Polygalacturonase45 cleaves pectin and links cell proliferation and morphogenesis to leaf curvature in *Arabidopsis thaliana*

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**SUMMARY**

Regulating plant architecture is a major goal in current breeding programs. Previous studies have increased our understanding of the genetic regulation of plant architecture, but it is also essential to understand how organ morphology is controlled at the cellular level. In the cell wall, pectin modification and degradation are required for organ morphogenesis, and these processes involve a series of pectin-modifying enzymes. Polygalacturonases (PGs) are a major group of pectin-hydrolyzing enzymes that cleave pectin backbones and release oligogalacturonides (OGs). PG genes function in cell expansion and separation, and contribute to organ expansion, separation and dehiscence in plants. However, whether and how they influence other cellular processes and organ morphogenesis are poorly understood. Here, we characterized the functions of Arabidopsis PG45 (*PG45*) in organ morphogenesis using genetic, developmental, cell biological and biochemical analyses. A heterologously expressed portion of *PG45* cleaves pectic homogalacturonan *in vitro*, indicating that *PG45* is a *bona fide* PG. *PG45* functions in leaf and flower structure, branch formation and organ growth. Undulation in *pg45* knockout and *PG45* overexpression leaves is accompanied by impaired adaxial–abaxial polarity, and loss of *PG45* shortens the duration of cell proliferation in the adaxial epidermis of developing leaves. Abnormal leaf curvature is coupled with altered pectin metabolism and autogenous OG profiles in *pg45* knockout and *PG45* overexpression leaves. Together, these results highlight a previously underappreciated function for PGs in determining tissue polarity and regulating cell proliferation, and imply the existence of OG-based signaling pathways that modulate plant development.

**Keywords:** Arabidopsis thaliana, cell proliferation, leaf curvature, oligogalacturonides, pectin, polygalacturonase.

**INTRODUCTION**

In plants, lateral organs initiate at the shoot apical meristem (SAM) and grow into myriad structures, including leaves, lateral branches and flowers. Leaf development involves primary and secondary morphogenesis (Bar and Ori, 2014). During primary morphogenesis, the leaf primordium differentiates from the peripheral zone of the SAM, with polarity being determined along proximodistal, adaxial–abaxial and mediolateral axes (Du et al., 2018). During this stage, cells divide without undergoing extensive expansion. After primordium formation, secondary morphogenesis involves leaf blade outgrowth (Bar and Ori, 2014). During this phase, cell division and expansion occur with partial temporal overlap. Cell division diminishes along a basipetal gradient, followed by a basipetal wave of cell expansion (Andriankaja et al., 2012; Gonzalez et al., 2012).

Organ morphogenesis is controlled by multiple internal and external cues during development. Previous studies have revealed that leaf morphogenesis involves spatiotemporal auxin gradients and complex networks of regulatory gene activities. During leaf polarity, pre-patterning, adaxial domain formation requires a temporary auxin minimum, which is achieved by PIN-FORMED1-mediated auxin transport from leaf primordia to the SAM (Qi et al., 2014). Multiple transcription factors and small RNAs control leaf adaxial–abaxial polarity (Braybrook and Kuhlemeier, 2010; Du et al., 2018; Heisler and Byrne, 2020). These regulators...
are expressed in specific domains and activate or repress the expression of downstream genes, helping maintain and promote leaf polarity. Perturbed adaxial-abaxial polarity can result in altered leaf curvature, and even cause trumpet-shaped leaves (Liu et al., 2010; Qi et al., 2014). During leaf blade outgrowth, secondary organ morphogenesis is also regulated by a complex network involving small RNAs, transcription factors, gibberellins and brassinosteroids (Du et al., 2018; Xiong et al., 2021). These and other studies lay a foundation for understanding the molecular mechanisms of organ patterning, but how cell growth is controlled and coordinated to achieve leaf morphogenesis is incompletely understood.

Organ morphogenesis is determined by a balance between cell proliferation and cell expansion, which is modulated by the cell wall. Pectins are major components of the primary cell walls of eudicots, and pectin status influences wall structure and extensibility, which can in turn regulate organ morphology (Palin and Geitmann, 2012; Wolf and Greiner, 2012). Pectins, which are synthesized in the Golgi apparatus, are secreted to the wall in a methyl-esterified and acetylated state, and can be modified and degraded in the wall (Sénéchal et al., 2014). The most abundant pectin domain, homogalacturonan (HG), is the best understood in terms of its modification and degradation (Sénéchal et al., 2014). Previous studies revealed that pectin methylsterification levels modulate wall mechanics and are closely linked with organ morphogenesis; during leaf outgrowth, pectin methylsterification status is related to asymmetries in wall mechanics in different domains, resulting in the differential growth of the adaxial, middle and abaxial domains (Qi et al., 2017). Flower primordium initiation and phyllotaxis require pectin demethylsterification, which leads to an increase in wall elasticity (Peaucelle et al., 2008, 2011). Regulating pectin methylsterification status is accomplished by pectin methylsterases (PMEs) and PME inhibitors. PMEs remove methylster groups from HG, producing negatively charged, demethylsterified pectin, and PME inhibitor proteins negatively regulate PME activity. Blockwise demethylsterification HG molecules can be crosslinked by calcium, increasing wall stiffness. In contrast, randomly demethylsterified pectin can be further cleaved by pectin degrading enzymes, such polygalacturonases (PGs) and pectate lyases (PLs), facilitating cell growth and producing oligogalacturonides (OGs) (Hocq et al., 2017). OGs with specific degrees of polymerization (DP) can act as regulators during plant development, as shown by exogenous OG treatment experiments (Ferrari et al., 2013; Hoqc et al., 2020; Savatin et al., 2011). However, whether and how autogenous OGs are coupled with organ morphogenesis remains unknown.

PGs belong to glycoside hydrolase family 28, which often includes numerous genes per plant species but shares four conserved functional motifs (Liang et al., 2016; Park et al., 2008; Yang et al., 2013). Based on their modes of action, PGs can be further divided into three different types: endo-PGs, exo-PGs and rhamno-PGs (Park et al., 2010; Yang et al., 2018). Endo-PGs randomly cleave HG backbones and produce OGs with various DPs, whereas exo-PGs hydrolyze HG chains only from the non-reducing end and release GalA monomers (Abbott and Boraston, 2007; Tu et al., 2015). Genetic characterization has revealed functions for PG genes in cell expansion and separation across multiple developmental stages, including hypocotyl elongation, leaf expansion, stomatal opening, microspore separation, pollen tube growth, organ abscission and fruit ripening (Hocq et al., 2020; Huang et al., 2009; Ogawa et al., 2009; Paniagua et al., 2017; Rhee et al., 2003; Rui et al., 2017; Villarreal et al., 2008; Xiao et al., 2014). Altered expression of POLYGALACTURONASE INVOLVED IN EXPANSION1 (PGX1) results in more flowers with extra petals and more variable angles between sequential floral primordia, suggesting that PG genes can also function in organ morphogenesis (Xiao et al., 2014). However, the functions of PGs in determining organ shape and how they influence cell proliferation and expansion in planar organs such as leaves remain largely mysterious. To bridge the gap between PG activity and organ growth, it is important to understand how PGs influence cellular behaviors during development.

In this study, we characterized a novel Arabidopsis PG gene, PG45, which is expressed in multiple tissues and functions in lateral organ morphogenesis. Subcellular localization and PG activity analysis of PG45 expressed in Escherichia coli confirmed that PG45 is a bona fide PG. Phenotypic characterization and cellular analysis revealed that PG45 modulates leaf flatness by influencing leaf adaxial-abaxial polarity and regulating the duration of cell proliferation in the adaxial epidermis. Biochemical characterization revealed that loss of PG45 leads to reduced total PG activity and uronic acid content in rosette leaves. Autogenous OG profiling was performed in rosette leaves, and showed that both knockout and overexpression of PG45 affect the composition of accumulated OGs. Together, our results indicate that PG45 encodes a PG that links cell proliferation with cell shape to perform unique functions in organ morphogenesis, and that the influence of PG45 on leaf flatness might be coupled with OG signaling.

RESULTS

**PG45 is expressed in meristematic and male reproductive tissues**

While screening a set of mutants for PG genes that are expressed in reproductive tissues in Arabidopsis thaliana, we found a mutant with reduced fertility and undulating rosette leaves, i.e., an insertional allele of At1g02790, which was initially identified from an EST library and named P4GA (Torki et al., 1999). Genotypes and insertion

site were confirmed by genotyping polymerase chain reaction (PCR) and DNA sequencing (Figure S1a,b). Transcript detection by quantitative PCR (qPCR) further confirmed that the pg45 mutant is a knockout mutant (Figure S1c). Based on phylogenetic analysis of the PG family, this gene is part of a largely uncharacterized clade of PG genes, and is distantly related to other members of that clade (Liang et al., 2015, 2016; McCarthy et al., 2014). At1g02790 is syntenic with Brassica rapa PG45/Brassica campestris MALE FERTILITY2, which functions in pollen development (Huang et al., 2009; Liang et al., 2015). Based on this homology with a functionally characterized PG, we named At1g02790 A. thaliana PG45 (AtPG45, hereafter referred to as PG45).

To investigate the temporal and spatial expression patterns of PG45, we performed qPCR on cDNA isolated from different tissues (Figure 1a). PG45 was highly expressed in inflorescences, and was expressed in siliques, rosette leaves, roots and stems, and at a low level in etiolated seedlings (Figure 1a). Expression in different organs and developmental stages was further analyzed using ProPG45:GUS reporter lines. In seedlings, GUS signals were found at shoot and leaf apices, but were not visible in hypocotyls or roots (Figure 1b; Figure S2). In the shoot apex, GUS signals were relatively weak in the SAM, were strong in young leaf primordia, and were prevalent only at the base of developing leaves (Figure 1e; Figure S2b). GUS signals were also detected at rosette leaf axils, which are branch initiation sites (Figure 1c,d). In inflorescences, GUS signals accumulated in anthers, but were not found in other floral organs (Figure 1f,g). During anther and pollen development, GUS signals were first detected in the tapetum at the uninucleate and bicellular stages (Figure 1h,i). After tapetum degradation, the PG45 promoter was highly active in mature pollen grains and in germinated pollen tubes in vitro (Figure 1j,k). Together, these results indicate that PG45 is expressed in multiple tissues and might function in aerial organ formation and male reproductive development.

**PG45 encodes a bona fide PG that localizes to the cell wall**

The protein sequence of PG45 is conserved in PG functional motifs I, II and IV, and contains conserved active-site residues in motif III (Figure S3). Based on the SMART (http://smart.embl-heidelberg.de/) and Phobius (http://phobius.sbc.su.se/) databases, PG45 is predicted to contain a...
signal peptide and a transmembrane domain at the N-terminus (Figure 2b). We next investigated the subcellular localization of PG45 by transforming PG45-tagRFP into the Col background. The localization of PG45-tagRFP was imaged in plasmolyzed roots. PG45-tagRFP signals localized to the cell wall, whereas little signal could be detected in Col control roots (Figure 2a).

To test whether PG45 encodes an active PG, we heterologously expressed PG45 and tested its enzymatic activity. To improve protein solubility, a truncated coding sequence (CDS) including the GH28 domain and C-terminus was cloned into the pSUMO bacterial expression vector (Figure 2b). A band with the predicted size of the fusion protein was visible on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Figure 2c), and was visible after performing Western blots with an anti-His antibody (Figure S4a). Purified 6xHis-SUMO-truncated PG45 protein displayed PG activity when incubated with polygalacturonic acid, whereas almost no PG activity was detected from a 6xHis-SUMO negative control (Figure 2d). Together, these results show that PG45 encodes a bona fide PG.

**PG45 is required for lateral organ morphogenesis during several developmental stages**

To probe the function of PG45 during plant development further, we constructed PG45 overexpression lines driven by cauliflower mosaic virus 35S promoter (CaMV35S). Six single insertion lines were chosen to test PG45 expression levels, and PG45OE5-6, with the highest expression level, was used for further studies. Complementation analysis was also performed by transforming ProPG45:PG45-mNeonGreen into the pg45 mutant background. Five of 10 single insertion lines resembled Col plants, and one of these lines, PG45Comp3, was chosen for further analyses. Compared with Col controls, the expression level (Log2) of PG45 in rosette leaves was 3.6-fold lower in the pg45 mutant, and 8.3- and 7.9-fold higher in PG45OE5-6 and PG45Comp3, respectively (Figure S1d).

One major phenotype we observed in the pg45 mutant and PG45 overexpression lines was abnormal leaf curvature compared with Col controls, where mature rosette leaves were flat or slightly convex but could be flattened without introducing any cuts (Figure 3a). In the pg45 mutant, rosette leaves were often partially concave and curved toward the adaxial side, which was most obvious at the margin of the leaf apex. In PG45OE5-6 plants, rosette leaves were differentially curved from the midrib toward the blade margin, and contained concave domains within an overall convex shape.

Given that PG45 is highly expressed in inflorescences, we also analyzed flower structure in Col, pg45 and PG45OE5-6 plants (Figure 4a,e). Compared with Col controls, shorter open flowers and more flowers with aberrant morphology were observed in pg45 and PG45OE5-6 plants.
In Col control plants, stamens were tetradynamous, with four stamens being longer than the other two. In pg45 plants, 81.0% of flowers had reduced stamen numbers, with most flowers containing four or five stamens. In PG45OE#5-5 plants, 16.8% of flowers only had five stamens, whereas the percentage of flowers with abnormal stamens was 2.1% in Col flowers. Homozygous pg45 mutants had notably lower pollen viability, indicating that PG45 is required for normal pollen development (Figure S5). However, it is possible that this reduced fertility arises from abnormal vegetative growth in pg45 plants. In addition, reduced petal and sepal numbers and aberrant gynoecium structures were found in pg45 flowers, but not in Col or PG45OE#5-5 flowers. In total, over 95% of pg45 flowers were abnormal (Figure 4f). To investigate the early developmental stages of pg45 and PG45OE#5-5 flowers, we imaged dissected inflorescence meristems by scanning electric microscopy (SEM). No obvious difference was found between Col and PG45OE#5-5 floral meristems (Figure 4b). However, pg45 inflorescence meristems were abnormal, with poorly defined phyllotactic patterns and floral organ primordia that were more open, more variable in number, and sometimes stunted (Figure 4b). Together, these results indicate that altered PG45 expression impairs flower morphogenesis, possibly due to aberrant patterning in floral meristems.

Following the vegetative-to-reproductive transition, pg45 mutants also showed a bushy phenotype, with excess branches developing from rosette leaves and stem nodes (Figure 4c,d). In pg45 plants, secondary axillary buds were also found at stem nodes, but were not observed in Col or PG45OE#5-5 plants (Figure 4c). Four to five branches typically emerged from Col and PG45OE#5-5 rosettes, but branches emerged from most rosette leaves in pg45 mutant plants (Figure 4d). In addition, hypocotyl and stem elongation were affected in pg45 and PG45OE#5-5 plants (Figure S6). In 6-day-old dark-grown seedlings, pg45 and PG45OE#5-5 hypocotyls were stunted (Figure S6a,b). In 40-day-old plants, pg45 and PG45OE#5-5 stem heights were 35% and 72% of Col controls, respectively (Figure S6c,d). PG45Comp#3 plants had similar hypocotyl lengths and adult heights as Col controls. Although PG45 was more highly expressed in roots than in hypocotyls, we did not observe obvious differences in root growth in knockout or overexpression plants (Figure S6e). Together, these results suggest that PG45 can also function in organ elongation.

Altered expression of PG45 causes undulating rosette leaves with impaired leaf adaxial–abaxial polarity

Given that abnormal leaf structure is one of the earliest developmental phenotypes observed in knockout and

overexpression mutants of PG45, we next focused on leaf development to investigate how PG45 influences organ morphogenesis. As mentioned above, PG45 expression was detected in rosette leaves (Figure 1a). We first compared the expression level of PG45 and three other PG genes related to leaf development in mature rosette leaves by performing qPCR. PG45 expression levels in Col rosette leaves were comparable with or higher than expression levels of PGX1, PGX2 and PGX3, indicating that PG45 might be important for leaf development (Figure S7c). Leaf undulation was first quantified by measuring leaf curvature index in longitudinal and transverse directions. Compared with Col controls, pg45 and PG45OE#5-5 leaves showed a significantly higher longitudinal curvature index (Figure 3c). A significantly higher transverse curvature index was also found in PG45OE#5-5 leaves (Figure 3d), whereas no significant difference in curvature index in either direction was found between PG45Comp#3 and Col leaves (Figure S7a,b). These quantitative results confirmed that PG45 influences leaf curvature.

To determine whether impaired leaf flatness is caused by altered leaf polarity, we examined the arrangements and shapes of different layers of leaf cells (Figure 3b). In cross-sections of Col leaves, palisade and spongy mesophyll cells showed distinctive morphologies and arrangements: palisade mesophyll cells were vertically elongated and densely packed, whereas spongy mesophyll cells were less regularly shaped and loosely packed. However, in cross-sections of pg45 and PG45OE#5-5 leaf blades, it was much harder to distinguish palisade mesophyll cells from...
spongy mesophyll cells, leading us to name these cell layers “adaxial mesophyll” and “abaxial mesophyll” instead. In total, 89% and 33% of pg45 and PG45OE#5-5 plants, respectively (n = 27 sections total from nine plants per genotype) had densely packed abaxial mesophyll cells. Mesophyll cell shape was quantified by calculating cell height/width ratio. In adaxial mesophyll cells, a significantly higher percentage of cells with height/width ratios < 1 was found in pg45 and PG45OE#5-5 leaves, which had 6.9- and 5.3-fold the number of round adaxial mesophyll cells found in Col, respectively (Figure 3e). Abnormal mesophyll cell shape and arrangement in PG45 knockout and overexpression leaves indicates that PG45 influences leaf adaxial–abaxial polarity.

**PG45 modulates epidermal growth without affecting cell size trends between leaf epidermis**

Given that epidermal growth can restrict leaf shape, we tested whether epidermal growth was correlated with abnormal leaf curvature in pg45 and PG45OE#5-5 leaves. We first compared leaf parameters in fully developed rosette leaves between different genotypes, which were flattened by introducing minimal cuts (Figure 5a). Compared with Col leaves, pg45 and PG45OE#5-5 leaves showed similar lengths and areas, but pg45 leaves were significant smaller than PG45OE#5-5 leaves (Figure 5c). Leaf width and leaf length/width ratio were similar between Col and pg45 mature leaves, whereas PG45OE#5-5 leaves had wider blades and a leaf length/width ratio closer to 1 (Figure 5d–f). We next analyzed whether altered expression of PG45 influences epidermal cell expansion between the adaxial and abaxial epidermis. Compared with Col leaves, smaller epidermal cell area was found on both sides of pg45 leaves, whereas PG45OE#5-5 leaves showed smaller cell area in the adaxial epidermis (Figure 5b,g). However, the reduced cell size in the adaxial epidermis of pg45 and PG45OE#5-5 leaves did not affect the fact that cell size was significantly larger in the adaxial epidermis than the abaxial epidermis for all genotypes (Figure 5g). Together, these results indicate that overexpressing PG45 leads to rounder leaves, but that cell expansion between top and bottom epidermal layers remains coordinated when PG45 expression is altered, suggesting that a loss of this coordination is not responsible for the undulating leaf shapes in PG45 mutant and overexpression lines.

**Loss of PG45 function causes an imbalance in the duration of cell proliferation between the adaxial and abaxial epidermis of developing leaves**

Given that cell expansion and proliferation together influence epidermal patterning, we next investigated whether PG45 influences cell proliferation in developing leaves. Cell division is accompanied by the reorganization of microtubules, which mark the division plane in a preprophase band, form a mitotic spindle at prometaphase, and contribute to the formation of a phragmoplast during cytokinesis (Smith, 2001). The mitotic index was quantified as the percentage of epidermal cells with either a mitotic spindle or phragmoplast, detected by the microtubule marker GFP-MAP4 (Figure 6a). Initial leaf pairs in growing seedlings...
were selected to analyze cell proliferation in the adaxial and abaxial epidermis because these leaves develop synchronously. Given that epidermal cells proliferate and expand in sequential basipetal waves, cells in regions approximately 25% and 75% from the leaf apex were selected to monitor cell division and expansion (Figure 6b). In Col plants at 5 days after sowing (DAS), initial leaf pairs were too small to quantify the mitotic index or cell size reliably, whereas by 11 DAS, the initial leaf pair had a very low mitotic index of 0.15% and 0.69% at 25% and 75% leaf regions, respectively, indicating that cell division was largely complete by then. Therefore, GFP-MAP4 was imaged in 7- and 9-day-old Col and pg45 seedlings.

To determine the developmental phase of the first leaf pairs, we first compared the mitotic index between 25% and 75% regions, which in most cases was significantly lower in 25% regions than in 75% regions for both 7- and 9-day-old Col and pg45 leaves (Figure S8a,b). This result suggests that initial leaf pairs were undergoing diminishing cell proliferation at 7 and 9 DAS. No significant differences in mitotic index between genotypes were found between matched regions on either day, indicating that PG45 does not influence the proportions of cells undergoing mitosis within a specific leaf region at a given developmental stage (Figure S8a,b). To analyze the duration of cell proliferation in the epidermis, we calculated ratios of mitotic index between 25% and 75% regions (Figure 6c). The 25%-75% mitotic index ratios from 7- and 9-day-old plants were combined to analyze the duration of cell proliferation, as there was no significant difference between these two time points (Figure S8c). The 25%-75% mitotic index ratio was significantly lower in the adaxial epidermis than the abaxial epidermis of pg45 leaves, whereas no significant difference was found between the adaxial and abaxial epidermis of Col leaves. Compared with Col leaves, the 25%-75% mitotic index ratio was significantly lower in the adaxial epidermis of pg45 leaves. Together, these results suggest that loss of PG45 leads to a shortened duration of the basipetal wave of cell proliferation in the adaxial epidermis, resulting in unbalanced cell numbers between epidermal sides and contributing to the undulating shapes of pg45 leaves.

In addition, we analyzed how cell expansion is coordinated during the overlapping transition from cell proliferation to cell expansion along the leaf axis. Larger cell size was found in pg45 leaves than in Col leaves in 25% regions at 7 DAS, and in 75% regions at 9 DAS (Figure S8d,e). These differences in cell size could result from either asynchronous transition rates or differential cell expansion rates. Therefore, we calculated relative cell expansion rates between 9- and 7-day-old plants. Relative expansion rates at 25% and 75% regions from the same epidermis side were combined, as no significant difference was found between these two regions (Figure S8f). No significant difference was evident in cell expansion rates between genotypes or between the adaxial and abaxial epidermis (Figure 6d). These results indicate that instead of differences in cell expansion rate, differing paces of

Figure 6. PG45 regulates the duration of pavement cell proliferation in seedlings. (a) Phragmoplast (left) and mitotic spindle (right) from the abaxial epidermis of a rosette leaf. Microtubules (green) and propidium iodide staining (magenta) are shown. Maximum projection of Z-stack; Bar = 10 μm. (b) Schematic of imaging regions of the first leaf pairs (dotted lines). (c) 25%-75% mitotic index ratios of the first leaf pairs in seedlings. n = three biological replicates from 7- and 9-day-old plants, respectively. In each biological replicate, 12–16 fields of view per region were imaged for three plants. For each genotype, at least 1431 and 3134 pavement cells were analyzed from 25% and 75% regions, respectively. Lines are medians. *P < 0.05 (Mann-Whitney test). (d) Relative expansion rates of the first leaf pairs in seedlings. n = three biological replicates from 25% regions and 75% regions, respectively. At least 270 pavement cells were measured from each region per genotype. Lines are medians. N.S., no significant difference (Mann-Whitney test).
transitioning from cell division to expansion cause the differences in cell size we observed in developing pg45 leaves (Figure S8d,e).

**PG45 influences total PG activity and uronic acid content in rosette leaves with altered autogenous OG profiling**

Given that PG45 displays PG activity in vitro, we next tested whether altered PG45 expression affects pectin metabolism in plant tissues. In protein extracts from rosette leaves, total PG activity was significantly lower in pg45 than in Col controls, whereas total PG activity did not differ between PG45OE#5-5 and Col controls (Figure 7a). Total proteins were also extracted from inflorescences, where PG45 is highly expressed. Consistent with the result from rosette leaves, total protein from pg45 inflorescences, but not from PG45OE#5-5 inflorescences, possessed lower PG activity than protein from Col inflorescences (Figure S4b). We also tested whether altered expression of PG45 affects pectin content in plant tissues. Total uronic acid content was measured in cell wall-enriched alcohol insoluble residue (AIR) extracted from 31-day-old rosette leaves (Figure 7b). We found lower uronic acid content in pg45 AIR, whereas no difference was observed between PG45OE#5-5 and Col AIR. Together, these results indicate that mutation of PG45 affects both total PG activity and pectin content in rosette leaves.

To analyze how PG45 influences pectin degradation in rosette leaves further, we next analyzed autogenous OGS, which are produced in planta by endogenous PGs and PLs and are hypothesized to regulate plant development. High-performance size exclusion chromatography–mass spectrometry (HP-SEC-MS) was used to distinguish OGS with different DPs and modification status. In 31-day-old rosette leaves, OGS of DP 2–15 and different modifications were detected from all genotypes (Figure 7c; Figure S9). Most of the OG products were saturated and would thus be expected to arise from hydrolysis by PGs. Unsaturated OGS such as GalA6Me2H2O and GalA6Me2AC2H2O were detected in pg45 leaves but not in Col and PG45OE#5-5 leaves, and are predicted to arise from β-elimination of HG by PLs. For OGS of the same DP, for example, GalA6 with methyl and/or acetyl groups (Figure 7c). In Col leaves, GalA6 was the most abundant OG, comprising 25.0% of the total OGS. In pg45 leaves, GalA6 was also the most abundant, comprising 31.0% of the total OGS. In PG45OE#5-5 leaves, the relative abundance of GalA6 was lower than in Col controls, and GalA6 instead accounted for the highest percentage, which comprised 21.5% of total OGS. OGS of DP 2–7 comprised 76.8%, 93.2% and 89.1% of total OGS in Col, pg45 and PG45OE#5-5 leaves, respectively. Within this range, knockout of PG45 led to higher proportions of longer OGS, whereas overexpressing PG45 led to the accumulation of shorter OGS. In addition, OGS of DP 8–15 were less abundant in both pg45 and PG45OE#5-5 leaves. Together, these results suggest that both knockout and overexpression of PG45 alter the OG profiles in rosette leaves, and that PG45 might be specifically capable of degrading GalA6, resulting in the accumulation of GalA2, GalA3 and GalA6.

**DISCUSSION**

As major constituents of primary cell walls in eudicots, pectins play a vital role in plant morphogenesis. Previous studies have highlighted the influence of pectin abundance and methylesterification status on cell shape and organ patterning (Haas et al., 2020; Peaucelle et al., 2008, 2011; Qi et al., 2017; Saffer et al., 2017). However, how pectin depolymerization, which is mediated by PGs and PLs, influences organ morphogenesis is less well understood. Here, based on the functional characterization of PG45, we generated new insights into how pectin degradation functions in organ morphogenesis. We demonstrated that PG45, which encodes a bona fide PG, is required for leaf and flower structure, as well as branch formation, in A. thaliana (Figures 3 and 4).

Up to now, only nine PG genes have been characterized from the large PG gene family in A. thaliana, and five of them have been demonstrated to have PG activity in vitro (Babu et al., 2013; Hocq et al., 2020; Ogawa et al., 2009; Rhee et al., 2003; Rui et al., 2017; Xiao et al., 2014, 2017). We confirmed that PG45 cleaves pectin in vitro (Figure 2d), and influences pectin degradation in planta (Figure 7a; Figure S4). Loss-of-function mutation of PG45 caused reduced total PG activity in rosette leaves and inflorescences (Figure 7a; Figure S4). Although total PG activity did not differ significantly between AtPG45OE#5-5 and Col leaves, higher proportions of small-sized OGS were found in PG45 overexpression lines (Figure 7c), suggesting that overexpressing PG45 also affects pectin degradation in vivo. A previous study showed that gene overexpression could induce silencing of homologous genes (Amack and Antunes, 2020). Therefore, one potential interpretation of the unchanged total PG activity detected in PG45 overexpression plants is that expression levels of closely related PGs might be diminished. Unexpectedly, in addition to the hypothesized decrease in total PG activity, lower uronic acid content was also observed in pg45 rosette leaves (Figure 7b). One possible explanation for this result is that decreased PG activity triggers a feedback loop to regulate pectin synthesis negatively.

Torki et al. (2000) proposed that PG45 encodes an exo-PG, because it is expressed in pollen and only exo-PG activity has been detected in pollen extracts. Exo-PGs cause the accumulation of GalA monomers (Tanaka et al., 2002). In contrast, knockout of PG45 results in the accumulation of GalA6, whereas overexpressing PG45 leads to a lower GalA6 level and overaccumulation of GalA2, GalA3 and GalA6 (Figure 7c). Similar with PG45 overexpression lines,
Figure 7. PG45 affects total polygalacturonase (PG) activity, uronic acid content and oligogalacturonide (OG) profiles in rosette leaves.
(a) Total PG activity is reduced in pg45 rosette leaves. Total plant proteins were extracted from 31-day-old rosette leaves. Lines are medians, \( n = \) three biological replicates (pools of leaves) per genotype, with each data point being an average of three technical replicates. Different letters denote \( P < 0.05 \) (one-way ANOVA and Tukey test).
(b) Uronic acid content is lower in pg45 rosette leaves. Alcohol insoluble residue (AIR) was prepared from 31-day-old rosette leaves. Lines are medians, \( n = \) three biological replicates (pools of leaves) per genotype, with each data point being an average of three technical replicates. Different letters denote \( P < 0.05 \) (one-way ANOVA and Tukey test).
(c) Altered expression of PG45 affects OG accumulation in rosette leaves. Autogenous OGs were extracted from 31-day-old rosette leaves and analyzed by high-performance size exclusion chromatography-mass spectrometry (three biological replicates per genotype). Saturated OGs are named as Gal\(A_y\)Me\(z\)Ac\(x\), unsaturated OGs are named as Gal\(A_y\)Me\(z\)Ac\(x\)-H\(2\)O. “\(x\)” denotes the degree of polymerization, and “\(y\)” and “\(z\)” denote the number of methylester and acetyester groups, respectively.
overexpressing a fungal endo-PG in Arabidopsis also leads to curly rosette leaves (Capodicasa et al., 2004). These results support the hypothesis that PG45 functions as an endo-PG to influence leaf shape formation. Further studies of enzymatic products from purified PG45 protein and determination of the atomic structure of PG45 might provide more clues concerning the mode of action of PG45.

In this study, both knocking out and overexpressing PG45 led to undulating rosette leaves. No PG knockout mutant has previously been reported to have undulating rosette leaves, and abnormally curved rosette leaves are found only in overexpression lines for another PG gene, QUARTET2 (QRT2) (Ogawa et al., 2009). Loss of PGX3 causes a higher proportion of concave cotyledons without influencing rosette leaf flatness (Rui et al., 2017). These results support the idea that PG genes are required to maintain normal leaf curvature, but the mechanisms by which they might do so have previously remained undefined. We found that PG45 mediates leaf flatness by influencing leaf adaxial–abaxial polarity and epidermal epidermal growth (Figures 3 and 6). The relationship between adaxial–abaxial polarity and leaf epidermal growth has been revealed by phenotypic analyses of hormone- and transcription-related genes (Yamaguchi et al., 2012). In maintaining leaf adaxial–abaxial polarity, auxin response factors (ARFs) such as ARF2, ETTIN (ETT/ARF3) and ARF4 function as repressors in the abaxial domain by binding to auxin-response DNA elements in gene promoters (Du et al., 2018; Li et al., 2016). Similar to ARF2-4, OGs of DP 9–16 repress the activity of the auxin-responsive promoter DR5, and inhibition by OGs is dose-dependent (Savatin et al., 2011). In pg45 knockout and PG45 overexpression lines, we found that OGs of DP 9–15 are less abundant than in Col plants (Figure 7c; Figure S9). Given that concave rosette leaves are also found in arf2 arf4 double mutants (Pekker et al., 2005), we speculate that PG45 might influence leaf polarity by producing OGs of specific DP ranges that trigger an OG feedback loop that feeds into ARF-mediated regulation of leaf polarity.

Apart from leaf polarity, PG45 also regulates leaf shape by influencing epidermal growth. Kinematic studies reveal that cell proliferation and expansion modulate epidermal growth (Andriankaja et al., 2012; Aasl et al., 2011). However, how adaxial and abaxial epidermal growth is coupled to leaf shape is not fully understood. In this work, we found that the adaxial and abaxial epidermis showed similar cell proliferation patterns and relative cell expansion rates in developing Col leaves, whereas the adaxial and abaxial epidermises of pg45 leaves were similar in relative cell expansion rate; however, the adaxial epidermis had a shortened duration of cell proliferation, which coincided with the undulating leaves observed in the mutant (Figure 6; Figure S8). Cytokinin negatively regulates the duration of cell proliferation during leaf initiation (Holst et al., 2011), and OGs can promote cytokinin-induced meristem formation and shoot growth in tobacco leaf explants (Falasca et al., 2020). To determine whether OGs modulate the duration of cell proliferation by regulating cytokinin levels will require further studies.

The functions of PGs in cell separation and expansion have been highlighted previously (Ogawa et al., 2009; Rhee et al., 2003; Xiao et al., 2014, 2017). Although PG45 is expressed in siliques, we did not observe dehiscence defects in pg45 and PG45ΔE45-6 siliques, suggesting that PG45 might not be essential for cell separation. Instead, PG45 functions in cell and tissue morphogenesis: pg45 plants had reduced hypocotyl lengths and adult heights (Figure S6), and PG45ΔE45-6 rosette leaves were wider than Col controls (Figure 5). Although overall leaf area did not differ between pg45 and Col rosette leaves, pavement cell size on both epidermal sides was smaller in pg45 rosette leaves (Figure 5). Unlike the excess growth of hypocotyls in PGX1 and PGX2 overexpression lines (Xiao et al., 2014, 2017), we observed growth defects in hypocotyls, flowers and stems of PG45 overexpression plants (Figure S6). Similarly, reduced plant height and dwarf rosettes are found in QRT2 overexpression lines, which also have curved leaves (Ogawa et al., 2009). Ectopically expressed ARABIDOPSIS-DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1) releases OG elicitors and triggers defense signaling, resulting in severe growth defects in lignin-modified mutants (Gallego-Giraldo et al., 2020), and exogenous OGs inhibit indole-3-acetic acid-induced elongation of pea stem segments (Branca et al., 1988). Therefore, a possible explanation for the growth defects in PG45 overexpression plants is that overaccumulation of PG45 releases excess OGs that elicit defense responses, inhibiting normal plant growth.

In conclusion, the functional characterization of PG45 in leaf and flower morphogenesis sheds light on how PGs and pectin degradation regulate organ morphogenesis. Our results demonstrate that apart from cell expansion and cell separation, PGs can regulate cell proliferation and cellular morphogenesis to influence tissue polarity. The functions of PG45 in plant growth could be linked with altered autogenous OG profiling, which further triggers developmental responses. Future studies of the molecular mechanisms underlying this regulation will add an additional dimension to the large and deeply conserved PG family and its functional interfaces with developmental regulatory pathways in plants.

**Experimental Procedures**

**Plant material and growth conditions**

Arabidopsis seeds were sterilized with 30% bleach + 0.1% sodium dodecyl sulfate in water for 20 min, then washed four times with sterile water. For vernalization, seeds were resuspended in 0.15% w/v agar (Sigma-Aldrich), and stored in the dark at 4°C for 3 days.
Seeds were sown on plates containing 1/2 strength 2.2 g L⁻¹ Murashige and Skoog salts (Caisson Laboratories), 0.6 g L⁻¹ MES monohydrate, 1% sucrose, 0.8% agar, pH 5.6. Plates were placed vertically in a 22°C growth chamber with 24-h light. For adult plant growth, 10-day-old seedlings were transplanted into soil, and plants were grown in a 22°C growth room (Conviron) with 16 h light/8 h dark. For dark-grown seedlings, seeds were sown on 0.7 Murashige and Skoog plates without sucrose and then plates were wrapped with aluminum foil before being placed vertically in a 22°C chamber. Elotted seedlings were scanned using a Scanjet 8300 flatbed scanner (HP), and hypocotyl length was measured using ImageJ software (https://www.imagej.nih.gov).

**Plasmid construction and plant transformation**

For constructing a promoter reporter vector, the 889-bp region between the PG45 (At1g02790) start codon and the upstream gene was amplified from Col genomic DNA and cloned into pENTR/D-TOPO (Invitrogen). The promoter fragment was then recombined into pBGWFP7.0 (Karimi et al., 2002). Transgenic lines expressing ProPG45:GFP-GUS were selected using 0.002% (w/v) Basta. An Spm transposon insertion line (Tissier et al., 1999) we named pg45 (stock number CS117498) was obtained from ABRC. For generating overexpression lines, the full-length PG45 CDS (1269 bp) was amplified from Col inflorescence cDNA and cloned into pBl21 (Chen et al., 2003). Transgenic lines containing ProCaMV35S:PG45 were selected with 50 μg ml⁻¹ kanamycin (Omega Scientific). For complementation tests, the promoter fragment and CDS of PG45 were ligated by overlap PCR and cloned into a modified pMDC10 vector (Curtis and Grossniklaus, 2003) with GFP replaced by mNeonGreen (Shaner et al., 2013). ProPG45:PG45-mNeonGreen was transformed into the pg45 mutant, and transgenic lines were selected with 25 μg ml⁻¹ hygromycin (Omega Scientific). To image microtubules, GFP-MAP4 (Marc et al., 1998) was transformed into Col-0 and pg45 mutant plants, and transgenic lines were selected with 25 μg ml⁻¹ hygromycin. All transformations mentioned above were performed with Agrobacterium tumefaciens strain GV3101 using the floral dip method (Clough and Bent, 1998). Primers for genotyping and cloning are listed in Table S1.

**Gene expression analysis**

For qPCR experiments, total RNA was extracted from different plant tissues using a NucleoSpin® RNA Plant kit (Machery-Nagel). Quanta qScript cDNA Supermix (Quantabio) was used to synthesize cDNA from 1 μg total RNA. qPCR was performed using Quanta PerfeCTa SYBR Green Supermix (Quantabio). Primer pairs listed in Table S1 were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/Toolbox/primer-blast). All primer sequences are listed in Table S1. For genotyping and cloning are listed in Table S1.

**Leaf parameter analysis**

Fifth rosette leaves were collected from 28-day-old plants to measure leaf parameters. Leaves were imaged before and after flattening, with minimal cuts introduced to flatten leaves. Leaf length and width before and after flattening were measured to calculate leaf curvature index. Longitudinal leaf curvature index was calculated as: (leaf length after flattening – leaf length before flattening)/leaf length before flattening. Transverse leaf curvature index was calculated similarly. The wand tracing tool in ImageJ was used to quantify leaf area, and color threshold was adjusted to 38–45 to remove background coloration.

Pavement cell number and cell size were measured in 30-day-old plants. Two longitudinally symmetric square segments were collected from middle regions of fifth rosette leaves. To label cell outlines, leaf samples were stained with 0.1 mg ml⁻¹ (w/v) propidium iodide (Life Technologies) for 5 min, then washed with water. Images were collected using a Zeiss Cell Observer SD spinning disk confocal microscope with a 561-nm excitation laser and a 617/673-nm emission filter, and a 20 × 0.5 NA objective. Pavement cell number and mitotic index were measured from plants expressing the GFP-MAP4 marker at 5, 7, 9 and 11 DAS. A 488-nm excitation laser and 525/550-nm emission filter were used to detect GFP-MAP4 signal on the spinning disk confocal microscope with an 83 × 1.4 NA oil and 40 × 1.3 NA oil objectives. Pavement cell size was measured from intact cells in fields of view in ImageJ. Relative cell expansion rate was calculated as: (cell size at 9 DAS/cell size at 7 DAS)/2 days.

For mesophyll cell parameter measurement, leaf sectioning was performed as described by Rui et al. (2017) with minor modifications. Rosette leaves from 35-day-old plants were cut into 7 × 5 mm segments and fixed in 4% paraformaldehyde in PEM buffer. 0.1 mol L⁻¹ piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2 mmol L⁻¹ ethylene glycol-bis[β-aminoethyl ether]-N,N',N',N'-tetraacetic acid (EGTA), 1 mmol L⁻¹ magnesium sulfate, pH 7.2) using vacuum infiltration. Leaf sections were rinsed in PEM buffer and dehydrated in an ethanol series (30%, 50%, 70% and 100% for 15 min each). Dehydrated samples were infiltrated with a dilution series of LR white resin (Electron Microscopy Sciences; 10%, 20%, 30%, 50%, 70%, 90%, 100% v/v in ethanol for 15 min each). For LR white resin polymerization, samples were embedded in gelatin capsules (Ted Pella) and incubated at 65°C for 3 days. Sections of 1-μm thickness were cut on a Leica UC6 ultramicrotome and stained with 0.05% (w/v) toluidine blue for imaging. Images were collected on a Zeiss Cell Observer SD spinning disk confocal microscope with a Nikon DS510 DSR camera. ImageJ was used to quantify mesophyll cell height and width, measured perpendicular and parallel to the epidermal plane, respectively.

**SEM analysis of floral meristems**

Dissected inflorescences from 5-week-old plants were vacuum infiltrated in fixation buffer (2.5% glutaraldehyde in 0.1 mol L⁻¹ sodium cacodylate) and incubated at 4°C overnight. Sample fixation and SEM analysis were performed using a modified protocol from Xiao et al. (2014). Fixed samples were rinsed with 0.1 mol L⁻¹ sodium cacodylate three times and dehydrated in an ethanol series (30%, 50%, 70%, and 100%, for 20 min each). Samples were critical-point dried (Leica EM CPD300), and imaged using a Zeiss SIGMA VP-FESEM SEM. Images were collected in variable pressure mode, with extra-high tension acceleration voltage set to 7.00 kV.

**Expression and purification of PG45**

To improve protein solubility, a truncated PG45 CDS was cloned into the pSUMO bacterial expression vector. Protein expression and purification were performed following Xiao et al. (2014). The generated 6xHis-SUMO-PG45 vector and a pSUMO empty vector...
control was transformed into E. coli strain BL21-DE3, and expression was induced with 1 mmol L⁻¹ isopropyl-β-D-thiogalactopyranoside (GoldBio) at 25°C for 4 h. Cells were harvested by centrifugation at 10,000 g for 10 min at 4°C, and resuspended in protein extraction buffer (10 ml g⁻¹ of harvested wet cells) containing 50 mmol L⁻¹ sodium phosphate, 300 mmol L⁻¹ sodium chloride, 10 mmol L⁻¹ 2-mercaptoethanol, 1 mmol L⁻¹ phenylmethylsulfonyl fluoride, 10 mmol L⁻¹ imidazole and 2.5 µl ml⁻¹ DNase, pH 8.0. Resuspended cells were sonicated in a probe sonicator for 3 × 10 sec pulses, and then centrifuged at 10,000 g for 20 min at 4°C. For protein separation, supernatants were loaded on to 12% Mini-PROTEAN precast gels (Bio-Rad) on a Mini-PROTEAN Tetra Cell (Bio-Rad). After electrophoresis, gels were stained with GelCode Blue (Thermo Fisher Scientific) for imaging. For protein purification, supernatants were incubated with Ni-NTA Agarose (5 g ml⁻¹) then mixed with 14% acetone and air-dried in a chemical fume hood. Washing with 70% ethanol (45 min each) at room temperature, followed with 70% ethanol overnight. Supernatants were collected and dialyzed in 0.15% sodium chloride and 2-mercaptoethanol, 0.1 mmol L⁻¹ phenylmethylsulfonyl fluoride, 0.1 mmol L⁻¹ imidazole and 2 mol L⁻¹ DTT, pH 8.0. Bound proteins were eluted using elution buffer (50 mmol L⁻¹ sodium phosphate, 300 mmol L⁻¹ sodium chloride and 250 mmol L⁻¹ imidazole, pH 8.0), and then dialyzed in 50 mmol L⁻¹ sodium acetate, pH 5.0, at 4°C overnight. SpectraPor/MWCO 12-14 kDa membrane and SpectraPor MWCO 3.5 kDa membrane (Spectrum Labs) were used for dialysis of 6xHis-SUMO-PG45 and 6xHis-SUMO, respectively.

**Plant protein extraction**

Plant proteins were extracted from 31-day-old rosette leaves and 35-day-old inflorescences. One gram of plant tissue was ground into powder in liquid nitrogen. Plant proteins were extracted using extraction buffer (50 mmol L⁻¹ Tris-HCl, pH 7.5, 3 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA), 2.5 mmol L⁻¹ DTT (Sigma-Aldrich), 2 mmol L⁻¹ phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich), 1 mol L⁻¹ sodium chloride and 10% (v/v) glycerol). Supernatants were collected and dialyzed in 50 mmol L⁻¹ sodium acetate buffer (pH 5.0) with SpectraPor/MWCO 12-14 kDa dialysis membrane (Spectrum Labs) at 4°C overnight.

**PG activity analysis**

Protein samples purified from E. coli or extracted from plant tissues were incubated with 0.5% (w/v) polygalacturonic acid (Sigma-Aldrich) in 37.5 mmol L⁻¹ sodium acetate (pH 4.4) at 30°C for 3 h. PG activity was determined by measuring increases in reducing end groups, which were labeled with 2-cyanoacetamide (Sigma-Aldrich), 1 mol L⁻¹ sodium chloride and 10% (v/v) glycerol. Supernatants were stained with GelCode Blue (Thermo Fisher Scientific) for imaging. For protein separation, supernatants were incubated with Ni-NTA Agarose (5 g ml⁻¹) then loaded on to a Pierce™ disposable purification column (Thermo Fisher Scientific). The purification column was washed with extraction buffer and washing buffer (50 mmol L⁻¹ sodium phosphate, 300 mmol L⁻¹ sodium chloride and 20 mmol L⁻¹ imidazole, pH 8.0). Bound proteins were eluted using elution buffer (50 mmol L⁻¹ sodium phosphate, 300 mmol L⁻¹ sodium chloride and 250 mmol L⁻¹ imidazole, pH 8.0), and then dialyzed in 50 mmol L⁻¹ sodium acetate, pH 5.0, at 4°C overnight. SpectraPor/MWCO 12-14 kDa membrane and SpectraPor MWCO 3.5 kDa membrane (Spectrum Labs) were used for dialysis of 6xHis-SUMO-PG45 and 6xHis-SUMO, respectively.

**AIR preparation**

AIR was prepared from 31-day-old rosette leaves: 1.5 g sample was ground into powder in liquid nitrogen and washed with 1.5 ml chloroform/methanol (v/v = 1:1) on a shaker four times (45 min each) at room temperature, followed with 70% ethanol three times (60 min each). Pellets were resuspended with 100% acetone and air-dried in a chemical fume hood.

**Uronic acid content determination**

One milligram of AIR was resuspended with 141 µl Milli-Q water, then mixed with 14 µl 4 mol L⁻¹ sulfamic acid-potassium sulfamate (pH 1.6) and 845 µl concentrated sulfuric acid in 75 mmol L⁻¹ sodium tetraborate. Samples were heated in boiling water bath for 5 min, and quickly cooled down to room temperature in a water bath. Initial absorbances at 525 nm were measured from 10× diluted samples using a NanoDrop 2000C. Samples were mixed with 28.2 µl 0.15% m-hydroxydiphenyl in 0.5% sodium hydroxide and then incubated at room temperature for 5 min. Final absorbance was measured at 525 nm. α-Galacturonic acid was used to make a standard curve. Uronic acid content of AIR was calculated based on the standard curve and absorbance difference between the final and the initial.

**OG profiling analysis from rosette leaves**

AIR was extracted from 0.1 g 31-day-old rosette leaves, then resuspended in ultrapure water (10 ml g⁻¹ of AIR) and incubated at 4°C overnight. After centrifuging at 18,000 g for 15 min, the pellet was collected and resuspended in 200 µl chelating solution (50 mmol L⁻¹ 1,2-cyclohexylenedinitrilo-tetraacetic acid (CDTA), 50 mmol L⁻¹ ammonium oxalate, 50 mmol L⁻¹ ammonium acetate, pH 5.5). Samples were incubated at 4°C overnight and centrifuged at 18,000 g for 20 min. The supernatant was mixed with an equal volume of 20% ethanol, and precipitated at 4°C overnight, followed by precipitation with 80% ethanol. The supernatant was dried by speed vacuum.

OG characterization and quantification were performed following the HP-SEC-MS method described by Voeue et al. (2019). Dried samples were dissolved in 80 µl mobile phase (50 mmol L⁻¹ ammonium formate, 0.1% formic acid) and 10 µmol samples were injected for HP-SEC-MS analysis. An ACQUITY UPLC Protein BEH SEC Column (125 Å pore size, 1.7 µm particle size, 4.6 mm inner diameter × 300 mm length; Waters Corporation, USA) was used for OG separation. The flow rate of the mobile phase was set to 400 µl min⁻¹ for performing elution. The SEC column oven temperature was set at 40°C. Vanquish HPLC and Q Exactive mass spectrometers (Thermo Fisher Scientific) were used for HP-SEC-MS analysis. OG analysis was performed with xCALIBUR™ software (Thermo Fisher Scientific). Relative abundance of OGs was quantified by calculating the areas under the curves.

**Statistics**

All statistical analyses were performed using PAST software (Hammer et al., 2001).

**ACCESSION NUMBERS**

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: PG45 (At1g02790), BETA-TUBULIN4 (At5g44340), ACTIN2 (At3g18780), PGX1 (At3g26610), PGX2 (At1g78400), PGX3 (At1g48100), ADPG1 (At3g57510), ADPG2 (At2g41850), NMA (At2g33160), and QRT2 (At3g07970). Mutant germplasm of pg45 (CS117489) was obtained from ABRC (https://abrc.osu.edu).

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AUTHOR CONTRIBUTIONS
YY, CTA and JC designed experiments YY performed experiments YY, CTA and JC analyzed data and wrote the paper.

CONFLICT OF INTERESTS
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
All relevant data can be found within the manuscript and its supporting information.

SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Polygalacturonase45 (PG45) genotyping and transcript detection. (a) Schematic gene structure of PG45. Exons shown as dark blue boxes, introns as lines, 5' and 3' regions as light blue boxes. Defective 5'mp insertion site for pg45 mutant is shown with green triangle, and binding sites of quantitative PCR (qPCR) primers are shown as arrows. (b) Genotyping PCR result in Col and homozygous pg45 plants. (c) Expression level of PG45 in Col and pg45 inflorescences by performing qPCR. (d) Expression level of PG45 in Col, pg45, PG45OE and PG45Comp plants by qPCR. Actin2 was used as an internal control. Error bars are standard error (SE). n = three biological replicates from three independent pools of tissues.

Figure S2. GUS staining in a 4 days and a 15-day-old ProPG45::GUS plant. (a) GUS staining in a 4-day-old seedling, with zoom-in view of shoot apical in (b) and leaf tip in (c). (d) GUS staining in a 15-day-old ProPG45::GUS plant. Bar = 50 μm in (a)-(d).

Figure S3. Protein sequence alignment of PG45 and seven characterized PGs in Arabidopsis thaliana. Characterized Arabidopsis polygalacturonases (PGs) includes: ADPG1, ADPG2, NIMNA (NMA), PGX1, PGX2, PGX3 and ORT2. Red rectangles show the conserved motifs in plant and fungal PGs. NTD, DD, GHG and RIK are the conserved amino acid segments in motif I-IV, respectively. Sequence data of these PGs can be found under accession numbers: PG45 (At1g02790), PGX1 (At3g26610), PGX2 (At1g78400), PGX3 (At1g48100), ADPG1 (At2g57510), ADPG2 (At2g41850), NMA (At2g33180) and ORT2 (At3g07970).

Figure S4. Immunoblotting of 6xHis-SUMO-PG45; total PG activity is reduced in pg45 inflorescences. (a) Immunoblotting using anti-His antibody shows the purified 6xHis-SUMO-truncated PG45 with predicted size. (b) Total PG activity is reduced in pg45 inflorescences. Total plant proteins were extracted from 35-day-old inflorescences. One unit of PG activity is the amount of enzyme releasing 1 μmol reducing end group per minute per gram total protein at 30°C. Lines are medians, n = four biological replicates per genotype, and each biological replicate has three technical replicates. Each biological replicate uses independent pool of inflorescences. Different letters denote P < 0.05 (one-way analysis of variance [ANOVA] and Tukey test).

Figure S5. Pollen activities in Col and pg45 anthers. Anthers of Col and pg45 plants were stained with Alexander stain, Bar = 50 μm.

Figure S6. PG45 functions in seedling growth and plant stature, but does not affect root growth. (a) 6-day-old etiolated seedlings of Col, pg45, PG45OE and PG45Comp, Bar = 0.5 cm. (b) Hypocotyl length of 6-day-old dark-grown seedlings. n ≥ 59 seedlings per genotype from two independent experiments. Different letters denote P < 0.05 (one-way ANOVA and Tukey test). (c) 40-day-old plants of Col, pg45, PG45OE and PG45Comp, Bar = 2 cm. (d) Plant heights of 40-day-old Col, pg45, PG45OE and PG45Comp plants. n ≥ 31 plants per genotype from two independent experiments. Different letters denote P < 0.05 (one-way ANOVA and Tukey test). (e) Roots of 10-day-old light-grown seedlings of Col, pg45, PG45OE and PG45Comp lines.

Figure S7. Measurements of leaf curvature of PG45Comp rosette leaves and expression level of PG genes in rosette leaves. (a,b) Leaf curvature quantification in fifth rosette leaves of 28-day-old Col, pg45 and PG45Comp plants. n ≥ 40 plants per genotype from three independent experiments. Different letters denote P < 0.05 (one-way ANOVA and Tukey test). N.S., no significant difference. Error bars are SE. (c) qPCR quantification of PG45, PGX1, PGX2 and PGX3 expression levels in Col rosette leaves. Actin2 (At3g18780) was used as an internal control, and expression of PG45 was normalized to 1. Error bars are SE, n = three biological replicates from three independent pools of tissues.

Figure S8. Mitotic index and cell size measurement in seedlings. (a,b) Mitotic index of Col and pg45 first leaf pairs. n = 9 plants per genotypes from three independent experiments. Four to six fields of view per region per plant were imaged for each genotype. At least 1431 and 3134 pavement cells were analyzed from 25% and 75% regions per genotype, respectively. Ad: adaxial epidermis; Ab: abaxial epidermis. Error bars are SE. *P < 0.05 (t-test). (c) 25%-75% mitotic index ratios between 7- and 9-day-old Col and pg45 first leaf pairs. n = 3 biological replicates. Lines are medians. No significant difference was found between 7- and 9-day-old plants within genotypes (Mann-Whitney test). (d,e) Pavement cell size of Col and pg45 first leaf pairs. n = 9 plants per genotypes from three independent experiments. For each region, cell sizes were quantified from at least 299 pavement cells per genotype. Ab, abaxial epidermis; Ad, adaxial epidermis. Error bars are SE. *P < 0.05 (t-test). (f) Relative cell expansion rates between 25% and 75% regions of first leaf pairs. N = three biological replicates. Lines are medians. No significant difference was found between 25% and 75% regions within genotypes (Mann-Whitney test).

Figure S9. Composition of OGs with different degrees of polymerization in rosette leaves. Data points represent means of three biological replicates from three independent experiments.

Table S1. Primers used in this study.

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