer integuments is altered in lug ovules, resulting in a recessed outer and protruding inner integument (Roe et al. 1997). lug-1 exhibited a strong synergistic interaction with tso1-3, leading to ovules with highly reduced integuments with variable and aberrant morphology (Figure 7N). The narrow organ phenotype of lug mutants suggests that LUG, like TSO1, may regulate directional expansion and cell division. Thus, the effects of these two mutations on direc-
tional processes could combine to produce cellular aberrations, which were more extreme than either single mutant and resemble cellular effects of strong tso1 mutants. These effects would be most visible in the structures most sensitive to reductions in TSO1 activity—the ovules. The synergistic effects could also explain the observation that deviations from normal ovule development appear to occur at earlier stages of this process in the double mutant than in either single mutant.

Analyses of the tso1 mutants indicate that these mutations not only affect cell division but also disrupt directional cell expansion and coordination of growth among meristematic cells in flowers. These results highlight the importance of regulation of both cell division and cell expansion in plant morphogenesis. That these mutations affect such a variety of directional processes in cells has led us to hypothesize a role for TSO1 in cytoskeletal function. Further studies on this gene and its product may aid in determination of which components of the cytoskeleton govern different aspects of movement and placement of cellular materials.

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