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Over-expression of *AtPPD2* in tomato represses shoot and root growth, fruit development and seed formation through inhibition of cell cycle gene expression

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ABSTRACT

Arabidopsis *AtPPD2* encodes a ZIM-domain containing protein that acts as a negative regulator in cell proliferation. We show here that over-expression of *AtPPD2* in tomato plants led not only to the significant reduction in size of cotyledons and developing leaves during vegetative growth phase, but also to the altered architecture of mature compound leaves through inhibition of cell division and expansion. Interestingly, the constitutive expression of *AtPPD2* in transgenic tomato plants resulted in the growth inhibition of roots, suggesting that *AtPPD2* is also a potential repressor for root cell proliferation. In Addition, over-expressing *AtPPD2* caused a significant reduction in fruit and seed size. We noted that the transgenic fruits had a thinner pericarp layer, suggesting an additional role of *AtPPD2* in carpel cell division and expansion prior to ripening. The expression levels of cell proliferation marker genes in the leaves and flower buds of transgenic tomato plants such as *LeCYCD3;1*, *LeCYCB2;1* and *LeCDKB2;1* were markedly reduced. Taken together, these results indicate that *AtPPD2* functionally acts as a negative regulator during vegetative and reproductive growth and development in transgenic tomato plants through repression of cell cycle-related gene expression. Our results also demonstrate the potential usefulness of *AtPPD2* gene in modulating fruit and seed setting processes in vegetable crop plants.

KEYWORDS: growth regulator, tomato, cell proliferation, fruit development, growth inhibition, seed formation

ABBREVIATIONS

MS	:	Murashige and Skoog medium
Kan	:	kanamycin
RT-PCR	:	reverse transcription polymerase chain reaction
PPD2	:	PEAPOD2
GAPDH	:	glyceraldehyde-3- phosphate dehydrogenase
CDKB2;1	:	cyclin-dependent kinase B2;1
CYCB2;1	:	cyclin B2;1
CYCD3;1	:	cyclin D3;1

INTRODUCTION

Repetitive and flexible specification of new tissues and boundaries is required for formation and development of new organs in higher plants, which leads to remarkable uniformity of organs within species [1-3]. Most of the studies about the patterning of shoot and root apical meristems and their stem niches concentrated on a few genetically tractable model organisms such as Arabidopsis, tomato, snapdragon, petunia, maize and rice, which have brought considerable progress in understanding

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regulatory mechanisms by which plant organ morphogenesis is formed [1, 3]. To gain insights into molecular basis of meristem formation and maintenance, it is necessary to identify the regulators as components of the molecular networks that pattern cell proliferation, differentiation and expansion phases with temporal and spatial precision.

Organ development in higher plants is a tightly regulated process that is orchestrated by balancing cell proliferation and differentiation through growth stimulators and repressors. Some of the major transcriptional regulators that promote lateral organ growth include the APETALA2 (AP2)-domain protein AINTEGUMENTA (ANT) [4], the related C2H2 zinc-finger proteins JAGGED (JAG) [5, 6] and NUBBIN (NUB) [7]. Mutations in *ANT* lead to formation of small organs in the mutant plants whereas over-expression of *ANT* increases organ size by prolonging the phase of cell proliferation [4]. *JAG* loss of function causes premature cessation of cell proliferation in lateral organs whereas *JAG* over-expression is sufficient to cause tissue overgrowth [5, 6]. *LYRATE*, the tomato *JAGGED* homolog, coordinates lateral outgrowth in the compound leaves of tomato by interacting with both the *KNOX* and auxin transcriptional networks [8]. Unlike Class I TCP proteins, Class II TCP proteins, including Antirrhinum CYCLOIDEA (CYC), DICHOTOMA (DICH) and CINCINNATA (CIN) and Arabidopsis TCP proteins such as TEOSINTE BRANCHED1 and CYCLOIDEA, act as negative regulators to restrain cell and organ growth [1, 9].

Besides Class II TCP proteins, a number of additional growth repressors have been identified over the past few years. The novel RING-finger protein BIG BROTHER (BB), exhibiting E3 ubiquitin-ligase activity *in vitro*, was identified as a central repressor of plant organ growth by limiting the period of proliferative growth [10]. It has been proposed that BB acts by targeting critical growth-promoting factors for proteasomal degradation. Loss of function mutations in *AUXIN RESPONSE FACTOR2* (*ARF2*) lead to larger leaves and seeds, indicating that ARF2 functions as a growth repressor to restrain shoot growth and seed formation [11-13]. The *arf2* mutant phenotype and the expression analysis of genes promoting cell division provide evidence that over-growth of organs results from prolonged expression of *ANT* and one of its target genes, *CycD3;1* [13]. Recently, the redundant *PEAPOD1* (*PPD1*) and *PEAPOD2* (*PPD2*) have been shown to limit the proliferation of dispersed meristematic cells that are thought to initiate the regular array of tissue layers within the leaf blade in Arabidopsis [14]. Deletion of both genes increases leaf lamina size, alters leaf curvature and causes excess growth of siliques whereas over-expression of *AtPPD* reduces lamina size by promoting the early arrest of dispersed meristematic cell proliferation during leaf and silique development [14].

Tomato fruit set is controlled by positive growth signals generated during fertilization and final fruit size is determined by coordination of cell proliferation and expansion at different phases of fruit-set processing [15]. The first objective of this study was to verify whether constitutive expression of *AtPPD2* driven by 35S promoter in transgenic tomato plants could modulate fruit size through inhibition of cell proliferation during fruit development. A major form variation between simple and compound leaves results from tuning the timing, duration and further patterning events during leaf developmental stages [2, 16]. Given that the Arabidopsis *ppd* mutant displays the altered leaf shape and curvature, the second objective was to determine the effect of PPD-dependent arrest of cell proliferation on leaf configuration changes of transgenic tomato plants. Here we present a genetic gain-of-function analysis of the Arabidopsis *AtPPD2* gene in transgenic tomato plants. The results show that in tomato, over-expression of *AtPPD2* causes a significant reduction of growth cycle during vegetative and reproductive growth through inhibition of cell division and cell expansion. We uncover new roles of *AtPPD2* in regulation of compound leaf development, fruit development, seed formation and root development, especially for lateral roots.

MATERIALS AND METHODS

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. VF36) were used for all experiments described in this article, and were grown in pots in greenhouse under

controlled temperature (25°C/22°C day/night) with 16 h of light and 8 h of darkness at 60% relative air humidity. For root developmental analysis, seeds were surface-sterilized, germinated and grown vertically on MS culture medium with 0.8% agar on Petri dishes in growth conditions as described above.

Gene constructs and plant transformation

The Arabidopsis *PPD2* cDNA was amplified by PCR using primer pairs: PPD2_CDS_F (5'-CTCGAGA TGGATGTAGGAGTTACTACG-3') and PPD2_ CDS_R (5'-GAATTCTTAATTATCTTCGCTGTT TAG-3'). The amplified DNA fragment, containing the *AtPPD2* open reading frame, was inserted into the pMD19-T vector. The resulting plasmid was then digested with *Xho* I-*Eco*R I, and the *Xho* I-*Eco*R I fragment was inserted into the binary pMON530 vector downstream of the 35S promoter.

The chimeric construct was transferred via *Agrobacterium tumefaciens* strain GV3101 to VF36 cotyledons using the transformation and regeneration methods according to [17] with minor modifications. Briefly, seeds were surface-sterilized and germinated in half-strength MS medium with 30 g L^{-1} sucrose. The cotyledons excised from nine-d-old seedlings were infected with Agrobacteria harboring the desired constructs. The infected cotyledon explants were grown for four weeks on a shoot regeneration medium containing MS salts, 30 g L^{-1} sucrose, 1.0 mg L^{-1} zeatin and 100 mg L^{-1} Kan. The shoots regenerated from calli were removed and transferred to a rooting medium containing MS salts, 30 g L^{-1} sucrose, 0.1 mg L^{-1} 1-naphthalene acetic acid and 100 mg L^{-1} Kan. The resulting plants were selected, planted in peat soil pots and grown in the greenhouse at the growth conditions as described above. The *AtPPD2* expression was confirmed in leaves detached from the transformants by RT-PCR analyses using the specific primers PPD2_ RT_F (5'-GTTACTACGGCGAAGTCTATAC-3') and PPD2_RT_R (5'-CCACCATCTTCTCTTTA C TCAT-3').

RT-PCR and real-time PCR

For all analyses, the harvested leaves or flower buds were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from leaf or flower bud samples frozen in liquid nitrogen using the TRIzol reagent (Takara) according to manufacturer's protocol. For RT-PCR or quantitative real-time RT-PCR analysis, DNA contaminated in total RNA samples was digested with RNase-free DNase (Takara). Complementary DNA was produced using 2 µg total RNA and an oligo (dT) 18 primer. PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Primers specific to *AtPPD2* for RT-PCR analysis were as described above. *LeGAPDH* was amplified as an internal control using primer pairs (GAPDH_F: 5'-GGTGCTGACTTCGTTGTTG-3'; GAPDH_R: 5'-GCTCTGGCTTGTATTCATTCT C-3').

Quantitative real-time PCR was performed with SYBR Premix Ex TaqII (Takara) using a MyiQ5 single color Real-Time PCR Detection System (Bio-Rad). The comparative threshold cycle (Ct) method was used for determining relative transcript levels (iQ5 admin, Bio-Rad) with *LeGAPDH* as an internal control. The Primers for cell cycle genes used in real-time RT-PCR were (5'→3'): *LeCDKB2;1* (CDKB2_F: CCTGAGGTTCTACTTGGAGC; CDKB2_R: CATCAAGACCAGGGACAACG); *LeCYCB2;1* (CYCB2_F: ATCTGGTGTTGATTT CGGAC; CYCB2_R: CACATTGAGCAGCCTT GAGA); *LeCYCD3;1* (CYCD3_F: TGTTGAGG GTCATTGCTTAC; CYCD3_R: AGCACCATC CAGACACAAAC).

Measurements of cell number and area

Leaves were fixed with ethanol/acetic acid (6:1) for 12 h, washed with 100% ethanol three times and then with 70% ethanol once. The tissues were cleared in chloral hydrate solution (8 g of chloral hydrate, 1 ml of glycerol and 2 ml of distilled water) overnight. The tissues were mounted and examined using DIC under OLYMPUS BX51 microscope (OLYMPUS Corporation, Japan). To determine cell and leaf area, the microscopic images were analyzed with Image J software (OLYMPUS Corporation, Japan).

Accession numbers

Sequence data from this article can be found in GenBank/EMBL database under the following accession numbers: *AtPPD2*, At4g14720; *LeGAPDH*, U97257; *LeCDKB2;1*, AJ 297917; *LeCYCB2;1*, AJ 243455; *LeCYCD3;1*, AJ245415.

RESULTS

Over-expressing *AtPPD2* inhibits vegetative and reproductive growth and development

A binary vector carrying the coding sequence of *AtPPD2* driven by CaMV 35S promoter was transformed into tomato plants. 13 independent primary (T0) tomato transformants were selected and T1 plants were obtained by self-pollination. Expression levels of *AtPPD2* in leaves from 13 T0 independent transformants were analyzed by RT-PCR (Fig. 1a). Eight transformants showed a significant increase in levels of *AtPPD2* expression compared to the absence of *AtPPD2* expression in wild type plants. Over-expression of *AtPPD2* was further validated by RT-PCR in T1 plant leaves of over-expression line1 (OE1) and line3 (OE3) that were used for phenotypic analysis (Fig. 1d). In order to investigate the effect of over-expressing *AtPPD2* on organogenesis of transgenic tomato plants after embryogenesis, wild type plants independently regenerated by *in vitro* culture were grown under greenhouse conditions together with the transgenic lines, OE1 and OE3. In transgenic lines, cotyledon and leaf laminae were much smaller, and the reduction of lamina area was due to decreases in both length and width compared with that of wild type control plants (Fig. 1b, 1c, 2a and 2c). With respect to mature plants, the heights of transgenic plants were reduced to 45-50% of that of wild type plants, and so were the internode length between the first and second leaves, and stem diameter (Fig. 2b and 2d), indicating that over-expressing *AtPPD2* remarkably inhibited the shoot growth and development of the transgenic plants. For leaf development, OE1 and OE3 plants only generated 6-7 compound leaves whereas 9-10 compound leaves were produced in wild type plants (Fig. 1c). In addition to dramatic inhibitory effect on leaf development, over-expressing *AtPPD2* also led to architecture alterations of mature compound leaves in transgenic plants. In contrast to wild type plants, the transformants were in general inhibited in blade outgrowth and leaflet outgrowth.

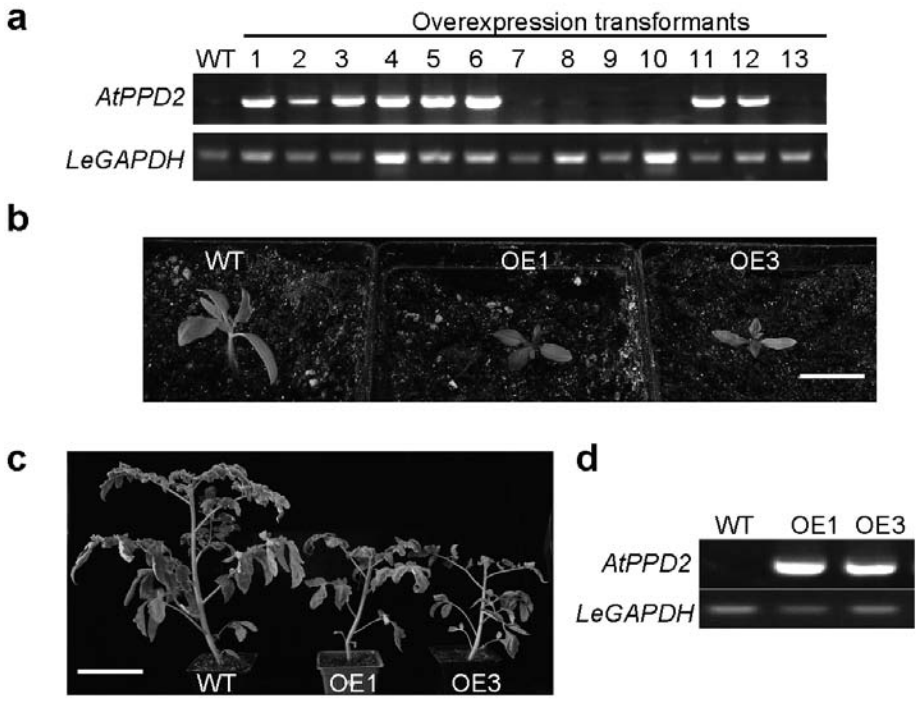

Fig. 1. Phenotypes of tomato plants over-expressing *AtPPD2*. (**a**) RT-PCR analysis of *AtPPD2* transcript levels in independent transformants. *LeGAPDH* was used as a loading control. (**b**) and (**c**) Phenotypes of wild type (WT) and transgenic tomato plants (over-expression lines, OE1 and OE3) at different developmental stage. (**d**) RT-PCR analysis of *AtPPD2* expression levels in leaves of OE1 and OE3 transgenic lines. Bar = 2.5 cm in (b) and 10 cm in (c).

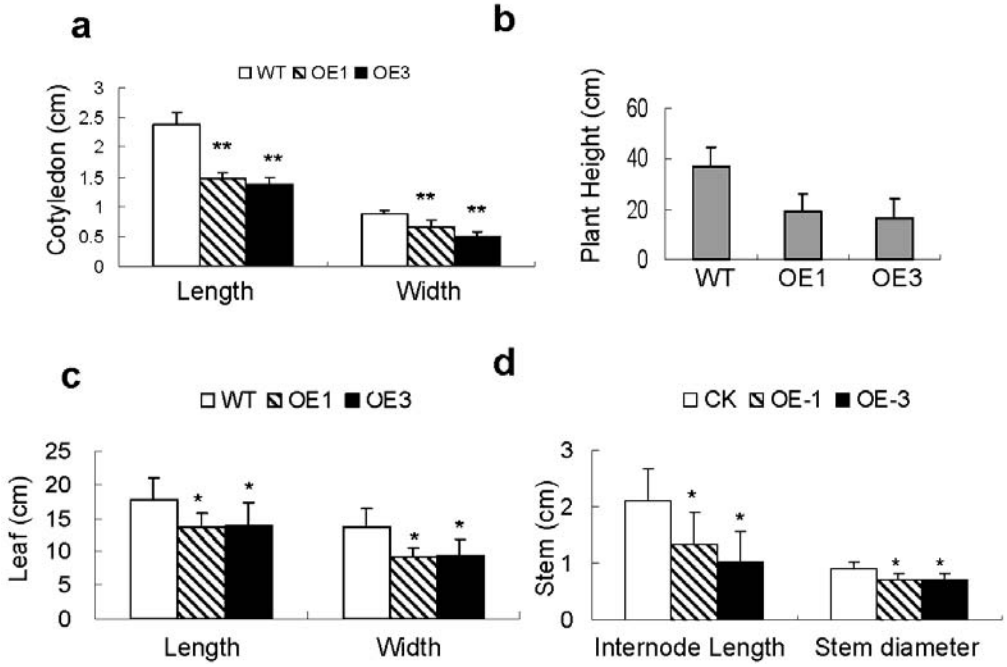

Fig. 2. Phenotypic analysis of transgenic tomato plants over-expressing *AtPPD2*. (**a**) Cotyledon growth analysis of 23-d-old seedlings after germination. Plant growth analysis of 60-d-old transgenic plants of OE1 and OE3 lines measured as plant height (**b**), the length and width of the fifth leaf (**c**), internode length between the first and second leaves, and stem diameter (**d**). Shown are averages ± SD (n = 6). Student's tests show that the means between WT and transgenic lines were significantly different in (a), (b), (c) and (d), $^{**}P<0.01$, $^{*}P<0.05$.

The mature compound leaves of wild type plants consisted of primary, secondary, intercalary and terminal leaflets with serrated margins whereas the compound leaves of transgenic tomato plants produced only terminal and primary leaflets with the absence of intercalary leaflets and secondary leaflets (Fig. 3a). In addition, over-expression of *AtPPD2* also led to severe fusion and abnormal serration of primary and terminal leaflets in transgenic plants, resulting in malformed leaves (Fig. 3a). Compared with the wild type plants, numbers of terminal leaflet lobe and lateral leaflet were reduced in transgenic lines, OE1 and OE3 (Fig. 3a and 3b). During floral development, the transgenic plants over-expressing *AtPPD2* did not show significant difference in flowering time with respect to wild type plants. However, the floral organ development of the transformants was in general inhibited as it was observed that the flower size was reduced due to decreases in both length and width of sepals and petals compared with wild type plants (Fig. 3c).

Over-expressing *AtPPD2* dramatically affects root development

The role of *AtPPD2* in root development has not been recognized before [14]. We examined the transgenic plants over-expressing *AtPPD2* in cell proliferation and differentiation in root organogenesis to determine if *AtPPD2* modulates root formation in tomato. In order to examine primary root elongation, adventitious root (AR) and lateral root (LR) formation, the seedlings of transgenic lines (OE1 and OE3) and wild type were grown vertically on agar media for nine days after sowing (Fig. 4a). We found that the primary root growth and adventitious root formation were inhibited in both transgenic lines, and this inhibitory effect was more pronounced in OE3 line, compared with wild type seedlings (Fig. 4a, 4b and 4c). Strikingly, lateral root formation was almost totally inhibited in the transgenic seedlings up to nine days post-germination with respect to three-four lateral roots produced and elongated in wild type seedlings (Fig. 4d).

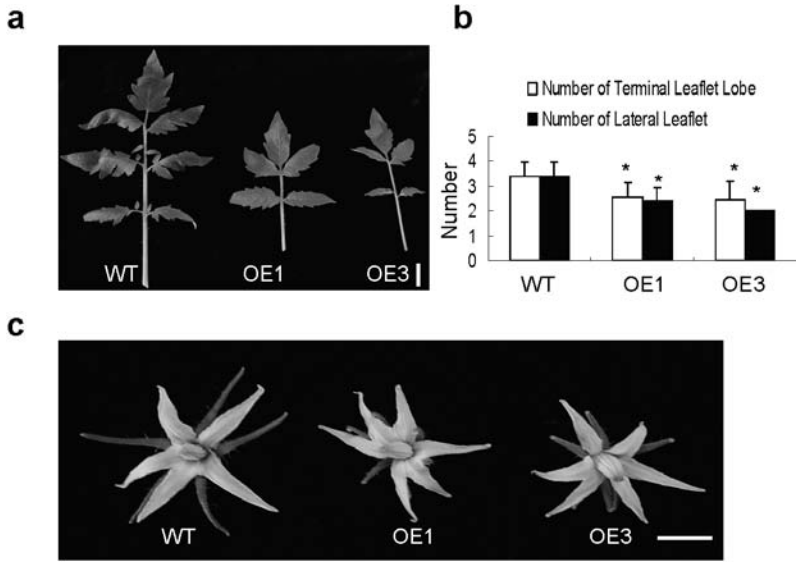

Fig. 3. Phenotypes of compound leaves and flowers in transgenic tomato plants over-expressing *AtPPD2*. (**a**) Morphology of the representative fifth compound leaves from WT and transgenic lines. (**b**) Phenotypic analysis of the fifth compound leaves between the transgenic lines and WT plants counted as number of terminal leaflet lobe and number of lateral leaflets. Shown are averages ± SD (n = 6). Student's tests show that the means between WT and transgenic lines were significantly different, **P*<0.05. (**c**) Photographs of the fully opened flowers harvested from WT and transgenic lines. Bar = 2 cm in (a) and 1 cm (c).

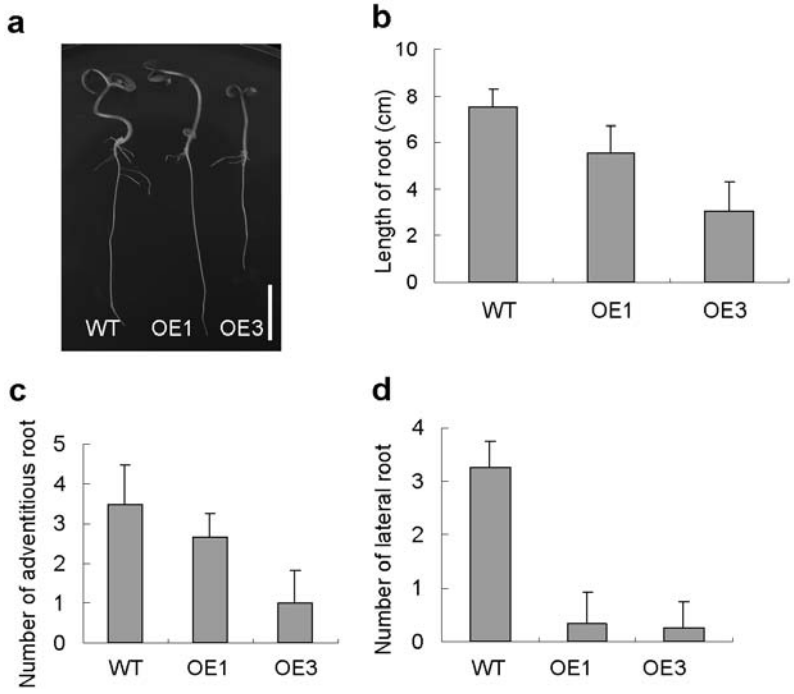

Fig. 4. Root development in transgenic tomato plants over-expressing *AtPPD2*. (**a**) Root phenotypes of nine-d-old seedlings of WT and transgenic lines. Scale bar represents 2 cm. Root growth analysis measured as primary root length (**b**), number of adventitious roots per seedling (**c**), and number of lateral roots per seedling (**d**). Error bars indicate standard deviations (n = 6).

Over-expressing *AtPPD2* negatively modulates fruit development and seed formation

All the transgenic fruits showed a similar ripening process to the fruits of wild type plants. Over-expression of *AtPPD2* resulted in a dramatic inhibition of the transgenic fruit development. It was observed that the fruits of OE1 and OE3 plants exhibited a strong reduction in size, but were very similar in appearance to the wild type fruits in terms of skin color, hardness, and flesh consistency (Fig. 5a). The fruit weight reduction of OE1 and OE3 plants was 30% and 23%, respectively, relative to those fruits of wild type plants (Fig. 5b). Interestingly, the fruits over-expressing *AtPPD2* showed a thinner pericarp layer and the outer wall thickness of OE1 and OE3 fruit pericarp was reduced to about 60% of that of the wild type fruits (Fig. 6a and 6b). These results indicate that *AtPPD2* governs morphological processes that occur prior to ripening, such as fruit size, pericarp thickness, and locular development throughout tomato fruit development in the transgenic plants. In addition to the reduction in fruit size, *AtPPD2* over-expressors also produced smaller seeds (Fig. 6c). The transgenic seeds taken from OE1 and OE3 fruits showed 24% and 59% decrease in dry weight with respect to that of the wild type seeds, respectively (Fig. 6d). Although a dramatic decrease in size and weight was observed in the transgenic seeds, no significant changes were observed in seed germination between the transgenic and wild type seeds under normal growth conditions (data not shown).

Over-expressing *AtPPD2* affects growth and development through inhibition of cell division and expansion

We performed detailed analysis of cell division and expansion between over-expression lines and wild type plants in order to determine how over-expression of *AtPPD2* inhibits vegetative and reproductive growth and development in transgenic tomato plants. To uncover the cause of inhibited lamina growth in OE1 and OE3 lines, the first leaves were excised from 27-d-old transgenic and wild type seedlings to examine the inhibited cell division rates by counting the number of palisade cells continuously along the large primary vein or midvein from base to top of the first leaves (Fig. 7a). The average number of the counted palisade cells in leaves of wild type leaves was around 1119, but the numbers were decreased to 827 (26% reduction) for OE1 and to 753 (33% reduction) for OE3, indicating that over-expressing *AtPPD2* causes arrest of cell division (Fig. 7b). We next quantified the effect of over-expressing *AtPPD2* on lamina outgrowth by measuring leaf areas. These results showed that the area of first leaves from both of 27-d-old OE1 and OE3 plants was 65-70% reduced in comparison with that of wild type leaves (Fig. 7c). Consistent with the leaf area data showed above, adaxial palisade cells in the first leaves of the transgenic plants were also dramatically reduced in size, and the transgenic line OE1 and OE3 showed 41% and 45% decrease in cell size, respectively, in relative to that of wild type cells (Fig. 8a and 8b). Taken together, these

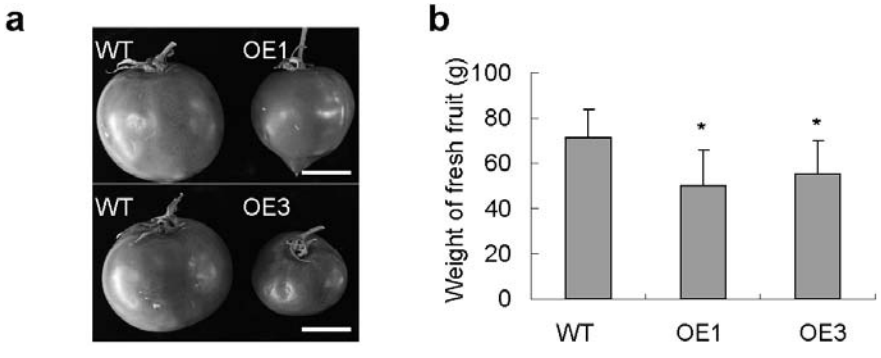

Fig. 5. Fruit development in transgenic tomato plants over-expressing *AtPPD2*. (**a**) Fruit size of WT and transgenic lines. Bar = 2 cm. (**b**) Difference in fruit weight between WT and transgenic lines. Error bars indicate standard deviations (n = 6). Student's tests show that the means between WT and transgenic lines were significantly different, **P*<0.05.

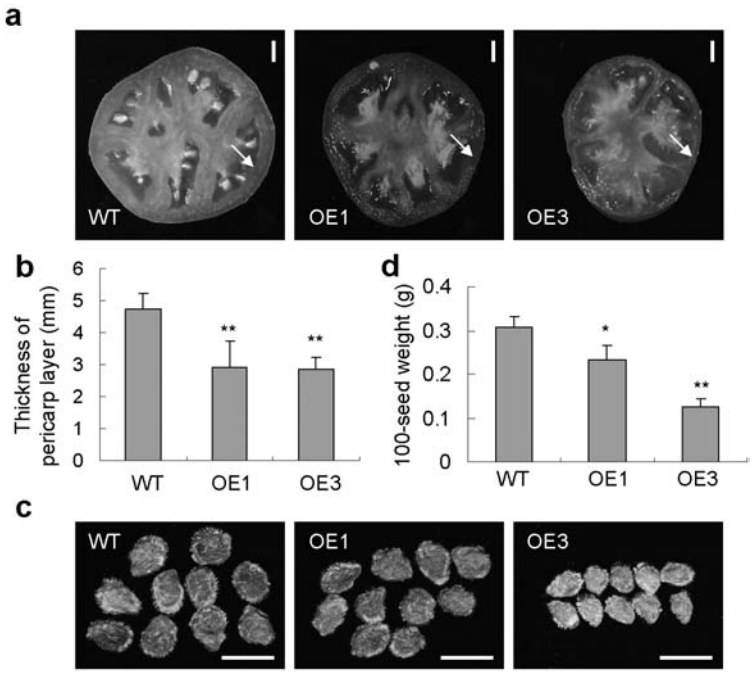

Fig. 6. Effect of *AtPPD2* over-expression on pericarp development and seed formation in transgenic fruits. (**a**) Cross-sectioning morphology of transgenic fruits showing a thinner pericarp layer compared to WT fruits. The arrows indicate pericarp layers. Bar = 5 mm. (**b**) Pericarp layer thickness of WT and transgenic fruits at breaker stage. Error bars indicate standard deviations (n = 6). (**c**) Photographs of mature seeds from WT and transgenic fruits. Bar = 5mm. (**d**) 100-seed weight of mature seeds as shown in (c) from WT and transgenic fruits. Error bars indicate standard deviation (n = 3). Student's tests show that the means of wild type and transgenic plants were significantly different in (b) and (d), $^{**}P<0.01$, $^{*}P<0.05$.

results suggest that over-expressing *AtPPD2*-induced growth and developmental inhibition is attributable to both reductions in cell proliferation and cell expansion in the transgenic tomato plants.

Over-expressing *AtPPD2* strongly represses expression of cell cycle genes

We found that the observed inhibitory effect of over-expressing *AtPPD2* on organ growth and development in tomato transgenic plants was partially related to repression of cell proliferation. To examine further the relationship of over-expression of *AtPPD2* with the expression levels of cell cycle genes, including *LeCDKB2;1*, *LeCYCB2;1* and *LeCYCD3;1*, in transgenic tomato plants, we performed qRT-PCR analysis in leaf and flower bud tissues. The levels of *LeCDKB2;1*, *LeCYCB2;1* and *LeCYCD3;1* transcripts were dramatically reduced in both leaves and flower buds of the transgenic lines in comparison with wild type (Fig. 9a and 9b). It was noted that over-expressing *AtPPD2* resulted in around 30-70% reduction in expression levels of *LeCDKB2;1*, *LeCYCB2;1* and *LeCYCD3;1* in the leaves of transgenic line OE1, and the inhibitory effect was even more pronounced in the leaves of transgenic line OE3 (77-95% reduction for these three genes), which could be a major reason that OE3 showed relatively stronger phenotypes in overall growth and developmental inhibition with respect to the line OE1.

DISCUSSION

In this study, over-expression of *AtPPD2* in tomato plants leads to a range of novel phenotypes, including deformed compound leaf, inhibited lateral and adventitious root development, reduced fruit size and seed weight, which are unexpected on the basis of the experience gained with Arabidopsis. In Arabidopsis, *AtPPD2* encodes a putative DNA-binding protein and is redundantly required for leaf and silique development [14].

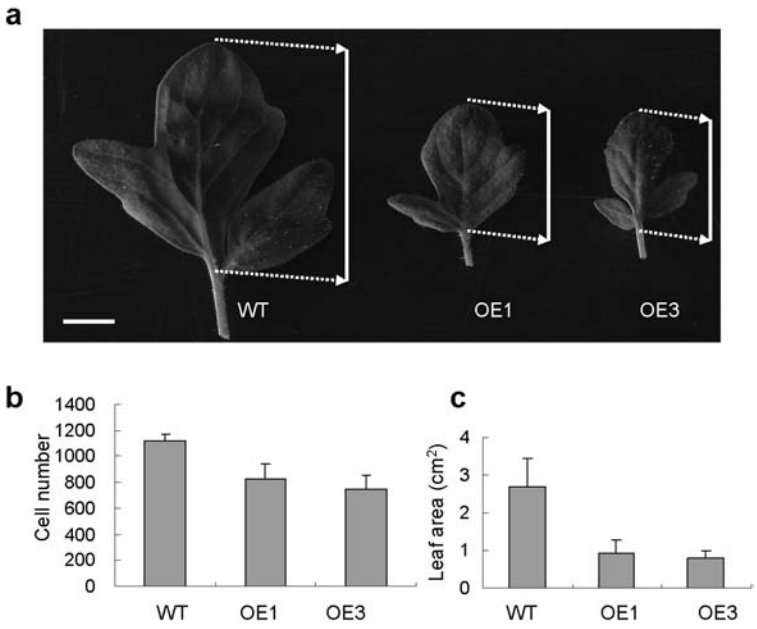

Fig. 7. Growth analysis of the first leaves in WT and transgenic plants over-expressing *AtPPD2*. (**a**) Phenotypes of the representative first leaves detached from WT and transgenic plants. The lines indicate the large primary vein or midvein from base to top of the first leaves used for cell-counting. Bar = 5 mm. (**b**) Average number of total palisade cells counted from leaf bottom to top along the large primary vein in the first leaves of WT and transgenic lines. Error bars indicate standard deviations (n = 6). (**c**) Area of the first leaves of WT and transgenic lines. Error bars indicate standard deviations (n = 6).

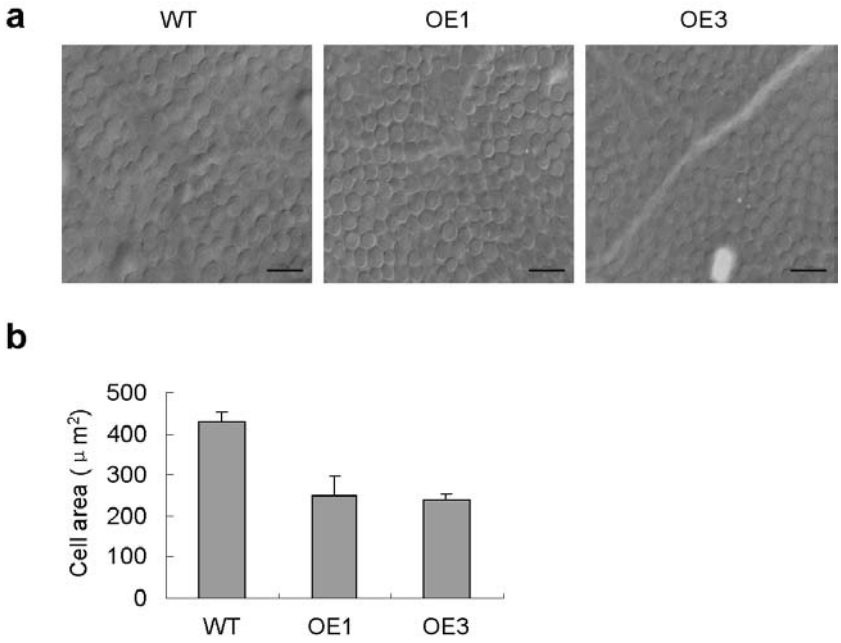

Fig. 8. Effect of *AtPPD2* over-expression on cell size of palisade cells in leaves of transgenic plants. (**a**) DIC images of adaxial palisade cells in the first leaves of WT and transgenic lines. Bar = 50 μm. (**b**) Cell area measurements of adaxial palisade cells in the first leaves. n = 1080 cells from six different leaves. Error bars indicate the standard deviations.

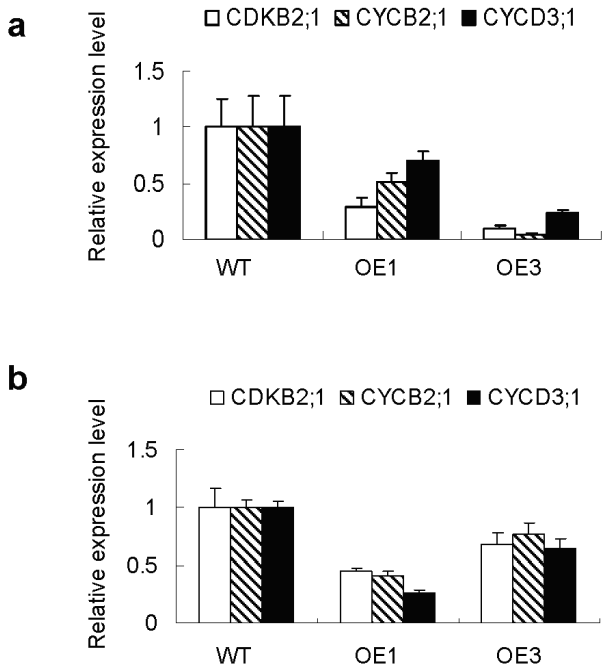

Fig. 9. Transcript analysis of cell cycle genes in WT and transgenic plants over-expressing *AtPPD2* by quantitative RT-PCR. Total RNA was extracted from the first leaves (**a**) and flower buds (**b**) of WT and transgenic lines, respectively. *LeGAPDH* was used to normalize the expression levels of *LeCDKB2;1*, *LeCYCB2;1* and *LeCYCD3;1*. Error bars indicate standard deviations of three technical replicates, and the results were consistent in two biological replicates.

Over-expressing *AtPPD2* as a growth repressor suggested a role in modulating fruit development, as transgenic tomato fruits showed reduced size and weight. Interestingly, fruits over-expressing *AtPPD2* had a thin pericarp layer and reduced size of seeds, suggesting that *AtPPD2* plays a previously unrecognized role in carpel expansion and seed formation prior to ripening. Although the role of *AtPPD2* in regulating leaf development has been established in Arabidopsis, our results uncover a novel role of *AtPPD2* in modulating compound leaf maturation, as the compound leaves of transgenic tomato plants produced only terminal and primary leaflets with the absence of intercalary leaflets and secondary leaflets, and some of primary and terminal leaflets in transgenic plants exhibited severe fusion and abnormal serration, resulting in malformed leaves (Fig. 3a). Moreover, over-expressing *AtPPD2* inhibits root development of transgenic tomato plants. Our findings demonstrate that *AtPPD2* acts as a negative regulator involved in the regulation of lateral organ development and contributes to cell proliferation and cell expansion in apical growth and development.

With respect to the blade structure, plant leaves can be divided into two leaf forms: simple leaves with a single undivided blade and compound leaves composed of multiple blade units termed leaflets. In simple leaf model species such as Arabidopsis and snap-dragon (*Antirrhinum majus*), final leaf shape and size are dictated by cell division that may, in part, guide subsequent cell expansion required to produce a mature leaf [18-22]. In comparison to simple leaf development, the prolonged activity of the marginal blastozone during primary morphogenesis is required for compound leaf development, although leaf development from the flanks of the shoot apical meristem and leaflet development from the leaf marginal blastozone share partial genetic mechanisms [18, 23, 24]. In tomato, a representative compound leaf is composed of a petiole, a terminal leaflet and four to six pairs of primary petiolate lateral leaflets [25]. In agreement with the notion that over-expression of *AtPPD* in Arabidopsis reduces leaf size by promoting the early arrest of dispersed meristematic cell (DMC) proliferation during leaf development [14], *AtPPD2* over-expression in tomato plants also causes the size of compound leaves dramatically reduced, confirming that *AtPPD2* acts as a negative regulator to control compound leaf development in tomato transgenic plants. Surprisingly, *AtPPD2* over-expression eliminates the development of some of primary petiolate lateral leaflets (Fig. 3a), suggesting that *AtPPD2* plays a negative role in cell proliferation at the leaf marginal blastozone. In contrast to the effect of *AtPPD2* over-expression on generation of leaflets, over-expression of the maize homeobox containing knotted-1 (*KN1*) gene in wild-type tomato leads to generate super-compound leaves bearing thousands of leaflets [26]. Similar to the role of *AtPPD2* in repressing cell proliferation at the leaf marginal blastozone, TCP domain proteins, such as LANCEOLATE (LA) in tomato, promote differentiation by negatively modulating SAM and marginal blastozone

activity during the transition developmental stage from proliferative to expansive growth in compound leaf development [16, 25]. Collectively, we demonstrate that tomato plants over-expressing *AtPPD2* display the inhibited outgrowth of pairs of primary petiolate lateral leaflets, presumably by inhibiting cell division and cell expansion at the leaf marginal blastozone where KNOX1 proteins and TCP domain proteins antagonistically regulate the meristematic activity of the marginal blastozone critical for initiation of leaflets during compound leaf development. Based on the results described above, we propose that *AtPPD2* and TCP domain proteins might share a degree of functional similarity during compound leaf development.

Consistent with the role of *AtPPD2* in repressing the meristematic activity of the marginal blastozone during compound leaf development, over-expressing *AtPPD2* in tomato transgenic plants leads to inhibition of root development (Fig. 4a to 4d). Cell cycle activation is required for root development at the very onset of root growth. In the Arabidopsis root apical meristem (RAM), the quiescent center (QC) consists of four cells and is surrounded by meristematic initials [27]. In Arabidopsis, *AtPPD2* is supposed to function as growth repressors in regulation of organ development to reach their specialized size [14]. We show that over-expression of *AtPPD2* inhibits lateral root development more severely compared with the growth of primary root and adventitious root in transgenic tomato plants. In contrast to shoot branching, lateral root primordia originate at a distance from the mature pericycle of the parent root. Our data support the idea that over-expression of *AtPPD2* may disrupt the whole process of cell cycle progression and stimulation of the molecular pathway towards lateral root initiation by inhibiting anticlinal asymmetric divisions of pericycle founder cells in the formation of a new organ.

In addition to effects of *AtPPD2* over-expression on shoot and root development, we also show that over-expressing *AtPPD2* leads to inhibition of reproductive growth and development in transgenic tomato plants, as transgenic tomato fruits showed reduced size and weight, and had a thin pericarp layer and reduced size of seeds. In general, the developmental process of ovule is composed of four distinct phases, including the initiation of the ovule primordial from the placenta inside carpel, the specification of ovule identity, the formation of spatially defined patterns within the developing ovule and the final fourth phase, the formation of nucellus from the distal portions of ovule primordia [28]. In recent years, considerable progress has been made in investigating regulatory mechanisms modulating fruit development through regulation of cell division and cell expansion. For instance, *fruit weight 2.2* (*fw2.2*) appears to act as a negative regulator of developing fruits in tomato [29-31]. In maize, over-expression of *Cell Number Regulator1* (*CNR1*), a ortholog gene of tomato *fw2.2*, led to reduced plant and organ size due to changes in cell number, not cell size, indicating that *fw2.2-like* genes play a conservative role in regulating organ development [32]. The cell division and enlargement phases were found to overlap in early pea ovary (pericarp) development and cell division in the pea pericarp was highest from anthesis, then subsequently decreased until mitotic phase of fruit development [33]. Ovule growth and development generally consists of both cell division and cell enlargement following pollination and fertilization [34]. Our findings are consistent with the suggestion that *AtPPD2* negatively regulates pericarp development through inhibition of cell division and enlargement phases, which consequently results in reduced fruit size, thinner pericarp layer and reduced seed size and weight (Fig. 5 and Fig. 6).

Plant organ size reflects both cell number and cell size [35]. We show that over-expressing *AtPPD2* both affects cell division and expansion in transgenic tomato plants by counting leaf palisade cells continuously along the midvein from base to top of the first leaves and measuring the area of palisade cells in the transgenic leaves taken from the wild type control and transgenic lines (Fig. 7 and Fig. 8). These results indicate that over-expressing *AtPPD2* alters plant and lateral organ size primarily through negatively regulating cell proliferation and cell expansion. Although both of *AtPPD2* and *CNR1* act as negative regulator of plant growth, over-expressing *CNR1* reduced plant and organ size mainly through inhibition of cell proliferation, not cell expansion [32]. We also demonstrate that over-expressing *AtPPD2* results

in the general cell-cycle arrest due to the reduced expression levels of cell proliferation marker genes such as *LeCDKB2;1*, *LeCYCB2;1* and *LeCYCD3;1* in transgenic tomato leaves and flower buds, although a direct connection to the cell cycle remains unclear. Importantly, our findings indicate that tomato fruit size and thickness of pericarp layer can be made smaller and thinner, and compound leaves can be simplified by over-expressing *AtPPD2* in tomato plants. In conclusion, despite obvious differences between Arabidopsis and tomato plants in leaf and fruit development, they may ultimately share a common strategy for organ size control through antagonistically regulating the meristematic activity by positive and negative regulators such as *AtPPD2*.

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