The Protein Phosphatases ATUNIS1 and ATUNIS2 Regulate Cell Wall Integrity in Tip-Growing Cells

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INTRODUCTION

Fast tip-growing plant cells such as pollen tubes (PTs) and root hairs (RHs) require a robust coordination between their internal growth machinery and modifications of their extracellular rigid, yet extensible, cell wall (CW). Part of this essential coordination is governed by members of the Catharanthus roseus receptor-like kinase1-like (CrRLK1L) subfamily of RLKs with FERONIA (FER) and its closest homologs, ANXUR1 (ANX1) and ANX2, controlling CW integrity during RH and PT growth, respectively. Recently, Leucine-Rich Repeat Extensin 8 (LRX8) to LRX11 were also shown to be important for CW integrity in PTs. We previously reported an anx1 anx2 suppressor screen in Arabidopsis thaliana that revealed MARIS (MRI) as a positive regulator of both FER- and ANX1/2-dependent CW integrity pathways. Here, we characterize a suppressor that exhibits a weak rescue of the anx1 anx2 PT bursting phenotype and a short RH phenotype. The corresponding suppressor mutation causes a D94N substitution in a Type One Protein Phosphatase we named ATUNIS1 (AUN1). We show that AUN1 and its closest homolog, AUN2, are nucleocytoplasmic negative regulators of tip growth. Moreover, we demonstrate that AUN1DN and AUN1H127A harboring mutations in key amino acids of the conserved catalytic site of phosphoprotein phosphatases function as dominant amorphic variants that repress PT growth. Finally, genetic interaction studies using the hypermorph MRI R240C mutant in fast growing cells like tip-growing pollen tubes (PTs) and root hairs (RHs). PTs deliver the sperm cells to the female gametophyte. Emerging PT does not lose integrity before reaching the female gametophyte. Successful fertilization depends on the interaction of the pollen tube with the female gametophyte. Pollen germination is essential for the growth of the pollen tube, which penetrates the floral tissues to reach the female gametophyte. Once a pollen tube has successfully reached the female gametophyte, it continues to grow until it reaches the female gamete, initiating the process of fertilization.

PTs play a crucial role in the reproductive process of many plant species, facilitating the transfer of male gametes (sperm) from the pollen to the egg cells of the female gametophyte within the ovule. By doing so, PTs ensure the successful production of seeds and the propagation of plant species. The ability of PTs to grow and navigate through the female reproductive tissues is tightly regulated and is crucial for the survival and reproduction of plants. Understanding the mechanisms that control PT growth is essential for the development of strategies to improve plant productivity and breed new varieties with improved traits.
IN A NUTSHELL

Background: Plant cells are protected from their environment by a rigid, yet flexible barrier, the cell wall (CW). When plant cells grow, they meet the challenge of having to loosen up their CW to allow for expansion, while at the same time ensuring that it does not break. Hence, plant cells have developed signaling pathways that monitor the status of their CW and adapt their growth accordingly. In tip-growing cells such as root hairs (RHs) and pollen tubes (PTs) of the plant model Arabidopsis, these pathways are controlled by three members of the maelectin-like receptor kinase family, FERONIA, ANXUR1 (ANX1), and ANX2, in cooperation with their ligands, the RAPID ALKALINIZATION FACTORS. Loss of CW integrity in PTs, as seen for example in anx1 anx2 double mutants, results in PTs that burst upon germination, leading to male sterility.

Question: As the late stages of this CW integrity pathway are currently completely unknown, we wanted to test if the study of a weak anx1 anx2 suppressor mutant, whose PTs do not prematurely burst, could allow us to identify new components of this pathway.

Findings: We found that two type one protein phosphatases ATUNIS1 (AUN1) and AUN2 function redundantly in promoting RH and PT growth in Arabidopsis. If we switch off these phosphatases or express malfunctioning variants of them, PTs and RHs of wild-type plants struggle to elongate properly. However, if we express the same variants in the anx1 anx2 mutant background, PTs burst significantly less and male fertility is restored. Our results indicate that the phosphatases AUN1 and AUN2 promote tip growth and constitute late negative regulators of the CW integrity pathway.

Next steps: Several protein kinases have been reported to play a role during CW integrity signaling, and the phosphatases AUN1 and AUN2 could potentially counterbalance the activity of these kinases. Thus, one major step forward will be identifying the direct targets of phosphorylation and dephosphorylation of these protein kinases and phosphatases, respectively. This will further our understanding of the fascinating coordination between internal growth machinery and external CW that a plant cell accomplishes.

2018), LRX triple and quadruple mutants exhibit precocious pollen bursting and various degrees of male sterility, similar to bups1, bups1 bups2, and anx1 anx2 mutants, and are insensitive to RALF4-induced PT growth inhibition (Mecchia et al., 2017). However, how RALF4-LRX8 binding relates to the RALF4/19-ANX1/2-BUPS1/2 complex is currently unknown (Stegmann and Zipfel, 2017; Franck et al., 2018).

While the upstream part of the CWI pathways is being unraveled, available information on the downstream effectors is still very sparse (reviewed in Franck et al., 2018). ANX1/2 action depends on two partially redundant pollen-preferentially expressed NADPH oxidases, RESPIRATORY BURST OXIDASE HOMOLOG H (RBOHH) and RBOHJ, responsible for the tip-localized production of reactive oxygen species (Boisson-Dernier et al., 2013). While the precise role of the NADPH-oxidase-derived reactive oxygen species during PT growth is not completely understood, it was proposed to be essential for maintaining the stable tip-focused Ca2+ gradient, which is a characteristic of the steady growth of Arabidopsis PTs (Boisson-Dernier et al., 2013; Lassig et al., 2014).

To identify novel members of the PT CWI pathway, a forward genetic screen was performed earlier to identify suppressor mutations of the anx1 anx2 PT bursting phenotype (Boisson-Dernier et al., 2015). In vivo, the suppressor mutants were easily detectable, as reduction in PT bursting results in successful fertilization and, thus, silique elongation. Out of the 32 suppressor mutants that were named impotence rescue mutants (ipr), a strong suppressor mutant of the anx1 anx2 pollen bursting phenotype, ipr19, was previously described (Boisson-Dernier et al., 2015). Its suppressor mutation was identified as a nonsynonymous arginine-to-cysteine change at residue 240 (R240C) in the conserved catalytic domain of a receptor-like cytoplasmic kinase called MARIS (MRI) (Boisson-Dernier et al., 2015). Interestingly, this R240C substitution rendered the protein overactive. MRI was found to be a positive regulator of tip growth in both PTs and RHs. $\text{M} \text{R}_{\text{ip}240\text{C}}$ expression could rescue the anx1 anx2 precocious pollen bursting phenotype in vivo and in vitro. In PTs, MRI acts downstream of RBOHH/J. Knockout or knockdown mutants of mri exhibit pollen bursting, while overexpression of $\text{M} \text{R}_{\text{ip}240\text{C}}$ in the wild type triggers growth inhibition of PTs, similar to but stronger than observed for ANX1 overexpression.

In RHs, MRI plays a similar function (Boisson-Dernier et al., 2015). The RH CWI pathway is governed by the CrRLK1L member FERONIA (FER), the closest homolog of ANX1/2 (Duan et al., 2010). fer root hairs depict the same precocious bursting phenotype as anx1 anx2 PTs. Similarly as ANX1/2, FER is regulated by a RALF peptide, RALF1. RALF1 treatment of wild-type seedlings triggers rapid alkalinization of the root extracellular matrix and mediates root growth cessation (Haruta et al., 2014). fer roots, in contrast, are insensitive to RALF1 treatment. Interestingly, loss of function of RBOHC (Rhd2), a root hair-expressed homolog of RBOHH/J, also triggers precociously bursting RHs similarly as in fer mutants (Foreman et al., 2003) and thus is thought to act downstream of FER. Moreover, seedlings of mri mutants exhibit bursting RHs as well, just like fer mutants (Boisson-Dernier et al., 2015). In concordance with the finding that $\text{M} \text{R}_{\text{ip}240\text{C}}$ expression rescues anx1 anx2 precocious pollen bursting, $\text{M} \text{R}_{\text{ip}240\text{C}}$ expression also rescues fer RH bursting. Thus, MRI was proposed to act downstream of RBOHC in the FER-dependent RH CWI pathway (Boisson-Dernier et al., 2015).

Here, we report the characterization of ipr7, a weak suppressor mutant of the anx1 anx2 PT bursting phenotype. We show that the ipr7 causative mutation is a dominant-negative mutation that causes a D94N substitution in the conserved catalytic
site of a Type One Protein Phosphatase (TOPP) that we named ATUN1 (AUN1), rendering AUN1 inactive. We show that AUN1 and its closest homolog, AUN2, are negative regulators of CWI maintenance during tip growth. Similarly to MRI, they function downstream of RALF4/19, ANX1/2, and RBOHH/J. However, our genetic interaction analyses show that RALF4/19-LRXs act through AUN1/2 but not through MRI, thus suggesting that the LRXs do not participate in the RALF4/19-ANX1/2-BUPS1/2 receptor complex.

RESULTS

ipr7 Phenotypic Characterization

To determine the extent of seed set rescue, we first compared the seed set of the original Arabidopsis suppressor mutant ipr7 with that of wild-type and anx1-2 anx2-2 plants. Henceforth, ipr7 refers to anx1-2 anx2-2 ipr7/AUN1 plants unless otherwise stated. Heterozygous and homozygous ipr7 plants depicted a mild seed set rescue of 8 and 14 seeds/silique, respectively, compared with anx1 anx2 plants (approximately two seeds/silique) (Figure 1A). To determine if this rescue originates from a reduction in pollen bursting, we conducted in vitro pollen germination assays (Figure 1B). Indeed, ipr7 pollen exhibited a mild, yet significant reduction of pollen bursting from 100 to 89%. Thus, the observed mild seed set rescue is due to a reduction of precocious pollen bursting, enabling more PTs to fertilize female gametophytes.

We also monitored RH growth in homozygous ipr7 plants, as the strong ipr19 suppressor characterized previously showed a strong short RH phenotype (Boisson-Dernier et al., 2015). Interestingly, we found that ipr7 mutants also exhibited a strong decrease in RH length to ~55% of the length of anx1 anx2 control RHs (Figure 1C), albeit not as strong as the decrease observed for ipr19 mutants.

The ipr7 Causative Mutation Is aun1D94N

To identify the single-nucleotide polymorphism (SNP) responsible for the ipr7 phenotype, we applied the same SNP-ratio mapping approach combined with next-generation sequencing previously used for ipr19 (mirD242) (Lindner et al., 2012; Boisson-Dernier et al., 2015). This approach relies on the fact that after two rounds of backcrosses from the M1 suppressor mutant to the parental line anx1 anx2, unlinked EMS-generated SNPs are either lost or maintained in a 1:3 ratio, while the causative SNP is conserved in a 1:1 ratio as plants are selected for the suppressor phenotype (see Methods). For ipr7, the most promising SNP among the exonic candidate SNPs displayed a ratio of alternate to reference reads of 0.504, which is close to the theoretically expected ratio of 0.500. Also, the SNP occurred in the coding sequence of a gene highly expressed in pollen (Supplemental Data Set 1). This SNP (G280A) mapped to the gene AT3G05580, also known as TOPP9. TOPP9 encodes a putative Ser/Thr phosphatase of the protein phosphatase type one (PP1) subfamily with Mg²⁺/Mn²⁺-dependent activity. The PP1 subfamily is included in the larger phosphoprotein phosphatase (PPP) family characterized by an identical catalytic mechanism (Uhrig et al., 2013). This SNP converts an aspartate into an asparagine at residue 94 (D94N) in the conserved catalytic subunit, and its presence was confirmed with a derived cleaved/cut amplified polymorphism sequence marker assay (see Methods). We named the gene AUN1, after an Etruscan deity of death and rebirth, as the mutation enables anx1 anx2 “dead” PTs to be “reborn.” Interestingly, and similarly to the previously described MRI identified from ipr19 (Boisson-Dernier et al., 2015), AUN1 is not exclusively expressed in pollen but also shows mild expression in root tissue (Figure 2A; Supplemental Table 1). This is in good agreement with the ipr7 short RH phenotype observed previously. Unlike MRI, however, AUN1 has a close homolog, TOPP8 (AT5G27840), which is also expressed in pollen and roots (Figure 2A; Supplemental Table 1). TOPP8 shares 89.8% identity with AUN1 at the amino acid level and was thus named AUN2. Interestingly, the D94, located in the core catalytic domain, mediates metal ion coordination during catalysis and is perfectly conserved in all eukaryotic Ser/Thr phosphatases (Zhang et al., 1996; Uhrig et al., 2013; Figures 2D and 2E).

To confirm that the ipr7 causative mutation indeed corresponds to aun1D94N, we transformed anx1-1/anx1-1 anx2-1/ANX2 plants with AUN1-YFP and AUN1D94N-YFP protein fusions driven by a pollen-specific promoter. Among 141 individual T2 progenies pooled from two independent T1 lines of selfed anx1-1/anx1-1 anx2-1/ANX2 plants expressing AUN1-YFP, no double homozygous anx1-1 anx2-1 plants could be retrieved. In contrast, among 127 individual T2 progeny pools from two independent T1 lines of selfed anx1-1/anx1-1 anx2-1/ANX2 plants expressing AUN1D94N-YFP, three anx1-1 anx2-1 double homozygous plants were identified. This suggests that AUN1D94N-YFP expression can rescue the anx1 anx2 male sterility, albeit at lower rates than introduction of ANX1-YFP, ANX2-YFP, and MRI-FLAG-CFP (Boisson-Dernier et al., 2015). To confirm this rescue, two independent anx1-1 anx2-2 lines homozygous for AUN1D94N-YFP were subjected to seed set analysis and in vitro pollen germination assays. They will be referred to as anx rescue line1 (arl1) and arl2 henceforth. arl1 and arl2 depicted increased seed sets of ~19 seeds/silique (Figure 2B), comparable to the ~14 seeds per silique in ipr7/plr7 plants (Figure 1A). In pollen germination assays, both arl lines depicted 13% and 15% lower pollen bursting rates than untransformed anx1 anx2 plants (Figure 2C), similar to the observed ~11% bursting rate reduction observed for the original ipr7 mutant (Figure 1B). Thus, AUN1D94N-YFP, but not AUN1-YFP, expression is sufficient to phenocopy the original ipr7 phenotype, and the G280A SNP causing the D94N amino acid change in AUN1 is indeed the ipr7 causative mutation. Finally, it also shows that AUN1D94N is dominant over AUN1 and acts downstream of ANX1/2.

AUN1 and AUN2 Positively Impact Pollen Germination and Pollen Tube Growth

As mutations in the catalytic core region of protein phosphatases can result in dominant-negative forms of the phosphatases (Jurczak et al., 2010; Qin et al., 2014), we hypothesized that AUN1D94N could be a dominant-negative form of AUN1, which could be a negative regulator of the CWI pathway in PTs. To
test this, we ordered T-DNA insertion lines for **AUN1** and **AUN2**, namely, **aun1-1** (SALK_045433C), **aun1-2** (GABI_600E08), **aun2-1** (SALK_137888), and **aun2-2** (SALK_125184) (Figure 3A).

According to our model, knocking out a negative regulator downstream of **ANX1/2** would trigger an overactivation of the PT CWI pathway, resulting in growth inhibition, as observed for overexpression of **ANX1** and **MRIR240C** (Boisson-Dernier et al., 2013, 2015).

**aun1** and **aun2** plants exhibited wild-type-like seed sets, and their pollen germination and growth were indistinguishable from the wild type in vitro (Figures 3B and 3C; Supplemental Figure 1). As **AUN1** and **AUN2** share such a high degree of identity, we expected at least partial functional redundancy. Consequently, we crossed **aun1-1** with **aun2-1** as well as **aun1-2** with **aun2-2** single mutant lines in search for **aun1 aun2** double homozygous plants. Out of 147 selfed **aun1-1/AUN1 aun2-1/aun2-1** F3 plants, we found 19 **aun1-1 aun2-1** double homozygous plants, 80 **aun1-1/AUN1 aun2-1/aun2-1**, and 48 **AUN1/AUN1 aun2-1/ aun2-1** plants that significantly differs from the expected Mendelian segregation ratio of 1:2:1 ($\chi^2 = 12.592$, $P = 0.0018$, two-tailed $\chi^2$ test). This suggests a mild transmission defect of the **aun1-1 aun2-1** double mutant allele. To precisely determine the transmission efficiency of the mutant allele, we performed reciprocal crosses of **aun1-1/AUN1 aun2-1/aun2-1** mutant plants with the wild type and genotyped the F1 generation for the presence of **aun1-1 aun2-1**. When **aun1-1/AUN1 aun2-1/aun2-1** was used as pollen donor, the **aun1-1 aun2-1** transmission efficiency was significantly decreased to 50% (32 **aun1-1/AUN1 aun2-1/
Figure 2. The ipr7 Causative Mutation Corresponds to an aun1D94N Mutation in TOPP9.

(A) Phylogenetic tree of the Arabidopsis TOPP family combined with relative gene expression data. Multiple alignments of Arabidopsis TOPP proteins were made in ClustalW 2.0 and with MEGA7, and the phylogenetic tree was reconstructed under usage of the protein sequence parsimony method (bootstrap test, 1000 replicates). Thereafter, the tree was combined with the relative gene expression data of Arabidopsis TOPP family members in various plant tissues according to the Genevestigator microarray database with the Meta-Profile Analysis tool Anatomy Profile (Hruz et al., 2008).
aun1-1 and 64 AUN1/AUN1 aun2-1/aun2-1 out of 96 F1 plants; two-tailed Fisher's exact test, \( P = 0.0278 \). When the wild type was selected as pollen donor, the transmission efficiency was close to normal with 78% (39 aun1-1/AUN1 aun2-1/aun2-1 and 50 AUN1/AUN1 aun2-1/aun2-1 plants out of 89 F1 plants; two-tailed Fisher's exact test, \( P = 0.4529 \)). Thus, the transmission of \( \textit{aun1-1 aun2-1} \) mutant allele is mildly decreased through the male gametophyte but fairly normal through the female gametophyte. This indicates that AUN1 and AUN2 are important for pollen development and/or function.

Interestingly, the double homozygous \( \textit{aun1 aun2} \) plants exhibited a wild-type-like seed set as well. However, in clear contrast to the wild type, \( \textit{aun1 aun2} \) pollen showed a drastic reduction of pollen germination in vitro to only 10 to 20% (Figures 3B and 3C), a phenotype that is fully complemented by expression of AUN1-YFP and AUN2-YFP in pollen (see below). We frequently observed pollen grains unsuccessfully trying to initiate PT outgrowth through two or more colpi. Also, some pollen grains either only formed little bulges instead of PTs or short PTs with wide tips displaying CW material overaccumulation (Figure 3C). This is reminiscent of the ANX1/2 and MR\textsuperscript{GODIC} overexpressor phenotype (Boisson-Dernier et al., 2013, 2015). Thus, in the absence of both AUN1 and AUN2, the CWI maintenance machinery appears to be overactivated, resulting in CW material overaccumulations that trigger a strong inhibition of PT growth and render \( \textit{aun1 aun2} \) pollen grains unable to properly develop PTs. Oddly, \( \textit{aun1 aun2} \) pollen also depicted an increased pollen bursting rate of 25 to 48% in vitro (Figure 3B). However, this increase turned out to be a sampling artifact. Indeed, when imaging pollen germination assays, the zones with the best pollen germination rates—normally comprised of PTs and burst pollen grains—are consistently chosen for image capturing and subsequent analyses. For genotypes or conditions with low pollen germination rates, the bursting fraction (BF in the figures), defined as 100% × (number of burst pollen grains/total number of pollen grains), is calculated as PTs are very sparse. Thus, to determine the true pollen bursting rate for these “low germination” conditions, we calculated the bursting fraction (BF in the figures), defined as 100% × (number of burst pollen grains/total number of pollen grains), in contrast to the traditional bursting rate defined as 100% × (number of burst pollen grains/number of germinated pollen grains) (Figure 3B). In the \( \textit{aun1 aun2} \) situation, the bursting fraction of 4 to 5% is similar to wild-type levels (Figure 3B), indicating that the elevated bursting rate indeed originates only from the very low germination rate of the mutant line pollen.

To visualize the impact of \( \textit{aun1-1 aun2-1} \) PT growth behavior in vivo, we pollinated emasculated wild-type pistils with either wild-type or \( \textit{aun1-1 aun2-1} \) pollen. After 4 h, the pistils were harvested, fixed, and subsequently stained with aniline blue. As expected, \( \textit{aun1 aun2} \) PTs depicted enhanced growth in vivo compared with in vitro. However, a clear significant delay in \( \textit{aun1-1 aun2-1} \) PT growth could be observed in comparison to wild-type PTs as the longest \( \textit{aun1 aun2} \) PT had reached on average 525 μm after 4 h, while wild-type tubes had already extended 777 μm (Figure 3D).

In summary, while \( \textit{aun1} \) and \( \textit{aun2} \) single mutants do not display any fertilization-related phenotypes, the \( \textit{aun1 aun2} \) double mutants exhibit inhibited pollen germination and PT growth, which is most likely responsible for the observed mild reduction of the transmission efficiency through the male gametophyte. Thus, AUN1 and AUN2 function redundantly as negative regulators of PT growth.

**AUN1 and AUN2 Also Positively Impact RH Growth**

To test whether the growth inhibitory effect on PTs also applies to other tip-growing plant cells, namely, RHs, we measured the RH length of \( \textit{aun1-1, aun2-1, and aun1-1 aun2-1} \) plants compared with the wild type. Both single mutants as well as the \( \textit{aun1-1 aun2-1} \) double mutant exhibited shorter RHs compared with wild-type seedlings (Figure 3E), similarly as the original ipr7 mutant (Figure 1C). This indicates that AUN1/2 function in growth control of both PTs and RHs. Also, while during PT growth the T-DNA disruption of either AUN1 or AUN2 does not trigger a phenotypic reaction, in RHs, disruption of one of the two genes is enough to obtain a growth inhibitory effect. This points toward a differential cell-type-specific regulation of the two genes.

**Overexpression of AUN1 and AUN1\textsuperscript{D94N} Triggers Opposite Phenotypes**

If AUN1/2 inhibit the CWI maintenance machinery and if AUN1\textsuperscript{D94N} indeed is a dominant-negative form of AUN1, expression of AUN1-YFP and AUN1\textsuperscript{D94N}-YFP in wild-type pollen...
Figure 3. Molecular and Phenotypic Characterization of T-DNA Insertional aun1, aun2, and aun1 aun2 Mutants.

(A) Representation of the AT3G05580 (AUN1) and the AT5G27840 (AUN2) loci with introns, exons, and positions of the mutant alleles. Light-green squares represent the untranslated regions and gray squares the coding regions. Black continuous lines represent splicing sites. Lb-related primers are indicated in dark red. The positions of the end of the primer binding sites are relative to the beginning of the 5’ untranslated region marked by a dashed black line with a 0. Transparent triangles with black borders indicate the T-DNA insertions. Both aun2-1 and aun2-2 harbor two T-DNA insertions in inverted repeat, respectively. The nucleotides between −37 and −9, as well as −38 and +58, were deleted during T-DNA integration, respectively.

(B) Pollen germination assays of the wild type, aun1-2, aun2-2, aun1-2 aun2-2, and aun1-1 aun2-1 with germination and bursting rates as well as bursting fraction (BF). Two asterisks denote significant differences in pollen germination with P < 0.01 (two-tailed unpaired Student’s t test). Error bars indicate the sd of three independent experiments with around 200 pollen scored per genotype and experiment.

(C) Representative images of wild type, aun1-2, and aun1-1 aun2-1 pollen germination assays 5 h after incubation. Magenta arrows indicate pollen grains that show attempted PT outgrowth through two colpi. Blue arrows indicate CW material overaccumulations. Right image is a close-up of aun1-1 aun2-1 pollen grain accumulating CW material precociously. Bars = 80 (left) and 25 (right) μm.

(D) Representative images of wild-type and aun1-1 aun2-1 pollen tube growth in vivo (left) and corresponding PT length measurements (right). Emasculated wild-type pistils manually pollinated by either wild-type or aun1-1 aun2-1 pollen. Aniline blue staining 4 h after pollination. Arrows indicate the PT traveling the furthest. Two asterisks indicate a significant difference in PT length with P < 0.01 (two-tailed unpaired Student’s t test). Error bars represent the sd with n > 17 per genotype. Bar = 200 μm.

(E) Representative images of wild-type, aun1-1, aun2-1, and aun1-1 aun2-1 roots (left) and corresponding RH length analysis (right). RH length measurements of wild-type (n = 153 from 14 individuals), aun1-1 (n = 181 from 16 individuals), aun2-1 (n = 161 from 18 individuals), and aun1-1 aun2-1 (n = 109 from 17 individuals) roots. Two asterisks indicate significant differences from the wild type with P < 0.01 (two-tailed unpaired Student’s t test). Error bars represent the sd. Bar = 200 μm.
could trigger opposite phenotypes during PT growth. Pollen of an AUN1 overexpressor would contain more functional AUN1, inhibiting the CWI maintenance machinery. Consequently, we could observe elevated pollen bursting. If AUN1\textsuperscript{D94N} is expressed in wild-type pollen, it would exert a dominant effect over the native AUN1 preventing its activity and thus would result in PT growth inhibition, as observed in aun1-1 aun2-1 T-DNA insertion mutants.

To test this hypothesis, we transformed wild-type plants with both ProLAT52:AUN1-YFP and ProLAT52:AUN1\textsuperscript{D94N}-YFP and subsequently selected two independent YFP-expressing lines homozygous for each of the respective constructs. Henceforth, the two wild-type lines homozygous for AUN1-YFP will be referred to as AUN1-overexpressor 1 (AUN1-ox1) and AUN1-ox2, whereas the two wild-type lines homozygous for AUN1\textsuperscript{D94N} will be called AUN1\textsuperscript{D94N}-overexpressor 1 (AUN1\textsuperscript{D94N}-ox1) and AUN1\textsuperscript{D94N}-ox2. During pollen germination assays, the two independent AUN1\textsuperscript{D94N}-ox lines depicted low pollen germination rates of 10 to 20% compared with 75% in the wild type (Figures 4A and 4B). In contrast, pollen from the two independent AUN1-ox lines germinated normally but showed mildly increased bursting fractions of 12 to 17% compared with 7% in the wild type and 1 to 4% in the AUN1\textsuperscript{D94N}-ox lines.

In summary, when AUN1\textsuperscript{D94N} is expressed in a wild-type background, pollen germination is strongly inhibited, similarly as described for the aun1 aun2 mutants. When AUN1 is overexpressed in a wild-type background, a mild elevation of bursting pollen grains is observed while germination is not affected. Thus, AUN1/2 indeed are negative regulators of CWI maintenance. These results confirm that AUN1\textsuperscript{D94N} is a dominant-negative form of AUN1.

**AUN1 Phosphatase Activity Is Required for Its Function during PT Growth**

Next, we compared the functionality of AUN1 and AUN1\textsuperscript{D94N} with that of AUN1\textsuperscript{H127A}—the phospho-dead version of AUN1 (Takemiya et al., 2006; Boevink et al., 2016)—by transforming aun1-1 aun2-1 plants with ProLAT52:AUN1\textsuperscript{H127A}-YFP, ProLAT52:AUN1\textsuperscript{D94N}-YFP, and ProLAT52:AUN1\textsuperscript{H127A}-YFP. H127, like D94, is located in the conserved core catalytic domain of PPPs and donates a proton to the dephosphorylated leaving substrate (Figures 2D and 2E; Shi, 2009). In the T3 generation, we conducted pollen germination assays on one representative line with good fluorescence in pollen grains for each construct and compared the transformed lines to the wild type as positive control and aun1-1 aun2-1 as negative control.

In vitro, the aun1-1 aun2-1 line hemizygous for AUN1-YFP readily displayed a clear rescue of the low pollen germination rate observed in untransformed aun1-1 aun2-1 plants (Figure 4C). By contrast, the aun1-1 aun2-1 lines homozygous for either AUN1\textsuperscript{D94N}-YFP or AUN1\textsuperscript{H127A}-YFP were indistinguishable from the untransformed aun1-1 aun2-1 mutant with a germination rate of below 20% (Figure 4C). These results show that AUN1-YFP, but not AUN1\textsuperscript{D94N}-YFP or AUN1\textsuperscript{H127A}-YFP, is a functional protein fusion. Furthermore, it suggests that AUN1’s function during CWI requires its phosphatase activity.

The finding that AUN1\textsuperscript{D94N}-YFP and AUN1\textsuperscript{H127A}-YFP expression are similarly unable to rescue the low germination phenotype of aun1-1 aun2-1 plants raised the question whether AUN1\textsuperscript{H127A}-YFP overexpression in a wild-type background would promote low pollen germination, similarly to AUN1\textsuperscript{D94N}-YFP overexpression. To test this, we transformed wild-type plants with ProLAT52:AUN1\textsuperscript{H127A}-YFP and isolated four independent homozygous transgenic lines. We performed pollen germination assays on these lines and compared the results to untransformed wild-type plants as well as an AUN1-YFP and an AUN1\textsuperscript{D94N}-YFP overexpression line, respectively (Figure 4D; Supplemental Figure 3). Interestingly, the four independent AUN1\textsuperscript{H127A}-YFP overexpressing lines all had similarly low germination rates (9–17%) and bursting fractions (2–6%) as the AUN1\textsuperscript{D94N}-YFP control line. This indicates that AUN1\textsuperscript{H127A} also constitutes a dominant-negative form of AUN1, similar to AUN1\textsuperscript{D94N}.

**AUN1/2 Are Localized to the PT Cytoplasm and Vegetative Nucleus**

We also were interested to know whether AUN2-YFP is likewise able to complement the aun1-1 aun2-1 low pollen germination phenotype. To address this, we cloned AUN2 without a stop codon into ProLAT52:GW-YFP and introduced the fusion into aun1-1 aun2-1 plants. Two independent aun1-1 aun2-1 AUN2-YFP triple homozygous lines were retrieved and pollen germination assays were performed (referred to as AUN2-YFP in the figure). As seen in Figure 4E, both AUN2-YFP-expressing lines were able to rescue the aun1-1 aun2-1 low pollen germination phenotype, indicating that the fusion protein is functional.

Finally, we made use of both AUN1- and AUN2-YFP-complemented lines to pinpoint the subcellular localization of AUN1 and AUN2. By live-imaging growing aun1-1 aun2-1 PTs expressing either AUN1-YFP or AUN2-YFP, we found that the two fusion proteins localized to both the cytoplasm and vegetative nucleus (Figure 4F; Supplemental Movie 1). This was further supported by transiently expressing the AUN1- and AUN2-YFP fusion proteins in Arabidopsis leaf epidermal cells (Supplemental Figure 4). This indicates that AUN1/2 could possibly function in both the cytoplasm and vegetative nucleus of the growing PT.

**AUN1 Acts Downstream of RALF4/19, LRXs, and RBOHH/J, but Not Downstream of MRI**

According to the suppressor screen and the anx1 anx2 rescue assays, AUN1 seems to act downstream of ANX1/2. To confirm this and deepen the genetic positioning within the CWI pathway, we transformed artificial microRNA lines of rapid alkalization factor 4 and rapid alkalization factor 19 (amiRRALF4/19, and rbohH-3 rbohJ-3 plants with ProLAT52:AUN1-YFP and ProLAT52:AUN1\textsuperscript{D94N}-YFP. amiRRALF4/19 and rbohH-3 rbohJ-3 expression lines exhibiting partial male sterility due to an elevated pollen bursting rate (Boisson-Dernier et al., 2013; Mecchia et al., 2017).

After transforming amiRRALF4/19 plants, eight and nine independent lines with clear YFP-derived fluorescence in pollen grains were selected in the T1 generation for ProLAT52:AUN1-YFP and ProLAT52:AUN1\textsuperscript{D94N}-YFP, respectively. In the T2 generation, the
Figure 4. The Nucleo-Cytoplasmic AUN1 and AUN2 Are Functional Negative Regulators of PT CWI.

(A) Representative images of wild-type, AUN1-ox1, and AUN1D94N-ox1 pollen subjected to germination assays, 5 h after incubation. Asterisks indicate burst pollen grains. Bar = 80 μm.

(B) Results of pollen germination assays of the wild type, AUN1-ox1, AUN1-ox2, AUN1D94N-ox1, and AUN1D94N-ox2, including germination rate and bursting fraction. Because our wild-type plants could only be used once during these particular assays due to insecticide treatment, the wild-type data here were pooled from all the different wild-type pollen germination assays performed during this study. Two asterisks denote significant differences from the wild type with P < 0.01 (two-tailed unpaired Student’s t test). Error bars indicate the se of three independent experiments with around 200 pollen scored per genotype and experiment.

(C) Germination rate and bursting fraction of wild-type, untransformed aun1-1 aun2-1 plants hemizygous for AUN1-YFP, aun1-1 aun2-1 plants homozygous for AUN1D94N-YFP, and aun1-1 aun2-1 plants homozygous for AUN1H127A-YFP. Two asterisks denote significant differences from the wild type with P < 0.01 (two-tailed unpaired Student’s t test). Error bars indicate the se of three independent experiments with around 200 pollen scored per genotype and experiment.

(D) Results of pollen germination assays of the wild type, AUN1-ox1, AUN1D94N-ox1, and four independent homozygous AUN1H127A overexpressor lines, including germination rate and bursting fraction. Single asterisk denotes significant differences from the wild type with P < 0.05 (two-tailed unpaired Student’s t test). Error bars indicate the se of three independent experiments with around 200 pollen scored per genotype and experiment.
seed set of two to three homozygous plants per line was ana-
yzed (Figure 5A). All nine T2 lines homozygous for AUN1D94N-
YFP showed a significant seed set rescue compared with untransformed amiRRALF4/19 plants (average 27 ± 10 seeds per silique compared with 8 ± 2 seeds for untransformed controls, P < 0.01). In contrast, the eight amiRRALF4/19 plants homozygous for AUN1-YFP displayed a mean seed set of 9 ± 2 seeds, similar to untransformed amiRRALF4/19 plants (P = 0.15). To test whether the observed seed set rescue in the AUN1D94N-YFP transformed lines originates from a reduction in PT bursting rate, we selected three representative AUN1D94N-YFP and one AUN1-YFP transgenic homozygous line/s (colored orange in Figure 5A) for further in vitro pollen germination assays. Interestingly, while the AUN1-YFP transformed line behaved like untransformed amiRRALF4/19, the three AUN1D94N-YFP lines exhibited both a significantly reduced germination rate and a reduced bursting fraction (Figure 5B; Supplemental Figure 3). Thus, similar to MIRG40C-YFP (Meccia et al., 2017), AUN1D94N-YFP is capable of rescuing the amiRRALF4/19 pollen bursting phenotype, unequivocally positioning RALF4 and RALF19 upstream of MRI and AUN1 in the CWI pathway.

Additionally, rbohH-3 rbohJ-3 plants were transformed with ProLAT52:AUN1-YFP and ProLATS2:AUN1D94N-YFP. Eight rbo-
hH-3 rbohJ-3 homozygous lines for AUN1D94N-YFP had a sig-
nificantly increased seed set of 36 ± 3 seeds as compared with 6 ± 1 seeds for the untransformed controls (P < 0.01) (Figure 6A). The three homozygous lines for AUN1-YFP also depicted significantly, albeit only slightly, increased seed sets of 14 ± 4 seeds per silique (P < 0.01). We chose three representative lines for AUN1D94N-YFP and one line for AUN1-YFP to conduct pollen germination assays in the T3 generation with rbohH-3 rbohJ-3 as pollen bursting control (Figure 6B). In vitro, the AUN1-YFP line depicted a slight decrease in pollen germination rate and bursting fraction compared with untransformed rbohH-3 rbohJ-3 plants. In comparison, all three AUN1D94N-YFP expressing lines showed a much stronger decrease of germination rates and bursting fractions (Figure 6B; Supplemental Figure 3). Thus, our results indicate that AUN1D94N, similar to MIRG40C (Boisson-Dernier et al., 2015), can rescue the male sterility of rbohH-3 rbohJ-3 and that RBOHH/J functions upstream of both MRI and AUN1.

To clarify the genetic relationship between MRI and AUN1, we also transformed mri-1/mri-1 plants with ProLATS2:AUN1D94N-YFP and screened for resurgence of mri-1 homozygous plants in the T2 generation. Out of 297 genotyped progenies originating from three independent mri-1/MRI T1 lines displaying YFP-derived fluorescence from AUN1D94N-YFP, only one homozygous mri-1/ mri-1 plant was recovered. This unique mri-1/mri-1 plant displayed complete male sterility as opposed to the 29 (out of 30

DISCUSSION

Recent studies have started to elucidate the upstream mecha-
nisms activating the CrRLK1L-dependent pathways that control CWI (Ge et al., 2017; Meccia et al., 2017; reviewed in Franck et al., 2018). However, our knowledge of the mechanisms through which the CrRLK1Ls relay the collected information to downstream intracellular molecular players remains scarce. A few years ago, a suppressor screen for the loss of CWI in PTs of anx1 anx2 was performed that led to the isolation of the ipr class of mutants in Arabidopsis (Boisson-Dernier et al., 2015). Study of the strong suppressor mutant ipr19 led to the identifi-
cation of MRI as a downstream component common to both the ANX1/2- and the FER-dependent pathway, governing CWI in PTs and RHs, respectively. Interestingly, the causative mutation for ipr19 corresponded to a gain-of-function mutation in the positive regulator MRI. In this study, the characterization of ipr7, a weak suppressor of anx1 anx2 male sterility, unraveled the role of Ser/Thr protein phosphatases AUN1 and AUN2 in CWI maintenance. AUN1 and its closest homolog, AUN2, share 89.8% identity at the amino acid level and belong to the family of TOPPs in Arabidopsis that display an Mg2+/Mn2+-dependent activity in vitro (Smith and Walker, 1993).

Figure 4. (continued).

(E) Results of pollen germination assays of the wild type, aun1-1 aun2-1, and two independent aun1-1 aun2-1 AUN2-YFP triple homozygous lines, including germination rate and bursting fraction (top) and representative images (bottom). Two asterisks denote significant differences from the wild type with P < 0.01 (two-tailed unpaired Student’s t test). Error bars indicate the se of three independent experiments with around 200 pollen scored per genotype and experiment.

(F) Subcellular localization of AUN1-YFP and AUN2-YFP in complemented aun1-1 aun2-1 pollen tubes. Note how the YFP fusion proteins are absent from sperm cells leaving black empty spots indicated by magenta arrows. The YFP-labeled vegetative nucleus is indicated by a white arrow. Bars = 7 µm (left) and 12 µm (right).
The TOPP family is related to PP1, a eukaryotic Ser/Thr phosphatase mediating various cellular processes in animals such as cell cycle progression, transcription, protein synthesis, carbohydrate metabolism, muscle contraction, and neuronal signaling (Bollen, 2001; Cohen, 2002; Ceulemans and Bollen, 2004; Takemiya et al., 2009). PP1 consists of a catalytic subunit (PP1c) and diverse distinct regulatory subunits that determine specificity of the catalytic activity, specificity of the substrate, and

Figure 5. AUN1 Acts Downstream of RALF4 and RALF19 Peptides.

(A) Seed set analysis (top) and representative silique images (bottom) of untransformed amiRRALF4/19, amiRRALF4/19 homozygous for AUN1-YFP, and amiRRALF4/19 homozygous for AUN1D94N-YFP plants. Orange marks the lines selected for pollen germination assays. Two asterisks denote significant increase in seed set compared with untransformed amiRRALF4/19 with P < 0.01 (two-tailed unpaired Student’s t test). Error bars depict the sd with n > 11 siliques per genotype.

(B) Representative images of pollen germination assays (top) of untransformed amiRRALF4/19, amiRRALF4/19 homozygous for AUN1-YFP, and amiRRALF4/19 homozygous for AUN1D94N-YFP plants. Bar = 80 μm. Corresponding germination rate and bursting fraction from three independent pollen germination assays (bottom). Magenta asterisks indicate burst pollen grains. Two black asterisks indicate significant differences in germination rate and bursting fraction compared with amiRRALF4/19 with P < 0.01 (two-tailed unpaired Student’s t test). Error bars indicate the se of three independent experiments with around 200 pollen scored per genotype and experiment.
subcellular localization. The PP1c itself is highly conserved throughout eukaryotes and has three isoforms in humans, four in potato, *N. benthamiana* and *V. faba*, five in rice, and nine in *Arabidopsis* that constitute the TOPP family (Kerk et al., 2002; Matsumoto and International Rice Genome Sequencing Project et al., 2005; Takemiya et al., 2006; Boevink et al., 2016). In *V. faba*, PP1c-1 has been proposed to mediate blue light-induced stomatal opening as expression of the dominant-negative mutant form PP1C-1H137N inhibits blue light-induced stomatal opening (Takemiya et al., 2006). In *N. benthamiana*, silencing of NbPP1c isoforms decreases the levels of *P. infestans* infection, indicating that PP1c activity is required for full development of the
disease (Boevink et al., 2016). Out of nine Arabidopsis isoforms, only TOPP1 and TOPP4 have been characterized genetically in planta, with TOPP1 negatively regulating ABA signaling (Hou et al., 2016), while TOPP4 plays diverse roles. TOPP4 is a positive regulator of gibberellin-dependent control of plant growth (Qin et al., 2014), promotes lobe formation in pavement cells (Guo et al., 2015), and negatively regulates red light-induced photomorphogenesis (Yue et al., 2016).

Here, we show that the Arabidopsis PP1c AUN1 (TOPP9) and AUN2 (TOPP8) are expressed in both PTs and RHs (Figure 2A; Supplemental Table 1). They function redundantly as negative regulators of the ANX1/2-dependent CWI pathway (Figure 7).
The simultaneous loss of function of AUN1 and AUN2 leads to strong and moderate inhibition of PT growth in vitro (Figures 3B and 3C) and in vivo (Figure 3D), respectively. Interestingly, the weak anx1 anx2 suppressor mutant ipr7 harbors a D94N mutation in AUN1. This aspartate is perfectly conserved among the PPPs and important for metal ion coordination (Figures 2D and 2E; Zhang et al., 1996; Uhrig et al., 2013). Intriguingly, an AUN1D94N equivalent mutation in either the small bacteriophage Ser/Thr λ-phosphatase (λ-PPDH; Zhuo et al., 1994) or the mammalian PP1c, PP1cD92N (Zhang et al., 1996), leads to severe decrease of catalytic efficiency by a factor comprised between 103 and 106. These results, coupled with the fact that AUN1D94N-YFP does not complement aun1 aun2 mutant phenotypes (Figure 4C), indicate that D94N substitution in AUN1 most likely impairs the phosphatase activity.

The other mutant form used in this study, AUN1H127A, could not complement the aun1 aun2 low germination phenotype either (Figure 4C). This perfectly conserved histidine functions as the proton donor for the leaving alcohol group during catalysis (Figure 2E). Mutation of this histidine in the human phosphatase leads to a 23-fold reduction in phosphatase activity without altering its binding ability to the substrate (Myles et al., 2001). Moreover, Takemiya et al. (2006) confirmed the importance of this residue for plant phosphatases as the VPP1pc1-1H127N shows no detectable activity toward [32P]-labeled myelin basic protein as opposed to the wild-type VPP1pc1-1 form (Takemiya et al., 2006). Altogether, these results illustrate the remarkable conservation of the important residues of the PPPs throughout the eukaryotic kingdom. Furthermore, mutations in the catalytic core of PPPs not only lead to drastic loss of activity but also generate dominant-negative forms of the protein phosphatases. This was observed for mutant forms of PP2As, another subgroup of PPPs, where HsPP2AcH118N or AtPP2A-C20112N, while showing no detectable activity, could inhibit yeast cell growth in a dominant way (Evans et al., 1999; Lizotte et al., 1999). For PP1s, over-expression of mutant forms VPP1PC-1H127N and VPP1PC-1H127N inhibited blue light-induced stomata opening (Takemiya et al., 2006). In tobacco, expression of mutant NbPP1c-1H123A decreases P. infestans infection levels similarly as silencing the wild-type NbPP1c isoforms (Boevink et al., 2016).

In this study, we show that expression of nonfunctional AUN1D94N and AUN1H127A in wild-type pollen is sufficient to strongly inhibit PT growth and germination in vitro (Figures 4B and 4D), similarly as observed for the T-DNA insertional double mutants aun1 aun2 (Figure 3C). Considering the perfect conservation of these residues in the PPP catalytic site across the eukaryotic kingdom, this also suggests that AUN1 phosphatase activity is required for its function in inhibiting CWI. Furthermore, the original suppressor ipr7 carrying the D94N mutation in the anx1 anx2 background, displays a similar RH growth inhibition phenotype (Figure 1C) as the one displayed by aun1 aun2 (Figure 3E). This not only suggests that AUN1 and AUN2 are likely downstream components of the FER-dependent pathway that control RH growth, but also allows us to conclude that AUN1D94N and AUN1H127A, just as seen for other PPP mutant forms, constitute dominant-negative variants of AUN1. Although the mechanism for this dominant-negative effect of protein phosphatase mutants is not clearly understood, it was suggested that these interfering mutant forms could constitute nonproductive complexes with regulatory subunits and/or substrates preventing wild-type phosphatases from performing normally (Evans et al., 1999).

Moreover, functional AUN1-YFP and AUN2-YFP fusion proteins localize to the nucleus and cytoplasm in both transiently transformed wild-type epidermal cells (Supplemental Figure 4) and growing PTs of complemented aun1 aun2 mutants (Figure 4F). This localization pattern is consistent with the previously reported localization of the nine mCherry-TOPP fusion proteins in transiently transformed V. faba guard cells (Takemiya et al., 2009). Qin et al. (2014) also found that TOPP4-GFP associates with the plasma membrane of root cells in Pro35S:TOPP4-GFP transgenic plants. A cytoplasmic and nuclear localization was also reported for the rice OsPP1a-GFP (Ogawa et al., 2011) and the wheat T0PP1a-GFP (Brada et al., 2018). So far, all the identified components of the CWI pathway in PTs have been shown to be delivered to the plasma membrane (ANX1/2, BUPS1/2, RBOHH/J, and MRI) (Boisson-Dernier et al., 2009, 2013, 2015; Ge et al., 2017) or to be associated with vesicles and the CW (LRX8, LRX9, and LRX10 [Wang et al., 2018] and possibly RALF4 and RALF19 [Ge et al., 2017]). Thus, it is unlikely that AUN1 and AUN2 would exert their CWI-related function controlling fast tip growth in the nucleus. However, these phosphatases could play other important roles in the nucleus. The future identification of regulatory partners and targets of AUN1 and AUN2 will surely help pinpoint the location of these PPPs’ action.

Unlike the increasing number of identified PP1 regulatory subunits and interacting partners (Uhrig et al., 2013), the targets of PP1 activity are mostly unknown. TOPP4 was shown to dephosphorylate DELLA proteins and the transcription factor PIF5 in the nucleus to regulate GA-dependent signaling (Qin et al., 2014) and photomorphogenesis (Yue et al., 2016), respectively. TOPP4 was also found to dephosphorylate the auxin transporter PIN1 at the plasma membrane to promote lobe formation in the
leaf epidermis (Guo et al., 2015). Finally, TOPP1 was reported to interact with several SnRK2s and could inhibit the kinase activity of SnRK2.6 (OST1), suggesting that TOPP1 could possibly dephosphorylate SnRK2.6 to negatively regulate ABA signaling (Hou et al., 2016). Our genetic interaction studies clearly show that AUN1 functions in Pts downstream of RALF4/19, ANX1/2, and RBOH/H/J (Figures 2C, 5, and 6) similarly as MRI (Boisson-Dernier et al., 2015). Intriguingly, however, AUN1 appears to act downstream of LRX8-11 while MRI does not (Figure 7). While the role of RALF4 binding to LRX8 remains puzzling (Mecchia et al., 2017; Stegmann and Zipfel, 2017; Franck et al., 2018), our data indicate that LRX8-11 is positioned in a signaling branch that does not depend on a functional MRI. Consequently, it also suggests that LRX8-11 are unlikely to be involved in the RALF4/19-ANX1/2-BUPS1/2 ligand-receptor complex. Considering that ANX1/2, BUPS1/2, and MRI are all positive regulators of the CWI pathway with putative Ser/Thr kinase activity, the identification of Ser/Thr protein phosphatases as negative regulators of CWI maintenance was awaited. AUN1 and AUN2 could possibly (1) counterbalance the kinase activity of MRI and regulate the phosphorylation status of common downstream targets or (2) be the target of MRI itself. However, these scenarios appear quite unlikely since AUN1D94N could not rescue moi-1 male sterility. Alternatively, the phosphatases could function in a parallel branch (Figure 8). At this stage, it remains difficult to predict the growth-related targets of AUN1 and 2 dephosphorylation, but they could well belong to families of Rho GTPase (ROP)-related signaling components, proton pumps, channels, and/or transporters. While for a long time protein phosphatases have been considered to play a passive housekeeping role as opposed to kinases (Brautigan, 2013), our study illustrates well that they can also play central, yet specific, roles in specialized cell types, hereby negatively impacting CWI maintenance in tip-growing cells.

METHODS

Plant Material and Growth Conditions

All the mutant lines, transgenic lines, primer sequences, and plasmids used in this study are listed in Supplemental Tables 2 to 4, respectively. The binary vectors constructed and described below were introduced into Agrobacterium tumefaciens strain GV3101 by electroporation, which was afterwards used to transform plants by floral dipping (Clough and Bent, 1998). Sterilized Arabidopsis thaliana seeds were sown on half-strength Murashige and Skoog (MS) basal medium (Duchefa Biochemie) with 1% (w/v) sucrose and vertically placed in a fridge at 4°C for 2 d. Subsequently, the seeds were transferred to a growth chamber under long-day conditions (16 h light, 8 h dark) at 22°C, with approximate relative humidity of 60% and 150 to 300 nmol m⁻² s⁻¹ light intensity provided by white light bulbs. After 7 d, the seedlings were transferred to soil in a greenhouse under long-day conditions.

Single Nucleotide Polymorphism Ratio Mapping for the ipr7 Mutation

To identify the EMS-generated causative ipr7 suppressor mutation, an SNP-ratio mapping approach with modifications was performed (Lindner et al., 2012). When selecting plants for the silique elongation phenotype after two rounds of backcrosses from the M1 mutant to the parental line anx1 anx2, unlinked SNPs are theoretically either lost or maintained in a 1:3 ratio, while the suppressor SNP is conserved in a 1:1 ratio. The M1 ipr7 plant was backcrossed as a pollen donor twice successively on the original anx1-anx2-2 and 96 F1 plants from the second backcross were grown. Out of 96 plants, 81 displayed a silique-elongated phenotype, thereby showing the beneficial effect of the ipr7 mutation for pollen growth in an anx1 anx2 background. Genomic DNA of 55 plants with elongated siliques was extracted with the DNeasy Plant Mini kit (Qiagen) and pooled. The Functional Genomic Center of the University of Zürich prepared the Illumina-adapted library and sequenced heterozygous ipr7 with the paired-end Illumina HiSeq 2000 platform. The raw sequencing data were mapped to the TAIR10 reference genome with NGM mapper (Sedlazeck et al., 2013) (http://citiv.io/NGMMap/). The unambiguously mapped reads with a mapping quality score over 20 were subjected to duplicate removal (Li et al., 2009) (samtools; http://www.htslib.org/) and overlap clipping (Barnett et al., 2011) (bamtools; https://github.com/pezmaster31/bamtools). The SNPs were called with Freebayes (Garrison and Marth, 2012) (https://github.com/ekg/freebayes) with a lower threshold of three alternative reads per locus. The obtained data were fed to a hidden Markov chain based algorithm that estimated the likelihood of the SNP given the read data assuming linkage between the SNPs within each chromosome. The SNPs were classified into intronic, intergenic, coding (synonymous and nonsynonymous amino acid substitutions), and splice-site variants with Ensemble Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep). Supplemental Data Set 1 displays the list of 489 filtered EMS-generated SNPs with alternative reads >7 and the ratio of alternative over reference reads comprised between 0.05 and 0.75, ranked by probability of being the causative SNP. The same list was filtered for SNPs with a ratio between 0.4 and 0.6 and occurring in exonic regions only (12 SNPs remaining).

dCAPS Marker Assay for Genotyping the ipr7 Mutation

To confirm the causative SNP (G280A in AT3G05580) in ipr7 mutant plants, a derived cleaved amplified polymorphic sequence (dCAPS) marker assay was conducted on ipr7/AUN1 and ipr7/ipr7 plants with primer pair ABD732/ABD733 designed with dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). TaqI (0.4 μL) and 2.27 μL of 10xUnique TaqI buffer (Thermo Fisher Scientific) were mixed and added per genomic DNA sample. The PCR products were then digested at 65°C overnight and subsequently separated in a 3% agarose gel by gel electrophoresis. While wild-type plants produce a 170-bp-long fragment, ipr7/ipr7 plants give rise to a 202-bp-long fragment. Genomic DNA from ipr7/ AUN1 plants generates both fragments.

Phylogenetic Analysis

Multiple alignments of Arabidopsis TOPP proteins were made in ClustalW2.0 (Supplemental Data Set 2) and with MEGA7. The phylogenetic tree was then reconstructed under usage of the protein sequence parsimony method (bootstrap test, 1000 replicates).

Generation of AUN1-, AUN2-, AUN1D94N-, and AUN1H127A-YFP Protein Fusions for Stable Expression in Arabidopsis Pollen Tubes

To obtain the AUN1 and AUN1D94N coding sequences, RNA was isolated from ipr7/AUN1 flowers with the RNeasy Plant Mini kit (Qiagen). For the AUN2 coding sequence, RNA was isolated from wild-type flowers. cDNA synthesis was then performed using the ReverTra HMinus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with Gateway-compatible primer pairs ABD776/ABD777 and ABD776/ABD796 to amplify AUN1 and AUN2, respectively, without stop codon. The 950-bp-long fragments
without stop codons were cloned into pDONR207 (Invitrogen) and trans-
formed into Escherichia coli DH5α cells. After sequencing positive clones 
with ABD743 and ABD744, AUN1, AUN2, and AUN1<sup>10936</sup> were remobil-
ized into the Gateway-compatible binary vector pABD34 (ProLat52:GW-YFP)
that confers Basta resistance, yielding pCMF3, pJW7, and pCMF5, respec-
tively (Supplemental Table 4). To obtain the AUN1<sup>11127α</sup> variant, 
primer pair ABD810/ABD811 was designed with PrimerX (http://www.
bioinformatics.org/primerx/) and used on AUN1 without a stop codon in 
pDONR207 (Invitrogen) as a template for site-directed mutagenesis by 
PCR. After DpnI digestion, the newly synthesized vectors were trans-
formed into E. coli and sequenced. Afterwards, AUN1<sup>11127α</sup> was remobi-
lized into pABD34 as well, giving rise to pCMF10.

Selection of Transformed Lines

After plant floral dip transformation, the T1 seeds were preselected on 
half-strength MS plates with Basta (glufosinate ammonium, 10 mg/L) se-
lection. Basta-resistant seedlings were picked, transferred to soil, and
allowed to grow until flower emergence. Subsequently, pollen of each line
was screened for fluorescence, indicative of the expression of the fusion
proteins. The lines with clear homogeneous fluorescence in half of 
the pollen grains were taken to the next generation. Then, several T2 
lines were subjected to Basta selection and screened for fluorescence in 
all pollen grains to identify homozygous lines.

In Vitro Pollen Germination Assays

Pollen germination medium (5 mM KCl, 1 mM MgSO<sub>4</sub>, 0.01% H<sub>3</sub>BO<sub>3</sub> 
[w/v], 5 mM CaCl<sub>2</sub>, and 10% sucrose [w/v], dissolved in double-distilled
water and adjusted to pH 7.5) was prepared as previously described (Boavida and McCormick, 2007). Pollen germination assays were con-
ducted as described by Franck et al. (2017) and imaged using a Leica
DM5500 fluorescence microscope equipped with differential interference 
contrast optics. Subcellular localization of AUN1-YFP and AUN2-YFP was 
investigated using a confocal laser scanning microscope (Leica TSC SP8).

Typically, three rounds of independent pollen germination assays were 
conducted per experiment. For each assay, 5 h after incubation, ~10 
images of ~20 pollen or more are consistently taken in the best germination 
zones for a total of more than 200 pollen grains per line per assay. The im-
ages are then later analyzed and the pollen grains are classified into three 
groups: bursting pollen grains characterized by a retracted cytoplasm 
in the pollen with traces of cytoplasm outside (Boisson-Dernier et al., 
2009), pollen grains forming PTs larger than the pollen grain diameter, and 
pollen grains that do not match these two groups. The first two groups 
are comprised as germinated pollen grains. The germination rate is then 
defined as 100% × (number of germinated pollen grains/total number 
of pollen grains). The bursting rate is defined as 100% × (number of burst 
pollen grains/number of germinated pollen grains).

Aniline Blue Staining of Pollinated Pistils and Seed Set Analyses

Wild-type flower buds with nondenhescent stamens were emasculated 
and allowed to recover for 2 d. Thereafter, nicely developed pistils with 
elongated papillae were pollinated with wild-type or mutant pollen. After 
4 h, the siliques were harvested and fixed in a 9:1 solution of 96% (v/v)
ethanol/glacial acetic acid overnight. The next day, the fixing solution was 
replaced with 70% ethanol for 5 min, 50% ethanol for 5 min, and 30% 
ethanol for 5 min with occasional mild agitation. Thereafter, the ethanol 
was removed and replaced by 10% (v/v) chloral hydrate solution (in 
24 mL; 64 g chloral hydrate, 16 mL double-distilled water, and 8 mL glycerol)
at 65°C for 5 min. Pistils were washed with sodium phosphate buffer (in 
100 mL; 9.3 mL of 1 M Na<sub>2</sub>HPO<sub>4</sub>, 6.8 mL of 1 M NaH<sub>2</sub>PO<sub>4</sub>, dissolved 
in double-distilled water; pH 8.0). Then, pistils were incubated at 65°C for
10 min in 5 n NaOH. Samples were washed twice with sodium phosphate
buffer. Before microscopy, the pistils were arranged parallel to each other 
on a glass slide and stained with 0.1% (w/v) Methyl Blue before a cover 
slip was gently applied on top. The samples were imaged using a Leica
DM5500 fluorescence microscope.

For seed set analyses, 8 to 15 green-yellowish siliques were harvested 
per plant and fixed in a 3:1 mixture of 96% (v/v) ethanol glacial acetic
acid. After 1 d, the siliques were opened with a needle and the seeds per 
silique were counted under a dissecting microscope.

Root Hair Growth Assays

Root hair growth assays in liquid conditions were conducted as previously 
described (Boisson-Dernier et al., 2015). Seedlings were grown vertically 
on half-strength MS plates with microagar for 4 d. Thereafter, they were 
transferred to glass slides and the roots were covered in 1/10 MS medium.
A cover slip was applied on top using two layers of Parafilm on each end
of the glass slide as spacer to prevent squashing of the roots. The 
seedlings were transferred to an in situ hybridization dish covered in liq-
uid 1/10 MS medium so the slides would not dry and allowed to recover 
vertically for 1.5 d. Thereafter, the RHs of the newly grown root zones 
were imaged with a Leica DM5500 fluorescence microscope.

Particle Bombardment of Arabidopsis Leaves

AUN1, AUN2, and MRI coding sequences without a stop codon in 
pDONR207 were recombinated in the Gateway-compatible binary vector
pXCSG-YFP (Pro35S:GW-YFP; Feys et al., 2005). For confirmation of 
the subcellular localizations of AUN1, AUN2, and MRI as a reference, 
epidermal cells of young Arabidopsis leaves were bombarded with gold
particles coated with AUN1-YFP, AUN2-YFP, and MRI-YFP respectively,
using a PDS-1000/He instrument (Bio-Rad). All three constructs were 
cobombarded with plasma membrane marker mCherry-NPSN12 (Geldner
et al., 2009) as a transformation control. For coating the 1.0-μm gold 
particles (Bio-Rad) with DNA, a precipitation mixture (25 μL total volume:
400 ng of each, the YFP-plasmid DNA and the transformation control 
DNA; 5 μL of a 30 mg/mL gold stock; 10 μL of 2.5 M CaCl<sub>2</sub>; 4 μL of 0.1 
M spermidine; filled up with double-distilled water) was prepared for 
each sample. The mixtures were then vortexed for 10 min. The samples 
were centrifuged at 13,300 rpm for 20 s, the supernatant was discarded, 
and the pellet was resuspended in 50 μL of 70% ethanol. This washing
step was repeated with 20 μL 100% ethanol. Afterwards, the pellet was 
resuspended in 12 μL 100% ethanol and transferred to a macrocarrier.
The standard settings for bombardments were applied (900 p.s.i., 6-cm 
target distance, 27 mm Hg vacuum pressure). Afterwards, the samples 
were incubated in the dark for 24 h at room temperature and subsequently 
analyzed on a confocal laser scanning microscope (Leica TSC SP8).

Image Analyses

Image analyses were conducted using Leica LAS AF Lite software or
ImageJ/Fiji software (Schindelin et al., 2012).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL 
data libraries under the following accession numbers: NP_187209 for
AUN1 (TOPP9), NP_568501 for AUN2 (TOPP8), NP_181728 for MRI,
NP_187120 for ANX1, and NP_198220 for ANX2.

Supplemental Data

Supplemental Figure 1. Average seed set of wild-type, aun1-1, aun2-1,
aun1-1 aun2-1, and aun1-2 aun2-2 plants.
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AUTHOR CONTRIBUTIONS

C.M.F. and A.B.-D. conceived the experiments. C.M.F., J.W., S.B., R.L., and A.B.-D. performed the experiments shown. C.M.F., J.W., S.B., D.S.L., and A.B.-D. analyzed the data. C.M.F. and A.B.-D. wrote the manuscript with contributions from the other authors.

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The Protein Phosphatases ATUNIS1 and ATUNIS2 Regulate Cell Wall Integrity in Tip-Growing Cells
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