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Tryptophan deficiency affects organ growth by retarding cell expansion in Arabidopsis

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Summary

Tryptophan (Trp) is an essential amino acid required not only for protein synthesis but also for the production of many plant metabolites, including the hormone auxin. Mutations that disrupt Trp biosynthesis result in various developmental defects in plant organs, but how Trp affects organ growth and development remains unclear. Here, we identify an Arabidopsis mutant, *small organ1* (*smo1/trp2-301*), which exhibits a reduction in the size of its aerial organs as a result of the retardation of growth by cell expansion, rather than by the retardation of growth by cell proliferation. *smo1/trp2-301* contains a lesion in *TSB1* that encodes a predominantly expressed Trp synthase β-subunit, and is allelic with *trp2* mutants. Further analyses show that in *trp2* leaf cells, the nuclear endoreduplication is impaired and chloroplast development is delayed. Furthermore, cell expansion and leaf growth in *trp2* can be restored by the exogenous application of Trp, but not by auxin, and the general protein synthesis is not apparently affected in *trp2* mutants. Our findings suggest that the deficiency in Trp or its derivatives is a growth-limiting factor for cell expansion during plant organogenesis.

Keywords: *Arabidopsis thaliana*, tryptophan, *trp2*, cell expansion, organ growth.

Introduction

During organogenesis, aerial organs in higher plants develop from primordia initiated from the shoot apical meristem (SAM). The growth of an organ to its specific size depends on both the number and the size of its cells, which are the consequence of cell proliferation and expansion: two successive processes referred to as growth by cell proliferation and growth by expansion, respectively (Anastasiou *et al.*, 2007; Mizukami, 2001; Tsukaya, 2006). Recent genetic studies have identified several factors that control either cell proliferation or expansion, and have begun to uncover the molecular basis of organ growth control (Anastasiou and Lenhard, 2007). When an organ undergoes growth by proliferation, a fundamental event is the duration of cell proliferation, which generates a sufficient number of cells to support further growth, and finally determines the overall size of the organ (Mizukami, 2001). This process is modulated by several factors, including AINTEGUMENTA (ANT), ARGOS, JAGGED/NUBBIN (JAG/NUB), BIG BROTHER (BB) and KLUH (Anastasiou *et al.*, 2007; Disch *et al.*, 2006; Hu *et al.*, 2003; Mizukami and Fischer, 2000; Ohno *et al.*, 2004), which function in a few genetic pathways to either maintain or terminate the meristematic competence of organ cells, thereby affecting the duration of proliferation and the final cell number (Anastasiou and Lenhard, 2007). In contrast to growth by proliferation, growth by expansion of an organ appears to be more complicated (Tsukaya, 2006). For example, in Arabidopsis, the size of the aerial organs, most typically the leaf, is determined not only by polar cell expansion, which has been demonstrated by the characterization of ANGUSTIFOLIA (AN) and ROTUNDIFOLIA3 (ROT3) (Tsuge *et al.*, 1996), but also by general cell expansion, as illustrated by the identification of ARGOS-LIKE (ARL) and BRASSINOSTEROID-INSENSITIVE4 (BIN4) (Breuer *et al.*, 2007; Hu *et al.*, 2006). Furthermore, although the molecular

basis is still unclear, the final size of cells within an organ is often, but not always, correlated with the ploidy level resulting from the nuclear endoreduplication, which occurs when cells undergo differentiation and expansion, and has been found to be an important factor in determining cell size (Sugimoto-Shirasu and Roberts, 2003; Tsukaya, 2008).

The growth of plant organs is not only defined by genetic program, but is also greatly affected by other factors, such as light, temperature, and nutrition, as well as primary and secondary metabolites, including hormones. These signals should mediate developmental progression through regulating cell proliferation and/or expansion, and thus control the organ size and final plant architecture. Several plant hormones, such as auxin, brassinosteroids (BRs), ethylene and gibberellin, have been found to play critical roles in mediating organ growth (Mizukami, 2001; Tsukaya, 2002). For instance, auxin may control the growth by proliferation through ARF2 and ARGOS, and the growth by expansion through ABP1 (Hu *et al.*, 2003; Jones *et al.*, 1998; Schruff *et al.*, 2006). BR may influence the cell expansion through ROT3, ARL and BIN4 during leaf development (Breuer *et al.*, 2007; Hu *et al.*, 2006; Kim *et al.*, 1998). In addition, plant metabolites are also critical for plant organ development. Deficiencies in primary or secondary metabolites, such as fatty acids or amino acids, also disrupt organ developmental progression, often resulting in distorted organ growth and development (Last and Fink, 1988; Mou *et al.*, 2000).

Tryptophan (Trp) is an essential amino acid for both prokaryotes and eukaryotes, but animals and some eubacteria are unable to synthesize Trp, and must obtain it from plants and microorganisms. Using biochemical and molecular approaches, the Trp biosynthetic pathway has been well established in bacteria, yeast and plants, and almost all catalytic enzymes and their encoding genes have been identified in both microorganisms and plants (Braus, 1991; Radwanski and Last, 1995; Yanofsky, 2003). Trp synthase, an enzymatic complex formed by α- and β-subunits, is responsible for the last step of Trp biosynthesis that converts indole-3-glycerol phosphate to Trp. Two homologous genes encoding the Trp synthase α-subunit (*TSA1* and *INS*), and at least two for the β-subunit (*TSB1* and *TSB2*), have been identified in Arabidopsis (Last *et al.*, 1991; Zhang *et al.*, 2008). Trp is not only essential for protein synthesis, but is also a very important precursor for many secondary metabolites critical for developmental processes and environmental responses. In animals, Trp is required for producing many compounds, including the neurohormone serotonin and the vitamin nicotinic acid. Plants also use Trp as a precursor to synthesize auxin, phytoalexins, glucosinolates and indole- and anthranilate-derived alkaloids, which play direct roles in regulating plant development, pathogen defense response and plant–insect interaction (Radwanski and Last, 1995; Woodward and Bartel, 2005). All mutants defective in Trp biosynthetic pathways, including *trp3* and *trp2*, exhibit developmental defects under normal growth conditions, such as small plants with pale-green leaves and dark veins (Last *et al.*, 1991; Radwanski *et al.*, 1996). Mutations in these genes also affect root waving (Rutherford *et al.*, 1998). Nevertheless, little has been reported about how Trp affects growth and development at the cellular or molecular level.

To gain insight into how growth by cell proliferation and/or expansion is controlled during organogenesis, we performed genetic screens to isolate mutants with altered leaf size, and other altered organs, in Arabidopsis. Here, we describe a mutant, *small organ1* (*smo1/trp2-301*), in which cell expansion is greatly impaired, but cell proliferation is not affected, in aerial organs. Position cloning reveals that *smo1/trp2-301* contains a mutation in the *TSB1* gene. Our further analyses of *smo1/trp2-301* and other *trp2* alleles indicate that Trp is required for cell expansion during organ development.

Results

Aerial organ growth defects in smo1/trp2-301

Small organ1 (*smo1*, but also designated as *trp2-301*, see below) was initially identified by screening a collection of transgenic Arabidopsis plants harboring T-DNA insertions in their genomes with an apparent reduction in leaf size. Further genetic analysis indicated that the phenotype of *smo1/trp2-301* did not co-segregate with the T-DNA insertion (data not shown). Back-crossed *smo1/trp2-301* without harbored T-DNA was further characterized. Grown in a glasshouse, *smo1/trp2-301* seedlings displayed obviously small and chlorotic cotyledons with dark veins (Figure 1a), and this phenotype was further observed in *smo1/trp2-301* leaves in almost all developmental stages, although the chlorotic phenotype was partially restored in leaves at a later stage (Figure 1b and Figure S1). The significant difference between wild-type (WT) and *smo1/trp2-301* plants was the size of the aerial organs. The areas of fully expanded cotyledons and fifth leaves in *smo1/trp2-301* were about 70% and 40% of those in the WT, respectively (Figure 1c), and the size of other aerial organs, including stems, flowers and siliques, was also reduced to some extent, resulting in smaller plants compared with WT ones (Table 1). However, root development did not appear to be affected in *smo1/trp2-301*. The primary root length and lateral root initiates of *smo1/trp2*-301 and WT seedlings were almost the same. In addition, no apparent difference was observed in the hypocotyls between de-etiolated *smo1/trp2-301* and WT seedlings (Table 1). These observations demonstrate that the mutation in *SMO1* mainly results in the retardation of aerial organ development.

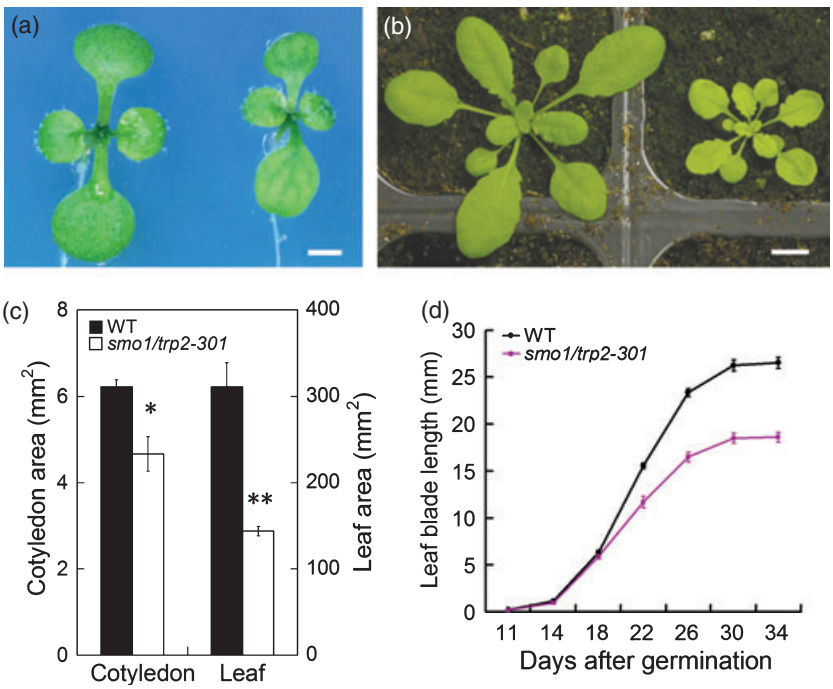

Figure 1. Morphological characterization of *smo1/trp2-301*.
(a, b) *Arabidopsis thaliana* Columbia-0 wild type (WT) (left) and *smo1/trp2-301* (right) plants: (a) 9-day-old seedlings; (b) 3-week-old plants. Scale bars: 1 mm in (a); 10 mm in (b).
(c) Area of fully expanded cotyledons and fifth leaves in WT and *smo1/trp2-301* plants. Cotyledons and leaves from at least 10 plants of each genotype were measured: average values ± SD are shown; Students' *t*-test, $*P < 0.05$, $**P < 0.01$.
(d) Growth rate of the fifth leaf of WT and *smo1/trp2-301* plants. The leaf blade length was measured from at least 10 plants for each genotype in 4-day intervals after emergence. Average values ± SE are plotted.

Table 1 Phenotype of *trp2* plants.[a]
50-day-old plants were used for the measurement of plant height and silique length, and 7-day-old seedlings were used for examining root length and lateral root initiates under a microscope. Start of anthesis of plants indicates the days from the seed germinating to the main stem bolting.

Measurements	Wild type	*smo1/trp2-301*	*trp2-8*	*trp2-1*
Plant height (cm)	32.5 ± 3	26.5 ± 2.6	23.5 ± 2.1	15.8 ± 2.7
Start of anthesis (day)	29.8 ± 0.5	30.2 ± 0.7	30.7 ± 0.6	29.7 ± 1.1
Silique length (mm)	15.9 ± 0.4	14.9 ± 0.4	13.3 ± 0.43	10.5 ± 0.24
Hypocotyl length (mm)[b]	14.6 ± 1.2	14.2 ± 1.4	14.6 ± 1.6	14.1 ± 1.2
Primary root length (mm)	22.9 ± 1.3	22.3 ± 1.3	23.5 ± 1.6	23.1 ± 1.4
Lateral root initiates	41 ± 4.2	41 ± 5.6	43 ± 3.6	40 ± 4.4

[a]At least 10 plants for each genotype were measured, and the data are shown as an average ± SD.
[b]The 5-day-old seedlings were grown in the dark.

The reduced size of smo1/trp2-301 *leaves results from a change in cell size rather than cell number*

As the most dramatic phenotypic alteration in *smo1/trp2-301* plants was found in the leaves, and the leaf has been found to be a good model to characterize organ morphogenesis (Tsukaya, 2003, 2008), we used the leaf as a representative organ to further investigate the effects of *SMO1* on organ development. Analysis of the growth kinetics of fifth leaves revealed that the size difference between *smo1/trp2-301* and WT occurred at comparatively late growing stages (Figure 1d), implying that the retarded leaf growth in *smo1/trp2-301* may result from an alteration in cell expansion and/or the duration of cell proliferation. To assess the contribution of cell proliferation and cell expansion to the smaller *smo1/trp2-301* leaf, we first compared the adaxial epidermal cells of WT fully expanded fifth leaves with those of *smo1/trp2-301*: obviously smaller epidermal pavement cells were observed in *smo1/trp2-301* (Figure 2a,b). We then cut thin sections in the leaf-width direction to compare leaf mesophyll cells, and a significant size difference was found in both palisade and spongy cells between the two genotypes (Figure 2c,d). The average area of palisade cells in *smo1/trp2-301* was dramatically decreased to ~30% of that found in the WT (Figure 2e), whereas the total mesophyll number counted with sections across a half-leaf (from the midvein to the leaf edge) remained almost unchanged between the two genotypes (Figure 2f). Consistent with the reduction in cell size, the volume of vacuoles, which contributes most to the size of plant cells, was also strikingly reduced in both the palisade and the spongy cells of *smo1/trp2-301* (Figure 2c,d). All of these findings indicate that the mutation in *SMO1* mainly impedes the process of cell expansion, rather than cell proliferation, during leaf growth.

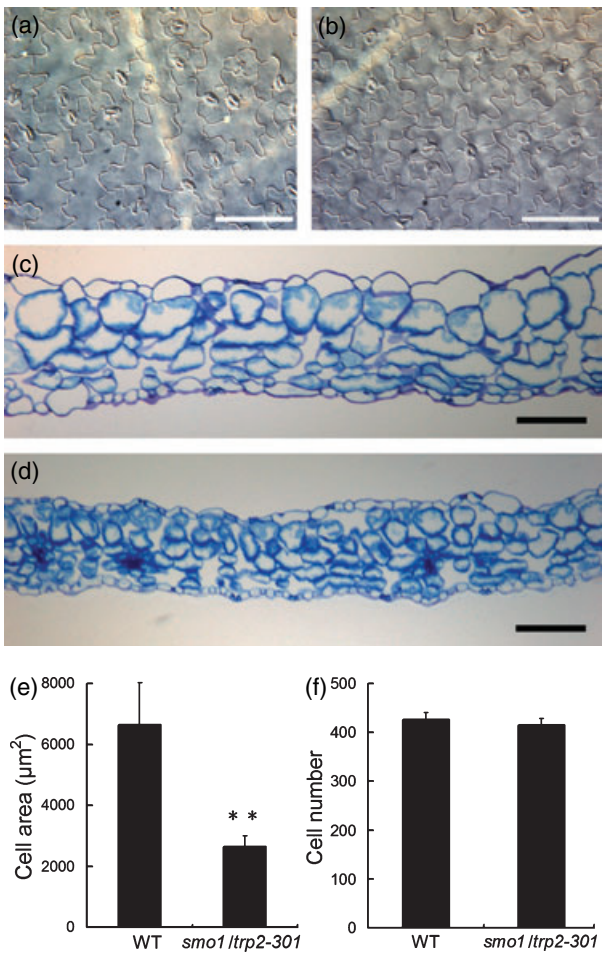

Figure 2. Anatomical analysis of wild-type (WT) and *smo1/trp2-301* leaves.
(a, b) Adaxial epidermal cells of the fully expanded fifth leaf of a WT plant (a), and of a *smo1/trp2-301* plant (b). Scale bar: 100 μm.
(c, d) Transverse sections of the fifth leaf blade in the WT (c), and in the *smo1/trp2-301* mutant (d). Scale bar: 50 μm.
(e) Area of palisade cells in WT and *smo1/trp2-301* leaves. Five blades of the fifth leaf from each genotype were cleared, and at least 50 palisade cells were measured in each leaf under a microscope. Average values ± SD are shown; $^{**}P < 0.01$.
(f) Mesophyll cell number in the WT and in the *smo1/trp2-301* mutant. At least three leaves were sectioned across the middle of the leaf-width direction for each genotype, and total mesophyll cells were counted from the midvein to the leaf edge. Average values ± SD are shown.

smo1/trp2-301 *contains a mutation in TSB1*

To define the genetic basis of *smo1/trp2-301*, we back-crossed *smo1/trp2-301* plants with WT Columbia-0 (Col-0) to generate F_1 and selfed F_2 progeny. All F_1 plants showed WT morphology, and F_2 seedlings exhibited a segregation ratio, WT:*smo1/trp2-301*, of 3:1 (data not shown), indicating that *smo1/trp2-301* is caused by a single-gene recessive mutation. We then crossed *smo1/trp2-301* with the Landsberg *erecta* (Ler) accession and isolated the gene by position cloning. *SMO1* was finely mapped to a ~70-kb region in chromosome 5, in which 18 genes were annotated or identified in the Arabidopsis genome, including *TSB1* (*At5g54810*). As all previously identified mutants in *TSB1*, named as *trp2*s, were reported to display a similar phenotype to *smo1/trp2-301* described above (Barczak *et al.*, 1995; Last *et al.*, 1991), we sequenced the *TSB1* gene in *smo1/trp2-301* and in the WT, and found a typical C → T transition in the *TSB1* coding region, causing a substitution of Thr by Ile in TSB1 (Figure 3a). However, the expression of *TSB1* was not affected in the mutant (Figure 3b).

To determine whether *smo1/trp2-301* is an allele of *trp2s*, two other finely characterized *trp2* alleles, *trp2-8* and *trp2-1*, were obtained from ABRC (CS8328 and CS8327). *trp2-8* contains a single base-pair deletion in *TSB1*, causing an open reading frame shift to yield a truncated protein with only 251 amino acids (Barczak *et al.*, 1995), whereas *trp2-1* has only ~10% TSB catalytic activity compared with that of WT (Last *et al.*, 1991). Both *trp2-8* and *trp2-1* exhibited the morphological and anatomical phenotype similar to that of *smo1/trp2-301* in all organs examined, and obviously *trp2-1* represented the most severe phenotype amongst these three mutants (Figure 3c,d and Figure S1; Table 1), suggesting that *smo1/trp2-301* is an allele of *trp2s*. We further crossed *smo1/trp2-301* with *trp2-8*, and finally verified that *smo1/trp2-301* is indeed an allelic mutant of *trp2s* (data not shown), and that *smo1* was thus re-designated as *trp2-301*, accordingly.

trp2 *cells are defective in nuclear endoreduplication*

In plants, the final size of a cell is often correlated with nuclear polyploidy, which results from nuclear endoreduplication when a cell undergoes differentiation and cell expansion (Sugimoto-Shirasu and Roberts, 2003). Given that cell size is responsible for growth defects in *trp2* organs, we examined whether nuclear endoreduplication was affected in cells of the three *trp2* mutants. Flow cytometric analyses with nuclei of fully expanded fifth leaves revealed obvious differences in the polyploidy among WT and *trp2* cells. Figure 4a showed that the ploidy of WT cells ranged from 2C to 32C, with a peak at 8C, whereas cells in all three *trp2* alleles were mainly from 2C to 8C, showing a peak at 4C. Quantitative analyses indicated that the decrease of ploidy level was highly consistent with the size reduction in cells and leaves of the three *trp2* mutants (Figures 4b and 3c,d), among which *trp2-1* contained the most 2C but the least 8C cells. These observations suggest that the mutation in *TSB1*, and the consequent Trp deficiency, impairs nuclear endoreduplication, and thus affects cell expansion and final cell size.

Chloroplast development is delayed in smo1/trp2-301 *mesophyll cells*

In plants, Trp biosynthesis occurs mainly in chloroplasts (Radwanski *et al.*, 1996). As a pale-green phenotype was

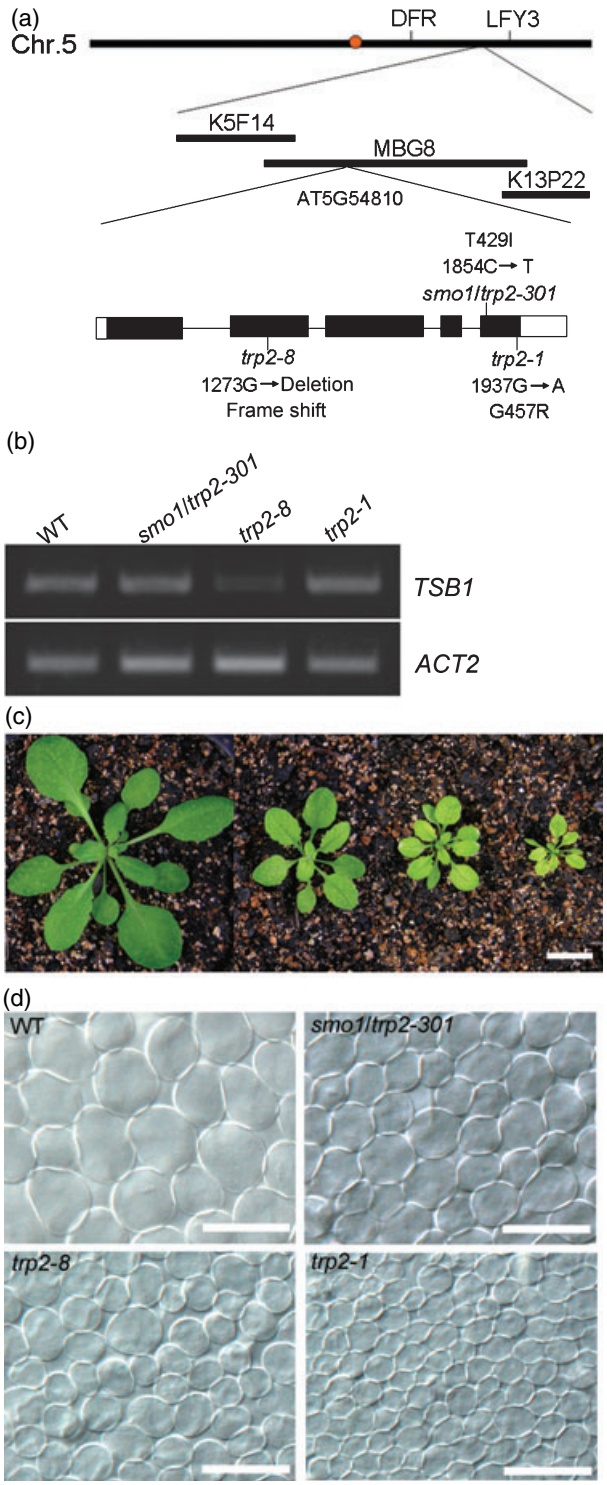

Figure 3. Structure of *SMO1/TSB1* and characterization of *trp2* alleles.
(a) Mapping and gene structure of *SMO1/TSB1*. The *SMO1* locus was mapped to *TSB1* (*At5g54810*). The gene structure of *TSB1* was shown schematically: the exons were represented as rectangles (filled, translated regions; open, non-translated regions), and the introns were shown as lines. The molecular lesions in *smo1/trp2-301*, *trp2-8* and *trp2-1* are indicated.
(b) *TSB1* expression in wild-type (WT), *smo1/trp2-301*, *trp2-8* and *trp2-1* plants. *ACTIN2* (*ACT2*) was used as an internal control.
(c) Comparison of 4-week-old plants of WT, *smo1/trp2-301*, *trp2-8* and *trp2-1* (from left to right). Scale bar: 10 mm.
(d) Palisade cells of the fully expanded fifth leaf in the WT and in the *trp2* mutants. Fifth leaves from 4-week-old plants were cleared and visualized under a microscope. Scale bar: 50 μm.

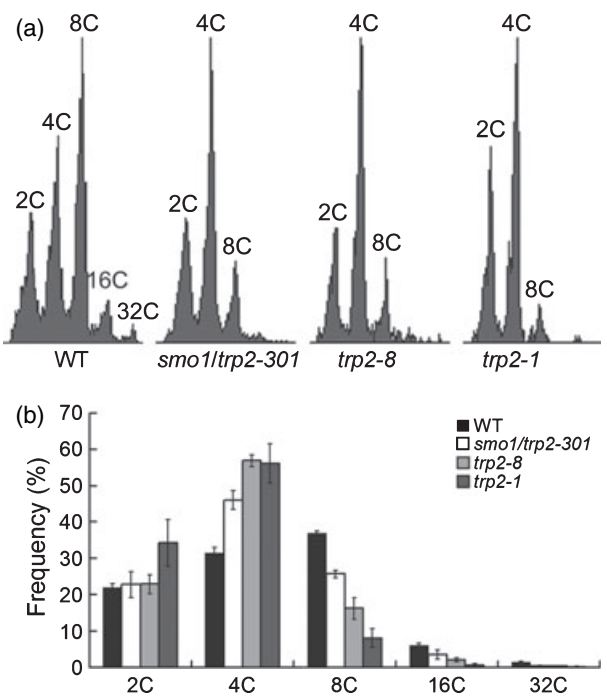

Figure 4. Polyploidization of wild-type (WT) and *trp2* leaf cells.
(a) Ploidy histogram for nuclei isolated from the fully expanded fifth leaf blades of WT and *trp2* mutants. Note that the proportion of nuclei in the WT peaks at 8C, and peaks at 4C in *trp2* mutants.
(b) Percentage of cells with different nuclear ploidy. Average values ± SDs are shown (n = 4).

observed in the interveinal regions of *trp2* cotyledons and young leaves, and the chlorophyll content and photosynthetic efficiency were found to be apparently decreased at this stage (data not shown), we suspect that this phenotype might be associated with a defect in chloroplast development in *trp2* mesophylls. We compared chloroplast ultrastructures in *smo1/trp2-301* and WT palisade cells of fifth leaves at early and late developmental stages (about 4 and 19 days after emergence, respectively), by transmission electron microscopy. Although detailed quantification was difficult to perform, the number of chloroplasts did not seem to differ between two genotypes. In contrast to the presence of more starch granules and thylakoids in WT chloroplasts, chloroplasts of *smo1/trp2-301* were much smaller, containing fewer starch granules and under-developed thylakoids at early stages (Figure 5a,b). Nevertheless, the size and structure of chloroplasts in *smo1/trp2-301* were similar to those in the WT at later stages, although a comparatively lower electron density was still observed in thylakoids

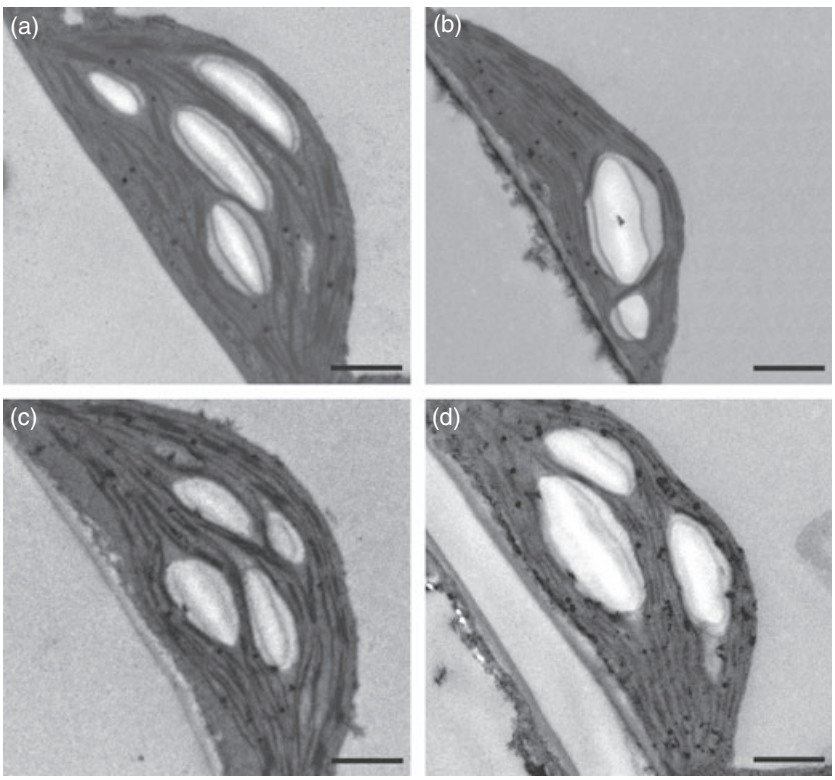

Figure 5. Chloroplast development in wild-type (WT) and *smo1/trp2-301* plants.
(a, b) Ultrastructure of the chloroplasts in young fifth leaves (4 days after emergence) of WT (a) and *smo1/trp2-301* (b) plants. Fifth leaves of 15-day-old plants were sectioned, and the chloroplasts were visualized under a transmission electron microscope. Scale bar: 1 μm.
(c, d) Ultrasturcture of developed chloroplasts in the WT (c) and in *smo1/trp2-301* (d). Fifth leaves from 30-day-old plants were sectioned. Scale bar: 1 μm.

(Figure 5c,d). These observations indicate that chloroplast development is also delayed in *trp2* mesophyll cells, which might be partly responsible for the pale-green phenotype in *trp2* cotyledons and young leaves.

Cell expansion and leaf growth in trp2 *alleles can be restored by the exogenous application of Trp, but not by auxin*

Two non-allelic *TSB* genes have been identified in the Arabidopsis genome, but *TSB1* produces >90% of the *TSB* mRNA in leaf tissues, and is responsible for most of the Trp production in Arabidopsis (Last *et al.*, 1991). The previously identified *trp2* mutants, including *trp2-1*, contained dramatically decreased Trp content (Normanly *et al.*, 1993; Ouyang *et al.*, 2000). To determine if the retarded cell expansion in *trp2* mutants is directly derived from a consequential shortage of Trp, we examined the seedlings of WT and three *trp2* alleles grown on media supplemented with/without L-Trp. Figure 6a showed that the exogenous application of Trp obviously restored leaf growth in the three *trp2* mutants, although the addition of Trp in the medium had a slightly negative effect on the growth of WT seedlings. Further examination of palisade cells in the third leaf revealed that the impeded cell expansion in these *trp2* alleles was indeed rescued (Figure 6b), demonstrating that the defect in cell expansion in *trp2*s is caused by a deficiency of Trp.

As plants use Trp as a precursor to synthesize the hormone auxin, which plays important roles in the regulation of cell division and cell expansion (Woodward and Bartel, 2005), we explored whether the cell growth defect is related to auxin. Neither the exogenous application of 2,4-dichlorophenoxyacetic acid (2,4-D) nor indole-3-acetic acid (IAA) restored the cell and leaf growth in the three *trp2* alleles (Figure 6a,b; data not shown). Quantification of free IAA contents revealed that the IAA levels in *trp2* plants were not decreased, but were instead elevated (Figure S2a), which is consistent with previous reports (Normanly *et al.*, 1993; Ouyang *et al.*, 2000) and our expression analyses of auxin responsive genes, including *IAA1*, *IAA5* and *IAA6* (Figure S2b). Nevertheless, the organ phenotype in *trp2* alleles described here was obviously not correlated with those observed in the mutants with enhanced IAA biosynthesis, such as epinastic cotyledons, elongated hypocotyls, excess adventitious and lateral root development, and reduced leaf number (Delarue *et al.*, 1998; Zhao *et al.*, 2001). All of these findings suggest that the organ growth defect in *trp2*s is independent of auxin.

General protein synthesis is not apparently altered in trp2 *alleles*

Because Trp is essential for general protein synthesis, it is likely that the shortage of Trp in *trp2* mutants affects general protein synthesis, thereby retarding cell expansion and organ growth. To investigate whether protein synthesis

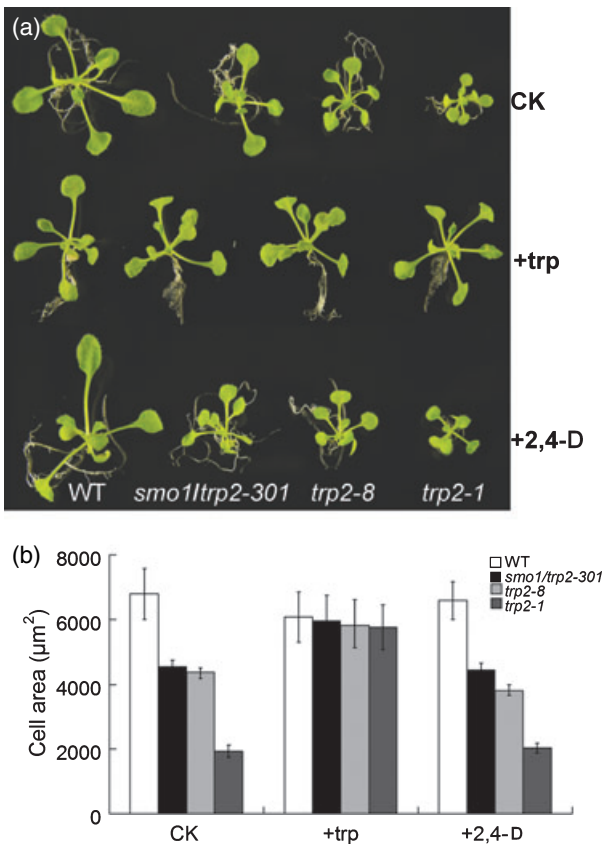

Figure 6. Leaf growth and cell expansion in *trp2* alleles are rescued by exogenously feeding plants with tryptophan, but not with auxin.
(a) Comparison of 3-week-old WT and *trp2* mutant plants grown on MS medium (upper panel, CK), and supplemented with L-Trp (middle panel, +trp) or 2,4-dichlorophenoxyacetic acid (bottom panel, +2,4-D). The 1-week-old seedlings germinated on MS plates were transferred to MS plates without/with 0.25 mM L-Trp or 0.1 μM 2,4-D, and were grown for a further 2 weeks.
(b) The area of palisade cells of third leaves from plants described above. At least four leaves were cleared for each treatment and genotype, and more than 20 cells were measured in each leaf under a microscope. Average values ± SD are shown.

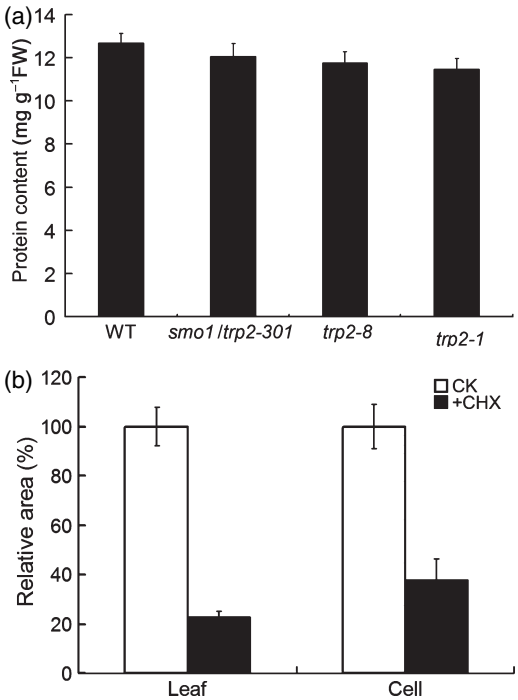

Figure 7. Total protein levels in *trp2* mutants, and the effect of inhibition of protein synthesis on leaf growth.
(a) Total protein levels of 4-week-old wild-type (WT) and *trp2* plants. Average values ± SE are shown ($n = 3$).
(b) Effect of inhibition of protein synthesis on leaf and cell growth. Leaf and palisade cell areas were determined with WT plants grown on the medium with or without the protein synthetic inhibitor cycloheximide (CHX). The 1-week-old seedlings were transferred to MS medium containing the same concentration of dimethyl sulfoxide, DMSO (CK) or 1 μM cycloheximide (+CHX), and were grown for a further 2 weeks. At least four third leaves were examined for each treatment, and more than 20 cells were measured in each leaf under a microscope. Average values ± SD are shown.

contributes to cell expansion defects in *trp2* plants, we first measured the total protein in WT and in the three *trp2* alleles. We found that the total protein levels among the WT and *trp2* mutants were almost the same (Figure 7a), implying that the decrease of Trp contents in these three *trp2* alleles has little effect on general protein synthesis. We next examined the impact of the inhibition of protein synthesis on cell and organ growth, by exogenous application of the protein synthetic inhibitor cycloheximide (CHX) to WT plants. As shown in Figure 7b, inclusion of 1 μM CHX in the medium greatly retarded the leaf growth, reducing the leaf area to about 21% of that of the control plants; however, the area of palisade cells was reduced to about 39% of that in the control, suggesting that the inhibition of protein synthesis alone impedes both cell expansion and proliferation during organ growth. Therefore, we conclude that the cell expansion defect in *trp2* mutants is not predominantly caused by an alteration in protein synthesis, but by a deficiency in Trp.

Discussion

Trp is required for cell expansion during organ growth

Genetic studies in Arabidopsis and maize have identified most of the genes participating in the Trp biosynthetic pathway, and previous studies revealed that the regulation of Trp biosynthesis is also important in biotic responses (Radwanski and Last, 1995; Zhao and Last, 1996; Zhao *et al.*, 1998). That *trp* mutants display morphological changes suggests that Trp is also required for and/or mediates developmental processes. Here, we provide the evidence that Trp is important for growth by cell expansion during plant organogenesis. The *smo1/trp2-301* mutant described here was initially identified by its decreased aerial organ

size. Our further analysis with *smo1/trp2-301* and other *trp2* alleles reveals that *trp2* mutants are defective in cell expansion and nuclear endoreduplication. Furthermore, supplementing the medium with L-Trp can restore the cell expansion and leaf development of *trp2* mutants. In addition, we also examined epidermal and palisade cells in *trp3*, and the same defect in cell expansion was found in *trp3-1* (data not shown). These results demonstrate that Trp deficiency impairs growth by cell expansion, but does not impact on growth by cell proliferation. As cell division and cell expansion are separately controlled, in general (Mizukami and Fischer, 2000), it is likely that a deficiency in Trp, and/or its derivatives, is a growth-limiting factor for the cell expansion process during plant growth and development.

Trp-mediated cell expansion in trp2 *mutants may not be attributable to general protein synthesis and auxin*

In any organism, the shortage of an amino acid may directly impact on general protein synthesis, which consequently may influence growth and development. Nevertheless, the general protein synthesis in *trp2* alleles is not obviously affected, and the partial inhibition of protein synthesis affects both cell proliferation and expansion at the same time (Figure 7), which was also reported in the mutant related to protein synthesis (Feng *et al.*, 2007). Therefore, the cell expansion and organ growth defect in *trp2* mutants is more likely to result from a deficiency in Trp rather than an alteration of the general protein synthesis.

The Trp biosynthetic pathway is also used for the synthesis of many important secondary metabolites in plants, including indole glucosinolates, phytoalexins, tryptomine derivatives and IAA, which is the most natural form of the plant hormone auxin (Radwanski and Last, 1995; Woodward and Bartel, 2005). In plants, auxin is essential for various growth and developmental processes, such as tropism, shoot and root formation, phyllotaxis and flower morphogenesis, and auxin also plays critical roles in the regulation of cell division and expansion, both in cell culture and in whole plant development (Davies, 2004). Although recent characterizations of *YUCCA* (*YUC*), *TAA* genes indicate that Trp-dependent IAA biosynthesis is indeed important for plant development (McSteen and Zhao, 2008; Stepanova *et al.*, 2008; Tao *et al.*, 2008; Zhao *et al.*, 2002), our studies on the *trp2* alleles and results from several previous reports have argued that IAA is not responsible for the phenotypic alteration in *trp* mutants. For example, in Arabidopsis *trp3-1* and *trp2-1*, the Trp content is remarkably decreased, whereas the IAA level is elevated (Normanly *et al.*, 1993; Ouyang *et al.*, 2000) (Figure S2). Similar changes were also reported in maize *orange pericarp* (*orp*), which is defective in the *TSB* orthologs (Wright *et al.*, 1991). This finding has led to the conclusion that a Trp-independent IAA biosynthetic pathway exists in plants (Woodward and Bartel, 2005). Nevertheless, *trp2* and other *trp* mutants do not develop any auxin-related phenotype in aerial architecture and lateral root formation, as do the mutants with either enhanced IAA biosynthesis, or defects in auxin biosynthesis or signaling (Cheng *et al.*, 2006; Delarue *et al.*, 1998; Zhao *et al.*, 2001) (Table 1; Figure S1). In addition, previous genetic and pharmacological analyses of *trp2-1*, *trp3-1* and *trp5-2* demonstrate that it is the deficiency in Trp, or its derivatives, rather than IAA that confers the compressed root waving phenotype of these mutants (Rutherford *et al.*, 1998). Taken together, we conclude that the Trp-related cell expansion growth is not attributable to auxin, but is attributable to Trp itself.

The role of amino acid homeostasis in plant growth and development

Our characterization of *trp2* mutants here raises the question of the role of amino acid homeostasis in plant development, and more specifically, whether Trp serves as a cue to regulate developmental progression. Recent identification of a few weak mutants defective in other amino acid biosynthetic pathways provides some evidence that such regulation may exist in plants. For example, a weak allele of *HISN3*, a gene involved in histidine biosynthesis, *albino and pale green 10* (*apg10*), exhibits small, narrow and pale-green leaves at the juvenile stage, but exhibits a short petiole, and small and round leaves, at a late stage (Noutoshi *et al.*, 2005). Some of these phenotypic alterations appear to be similar to those observed in *trp2*s. Another mutant, *hpa1*, a weak mutant of *HISN6*, is defective in root meristem maintenance (Mo *et al.*, 2006). A mutation in the threonine synthesis gene results in the retardation of both root and aerial organ development (Bartlem *et al.*, 2000). Obviously, the complete disruption of amino acid biosynthesis appears to be fatal to the plant (Muralla *et al.*, 2007).

In bacteria, some amino acids, such as Trp, appear to be signal molecules to regulate the transcription of genes. Starvation of Trp results in the inactivation of the TrpR repressor and the weakly charged $tRNA^{Trp}$, thereby elevating the transcription of Trp biosynthetic genes (Merino *et al.*, 2008). In yeast, starvation of Trp triggers a more complicated regulatory mechanism, including the activation of GCN4, a transcriptional co-activator that shares homology with the *jun* oncoprotein, and human transcoactivator protein AP-1. GCN4 is a regulator of a complex network, which involves the transcriptional derepression of at least 30 genes in multiple amino acid biosynthetic pathways (Braus, 1991). Mutant strains defective in the GCN4-dependent control system have a significantly reduced growth rate under Trp-starvation conditions (Niederberger *et al.*, 1981). In plants, mutations in genes involved in threonine or lysine biosynthesis in Arabidopsis cause an overaccumulation of methi-

onine or thrionine, respectively (Bartlem *et al.*, 2000; Craciun *et al.*, 2000), whereas the inhibition of histidine biosynthesis results in the activation of genes involved in multiple amino acid and other metabolic pathways, thus increasing the accumulation of at least 10 other amino acids (Guyer *et al.*, 1995; Stepansky and Leustek, 2006), suggesting that a general or cross regulation among amino acid homeostasis and/or other metabolic pathways may also exist in higher plants. The GCN4 ortholog has not been found in the Arabidopsis genome, but GCN4-like binding sites have been identified in a series of gene promoters involved in many metabolic pathways (Stepansky and Leustek, 2006). On the other hand, even if our evidence might exclude the involvement of auxin in Trp-related cell expansion and organ growth, homeostasis of amino acids does involve many metabolites that are essential for plant development. For instance, methionine is a precursor for the production of *S*-adenosyl-methionine, which is the main methyl donor in many transmethylation reactions in plants (Bartlem *et al.*, 2000). It is also possible that a shortage of a specific amino acid may result in changes in the accumulation of such a derivative or of other metabolites, which in turn influences developmental processes. Furthermore, although our work on *trp2* mutants reveals how Trp affects organ growth at the cellular level, the molecular basis underlying such regulation still remains unclear. Therefore, future work is necessary to further characterize the weak alleles defective in the biosynthesis of a specific amino acid, including Trp, to understand the molecular mechanism of how amino acid homeostasis coordinates plant growth and development.

Experimental procedures

Plant materials and growth conditions

The *Arabidopsis thaliana* accessions Col-0 and Ler were used in all experiments. The *smo1/trp2-301* mutant was initially identified from a mutant collection by screening for altered leaf size in the Col-0 background. *trp2-1* (CS8327) and *trp2-8* (CS8328) were obtained from the ABRC stock center (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm), and were described previously (Barczak *et al.*, 1995; Last *et al.*, 1991). Seeds were sterilized in 1% sodium hypochlorite for 15 min, and were then germinated on MS medium in a culture room at 22°C under a 16-h light/8-h dark photoperiod, at an intensity of 80–90 μmol m^{-2} s^{-1}. For measurements of hypocotyl length, primary root length and lateral root initiates, seedlings were grown vertically on MS medium containing 1.0% sucrose and 0.8% agar. For morphological and histological examinations, 7-day-old seedlings were transferred to soil, and were grown in a chamber at 22 ± 1°C under the same photoperiod and illumination as those grown in the culture room. For feeding and treatment assays, 7-day-old seedlings were transferred to MS plates supplemented with or without L-Trp, 2,4-D or cycloheximide (CHX), at the indicated concentrations, and kept in a growth room for 14 days. Plants were photographed, and the third leaf was cleared for examining the area of palisade cells.

Position cloning of smo1/trp2-301 *and gene expression analysis*

The *smo1/trp2-301* mutation was mapped with an F_2 population, generated by crossing *smo1/trp2-301* with Ler, by simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers. A point mutation in the *TSB1* gene (*At5g54810*) was identified when the candidate genes were sequenced. The allelic test was carried out by crossing *smo1/trp2-301* with *trp2-8* and then examining the phenotype of the F_1 plants.

Total RNA was prepared by a guanidine thiocyanate extraction method (Hu *et al.*, 2000), and the reverse-transcribed PCR (RT-PCR) was performed to monitor the expression of *TSB1* and *IAAs* in *trp2* mutants. The primers used for *TSB1* were 5′-TGGCAGCCTCAGGCACCT-3′ and 5′-TCAAACATCAAGATATTTAGCC-3′, and for the internal control *ACTIN2* (*ACT2*), the primers were 5′-GCCATCCAAGCTGTTCTCTC-3′ and 5′-GCTCGTAGTCAACAGCAACAA-3′. PCR amplifications were performed with 26 cycles for *TSB1* and 20 cycles for *ACT2*, and were repeated three times. The primers for the *IAA* genes were: *IAA1*F, 5′-GTCACCAATGGGCTTAACCTT-3′; *IAA1*R, 5′-AACATCACCGACCAACATCC-3′; *IAA5*F, 5′-ACCGAACTACGGCTAGGTCTT-3′; *IAA5*R, 5′-ACATCTCCAGCAAGCATCCA-3′; *IAA6*F, 5′-AGCTTCGATTGGGTCTTCCA-3′; *IAA6*R, 5′-TCTTCACGATCCTCAGCCTCT-3′.

Phenotypic analyses

To determine the size of the organ, excised fully expanded cotyledons and fifth leaves were photographed. To examine the growth kinetics of leaves, the blade length of fifth leaves was measured in 4-day intervals after emergence. We used 7-day-old seedlings for examining root length and lateral root initiates under a microscope, and 5-day-old seedlings grown in darkness were used for hypocotyl measurements. Palisade cells in the central area of a half leaf blade were used to determine the cell size. Areas of organs and cells were measured with ImageJ software (http://rsb.info.nih.gov/ij).

Light and transmission electron microscopy

An excised leaf was cleared with chloral hydrate, and the epidermal and palisade cells were visualized under a microscope (Leica Microsystems, http://www.leica-microsystems.com) as previously described (Raynaud *et al.*, 2005; Tsuge *et al.*, 1996). Thin sections of fully expanded fifth leaves were prepared according to the method described previously (Hu *et al.*, 2003).

To examine chloroplast development, the fifth leaves from 15- and 30-day-old plants were excised, and the fixation, dehydration, infiltration and sectioning of leaf tissues were performed according to the method described by Peng *et al.* (2006). Sections were stained with uranyl acetate and lead citrate, and were visualized under a transmission electron microscope (JEM-1230; JEOL, http://www.jeol.com).

Flow cytometric analysis

Fully expanded leaves of WT plants and *trp2* mutants were chopped with a razor, suspended in cold nuclear isolation buffer (Galbraith *et al.*, 1983) and filtered through a 20-μm filter. A 1-ml volume of 4′,6-diamidino-2-phenylindole (DAPI; 0.5 mg ml^{-1}) staining buffer was then added to the filtered solution. Nuclear ploidy was analyzed with a FACS calibur flow cytometer (BD Biosciences, http://www.bdbiosciences.com).

Total protein determination and free IAA measurement

Both 4-week-old WT and *trp2* plants were harvested, and the total protein was extracted and determined as described previously (Voll *et al*., 2003).

Leaves from 3-week-old plants of WT and *trp2* mutants were used for the measurement of free IAA levels. The extraction, purification of samples and analyses of free IAA by gas chromatography-mass spectrometry were performed according to the method described by Edlund *et al.* (1995), except that an Agilent/LECO gas chromatographer-mass spectrometer was used, with the separation performed in a DB-5ht column (Agilent, http://www.agilent.com). The internal standard [^{13}C]IAA was purchased from Cambridge Isotope Laboratories (http://www.isotope.com).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Morphology of aerial organs of *trp2* mutants.

Figure S2. Free indole-3-acetic acid (IAA) levels and expression of auxin-responsive genes in *trp2* mutants.

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