Patronus is the elusive plant securin, preventing chromosome separation by antagonizing separase

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Chromosome distribution at anaphase of mitosis and meiosis is triggered by separase, an evolutionarily conserved protease. Separase must be tightly regulated to prevent the untimely release of chromatid cohesion and disastrous chromosome distribution defects. Securin is the key inhibitor of separase in animals and fungi, but has not been identified in other eukaryotic lineages. Here, we identified PATRONUS1 and PATRONUS2 (PANS1 and PANS2) as the Arabidopsis homologs of securin. Disruption of PANS1 is known to lead to the premature separation of chromosomes at meiosis, and the simultaneous disruption of PANS1 and PANS2 is lethal. Here, we show that PANS1 targeting by the anaphase-promoting complex is required to trigger chromosome separation, mirroring the regulation of securin. We showed that PANS1 acts independently from Shugoshins. In a genetic screen for pans1 suppressors, we identified SEPARASE mutants, showing that PANS1 and SEPARASE have antagonistic functions in vivo. Finally, we showed that the PANS1 and PANS2 proteins interact directly with SEPARASE. Altogether, our results show that PANS1 and PANS2 act as a plant securin. Remote sequence similarity was identified between the plant patronus family and animal securins, suggesting that they indeed derive from a common ancestor. Identification of patronus as the elusive plant securin illustrates the extreme sequence divergence of this central regulator of mitosis and meiosis.

meiosis | mitosis | separase | securin

Balanced segregation of chromosomes at both mitosis and meiosis requires that chromatid cohesion complexes be removed after proper chromosome alignment in the spindle. Inaccuracies in this process cause chromosome missegregation and aneuploidy, contributing to cancer and birth defects. Separase, which is conserved in fungi, animals, and plants, triggers cohesion release by cleaving the kleisin subunit of the cohesin complex, opening the cohesin ring and allowing chromosome segregation (1). Securin is the primary regulator of separase activity in animals and fungi, forming a complex with separase and blocking substrate access to its active site. Securin, which is defined by its functional and biochemical properties, is a largely unstructured protein whose sequence is conserved only between closely related species (2–6). At the onset of anaphase, the anaphase-promoting complex (also known as the cyclosome) (APC/C) triggers the degradation of securin, releasing separase activity and allowing chromosome separation. In vertebrates, the Cdk1–CyclinB1 complex is an additional regulator of separase activity (7). At meiosis I, the cleavage of cohesins is also spatially controlled: Pericentromeric cohesins are protected from separase cleavage by shugoshin-PP2A through dephosphorylation of the kleisin subunit (8, 9).

In Arabidopsis, separase is essential for cohesion release and chromosome segregation at both meiosis and mitosis (10, 11). APC/C regulates the progression of division (12, 13) and the shugoshins SGO1 and SGO2 and PP2A are required for cohesion protection at meiosis (14–16). However, securin is missing from the picture, raising the intriguing possibility that securin has been lost in the green lineage and that plant separase is regulated by a different mechanism.

We previously characterized the two paralogues PATRONUS1 and PATRONUS2 in Arabidopsis (PANS1, PANS2) (14). PANS1 is essential for the protection of sister chromatid cohesion between the two meiotic divisions, through an unknown mechanism. In the pans1 mutant, sister chromatid cohesion is lost before metaphase II, leading to chromosome segregation defects at meiosis II (14, 17, 18). In addition, the pans1 mutant also has a slight growth defect, which is exacerbated under stress conditions, associated with a certain level of mitotic defects and aneuploidy in somatic cells (14, 17, 19). The pans2 mutant is indistinguishable from the wild type but has synthetic lethal interaction with pans1, suggesting that PANS1 and PANS2 have an essential but redundant role at mitosis (14). PANS1 and PANS2 share 42% identity and encode proteins of unknown function. PANS1 has been shown to interact with APC/C through its destruction-box (D-box) and KEN-box domains. Patronus proteins are well-conserved in dicots, one of the two major clades of flowering plants, with most species having one or two homologs (14). In monocots, the other major clade of flowering plants, PANS proteins share limited similarity with RSS1 (RICE SALT SENSITIVE 1, also known as Os02g39390), a protein that regulates cell cycle under stress conditions in rice (20). Although PANS1, PANS2, and RSS1 clearly play a role in plant development, their function remains to be elucidated.

Significance

Accurate chromosome segregation at mitosis and meiosis is crucial to prevent genome instability, birth defect, and cancer. Accordingly, separase, the protease that triggers chromosome distribution, is tightly regulated by a direct inhibitor, the securin. However, securin has not been identified, neither functionally nor by sequence similarity, in other clades that fungi and animals. This raised doubts about the conservation of this mechanism in other branches of eukaryotes. Here, we identify and characterize the securin in plants. Despite extreme sequence divergence, the securin kept the same core function and is likely a universal regulator of cell division in eukaryotes.

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important role in cell division, the molecular function of PANS1, PANS2, and RSS1 has remained elusive.

Results

Expression of Destruction-Box-Less PATRONUS1 Prevents Cohesion Release and Chromosome Segregation. The sequences of PANS proteins contain a conserved $\delta$-box domain (RxxLxxN), recognized by the anaphase-promoting complex (APC/C), which triggers the destruction of the targeted protein by the proteasome. A mutant version of PANS1 mutated in its $\delta$-box (PANS1Δ$\delta$; RxxL → LxxV) loses its capacity to interact with the APC/C activator subunit CDC20 in yeast two-hybrid (Y2H) assays (14). Expression of PANS1Δ$\delta$ under its own promoter is lethal, suggesting that PANS1 accumulation prevents plant development (14). To assess the function of the PANS1 $\delta$-box at meiosis, we expressed PANS1Δ$\delta$ under the meiosis-specific promoter DMC1. Wild-type PANS1 expressed under the DMC1 promoter was able to complement the meiotic defects of the pans1 mutant (n = 2/4) (SI Appendix, Fig. S1). In contrast, pDMC1::PANS1Δ$\delta$ caused full sterility when transformed in wild-type or pans1 plants (n = 15/15 and 2/2, respectively). In addition, pDMC1::PANS1Δ$\delta$ plants showed a variable growth defect, from barely developing plants to wild-type-like plants (SI Appendix, Fig. S2), suggesting that the DMC1 promoter can drive variable expression in somatic tissues, but all plants showed complete sterility. In pDMC1::PANS1Δ$\delta$ plants, meiotic chromosome spreads did not reveal any defect in prophase or early metaphase I (Fig. 1). However, among the 119 postprophase cells observed, none showed the configuration typical of anaphase I, metaphase II, anaphase II, or telophase II, suggesting that the meiotic cells do not progress beyond metaphase I. We observed normal metaphase I with five bivalents aligned on the metaphase plate (compare Fig. 1F with Fig. 1A), but also metaphase I with five overstretched bivalents (Fig. 1G). We also observed configurations resembling metaphase II, with chromosomes distributed in at least two groups separated by a dense band of organelles. However, a total of five bivalents aligned on the two metaphase II plates in pDMC1::PANS1Δ$\delta$ (e.g., three bivalents on one plate, and two on the other) (Fig. 1I), instead of five pairs of chromatids on each metaphase plate as observed in the wild type (Fig. 1D). At telophase II, we observed five nuclei, presumably each containing a decondensed bivalent. This observation suggests that pDMC1::PANS1Δ$\delta$ abolishes the separation of homologous chromosomes at meiosis I. Immunostaining revealed that the bivalents in pDMC1::PANS1Δ$\delta$ at a metaphase I- or metaphase II-like configuration were entirely decorated with the cohesin REC8, suggesting that the inhibition of chromosome separation is due to an incapacity to remove cohesins (Fig. 2). We then observed the effect on pDMC1::PANS1Δ$\delta$ on male meiocytes with live imaging of cells expressing a red fluorescent protein (RFP)-tagged tubulin (RFP:TUB4) and a green fluorescent protein (GFP)-tagged REC8 (21) (Figs. 3–5 and Movies S1–S3). In the wild type (Movie S1), we observed the progression of male meiosis from prophase, with tubulin surrounding the nucleus (Fig. 3A), to metaphase I with the formation of the spindle and alignment of the chromosomes (Fig. 3C), and to anaphase I with the disappearance of the REC8 signal and reorganization of the spindle (Fig. 3D and E). The first division, from the end of prophase (nuclear break down, Fig. 3B) to the onset of anaphase I, lasted 38 ± 5 min (mean ± SD, n = 29 cells). When analyzing pDMC1::PANS1Δ$\delta$ with live imaging (Movies S2 and S3), we observed cells progressing normally from prophase to metaphase I (Fig. 4A–C). However, the length of metaphase I was very variable, from 40 min (Movie S2 and Fig. 4) to more than 4 h (Movie S3). When anaphase occurred, as observed regarding microtubule reorganization (Fig. 4D and E and Movie S2), the REC8 signal was still detected. Further, five REC8:GFP bodies, presumably representing the five bivalents, were still observed at late anaphase I (arrows in Fig. 4E), and following interphase, a spindle polymerized around each of the five REC8:GFP bodies (Fig. 4F and Movie S2). Thus, live imaging confirmed that the expression of a $\delta$-box-less PANS1 at meiosis prevents the release of REC8 and the separation of chromosomes.

In Arabidopsis spo11-1 mutants, the formation of crossovers is eliminated and the resulting unconnected chromosomes (univalents) segregate randomly at anaphase I and progress...
through metaphase II and anaphase II with segregation of sister chromatids (22) (Fig. 5). Thus, the spo11-1 mutation should allow segregation of chromosomes at meiosis I in pDMC1::PANS1ΔD and entry into meiosis II, making it possible to assess the effect of PANS1ΔD on chromatid pairs at anaphase II. Our results indicated that spo11-1 pDMC1::PANS1ΔD meioocytes progressed to metaphase II (Fig. 5B), but appeared to be arrested at that stage with stretched chromosomes (Fig. 5D), showing that PANS1ΔD can prevent chromosome segregation of both homologs at meiosis I and sister chromatids at meiosis II. The lethality of PANS1ΔD expressed under its own promoter suggests that it can also prevent chromosome segregation at mitosis. Altogether, these results suggest that targeting of PANS by APC/C is required for the release of cohesion and chromosome distribution at anaphase of mitosis and meiosis, mimicking the regulation and function of securin (7, 23, 24).

**PATRONUS1 Acts Independently of SHUGOSHINs.** Shugoshin (SGO) is an evolutionarily conserved protein that protects sister chromatid cohesion against separase (8, 25). In Arabidopsis, both shugoshin genes (SGO1 and SGO2) are involved in the protection of pericentricmeric cohesion during meiosis, with the corresponding double-mutant leading to the complete loss of sister chromatid cohesion at anaphase I (14), without apparent somatic function. The pans1 mutant loses pericentricmeric cohesion during interkinesis. In both pans1 and sgo1 sgo2, metaphase I appears normal with five aligned bivalents (14). To test if PANS1 acts through or independently of SGO, we first combined sgo1 sgo2 and pans1 mutations (Fig. 6 A–D). In the pans1 sgo1 sgo2 triple mutant, only a small proportion of metaphase I showed five bivalents (11%, n = 321 cells, Fig. 6F), whereas the majority showed an almost complete (41%, Fig. 6C) or complete (48%, Fig. 6D) loss of cohesion, with 20 free chromatids. At diakinesis, the stage of prophase that immediately precedes metaphase I, five bivalents were systematically observed showing that crossovers occurred and sister chromatid cohesion was established (Fig. 6A). Thus, in pans1 sgo1 sgo2, sister chromatid cohesion was lost at prometaphase I or early metaphase I, dismantling the bivalent into free chromatids. This result shows that PANS1 and SGOs act in parallel to protect sister chromatid cohesion at metaphase I. This also reveals that SGO1 and SGO2 protect cohesion not only at pericentromeres, but also along the chromosome arms, redundantly with PANS1.

We then expressed pDMC1::PANS1ΔD in the sgo1 sgo2 mutant (n = 4 plants). In this context, meiotic chromosome spreads showed metaphase I with stretched bivalents (Fig. 6E) and aberrant metaphase II with five bivalents (Fig. 6F), similarly to what was observed when pDMC1::PANS1ΔD was expressed in the wild type (Fig. 1). This shows that the expression of PANS1ΔD at anthesis prevents chromosome segregation even in the absence of SGOs, confirming that the PANS1 function is independent of SGOs.

**Mutations in SEPARASE Can Restore Sister Chromatid Cohesion in** **patronus1.** With the aim of identifying antagonists of PATRONUS1 and to shed light on its function, we set up a genetic suppressor screen. We took advantage of the root-growth defect of pans1 when cultivated on medium supplemented with NaCl (14, 19). pans1-1 seeds were mutagenized with ethyl methanesulfonate and the two families obtained by self-fertilization of individual mutagenized plants were screened for (i) longer roots than pans1-1 on NaCl medium and, subsequently, (ii) longer roots than pans1-1 after transfer to the greenhouse. Plants satisfying both criteria were identified in 8 of the 200 independent families screened. Whole-genome sequencing of these plants revealed that four of the eight suppressors had a missense mutation in the Arabidopsis SEPARASE gene (AtESP; At4g22970, SI Appendix, Table S1), that we hereafter call esp-S606N, esp-P1946L, esp-A2047T, and esp-P2156S (Figs. 7 and 8 and SI Appendix, Fig. S3). Bulk genome sequencing of a segregating population identified esp-S606N as the mutation most strongly linked to the growth phenotype among the mutations segregating in that line, further supporting the conclusion that the mutations in ESP are causal. The esp-P1946L, esp-A2047T, and esp-P2156S mutations affected well-conserved residues of the protease domain of ESP (SI Appendix, Fig. S3). The S606 residue is in a less conserved helical domain and belongs to a stretch of serine, which may suggest regulation of ESP by phosphorylation. The previously described esp-2 mutation is null and lethal (10) but can restore pans1 growth in a dominant manner (Fig. 7C), confirming that decreasing ESP activity can suppress the pans1 somatic defect. Single mutants esp-S606N, esp-P1946L, esp-A2047T, and esp-P2156S are viable when homozygous, without apparent defects in growth or development, suggesting that they are hypomorphs. Quantification of root growth in pans1 mutants segregating for the esp-S606N or esp-P2156S mutations showed that these mutations restore root growth in a semidominant and dominant manner, respectively (Fig. 7D).

To test if the mutations in ESP suppress the pans1 1 meiotic defects, we quantified sister chromatid cohesion at metaphase II (Fig. 7 C and D). Although cohesion is almost completely lost in pans1 (Fig. 7D, 10 free chromatids indicating complete absence...
of cohesion), it is partially restored in a semidominant manner by the \(esp-S606N\) mutation and fully restored by the \(esp-P2156S\) mutation in a recessive manner. The \(esp-2\) heterozygous mutation did not restore the \(pans1\) sister chromatid defect, but \(pans1\) \(esp-2/esp-P2156S\) plants had restored cohesion, further confirming that \(esp\) mutations cause the suppression of the \(pans1\) phenotype. Thus, mutations in \(ESP\) can suppress the meiotic sister chromatid defect of \(pans1\). However, the \(esp\) mutations were not able to restore the gametophytic lethality of the \(pans1 pans2\) double mutant, suggesting that mutating \(ESP\) cannot compensate for the total absence of \(PANS\) (SI Appendix, Table S2). In addition, \(esp-S606N\) and \(esp-P2156S\) mutations were not able to suppress the meiotic sister chromatid defect of \(sgo1\) or of \(sgo1 sgo2\) (Fig. 7D). This result suggests that \(PANS1\) and \(ESP\) have a specific antagonistic function in regulating the release of sister chromatid cohesion at meiosis.

**PATRONUS Interacts Directly with SEPARASE and APC/C.** To better understand the role of \(PANS1\), we searched for interacting partners using pull-down protein purification coupled with mass
spectrometry using overexpressed GS\textsuperscript{rhino}-tagged PANS1 as bait in Arabidopsis cell culture (26). After filtering copurified proteins for false positives (see Methods and ref. 27), we recovered peptides from PANS1 itself and a series of additional proteins in three replicate experiments (Table 1). We recovered 10 subunits of the APC/C complex, confirming previous findings (14). Most importantly, the PANS1 pulldown identified numerous peptides of SEPARASE (ESP1) in all three purification experiments, showing that PANS1 interacts with SEPARASE in vivo. Y2H assays and bimolecular fluorescent complementation (BiFC) experiments both confirmed that PANS1 and PANS2 interact with SEPARASE (Fig. 8 and SI Appendix, Table S3). Y2H experiments with truncated PANS1 showed that the C-terminal half of PANS1 (which does not contain the conserved KEN and D boxes) is sufficient to mediate an interaction with SEPARASE. In yeast and animals, the securin C-terminal region also mediates the interaction with separase (4, 5) (Fig. 9). The N-terminal domain of Arabidopsis SEPARASE showed the strongest interaction with PANS1 and PANS2. In yeast and human, securin interacts along the entire length of separase (4, 5, 7, 28).

We previously showed that PANS1 interacts with the APC/C subunit CDC20 through its D box (14) and showed here that PANS2 also interacts with CDC20 (SI Appendix, Fig. S4). Yeast and animal securins have also been shown to bind directly CDC20 in a D box-dependent manner (29, 30).

PATRONUS Has Remote Sequence Similarity with Securins. The experimental data presented above suggest that PATRONUS may be the elusive plant securin. We thus investigated the degree to which PATRONUS is conserved and if any sequence similarity can be detected between PATRONUS and animal or yeast securin.

Position-specific iterated BLAST (PSI-BLAST) against the green lineage identified homologs of PATRONUS and RSS1 in flowering plants, gymnosperms, and basal vascular plants (e.g., mosses and ferns) as previously reported (14, 20). Further iterations then identified homologs in algae, including Ostreococcus lucimarinus (XP_001422400.1) (Methods). Reciprocal PSI-BLAST analyses starting from the O. lucimarinus protein recovered the entire plant protein family, including RSS1, PATRONUS1, and PATRONUS2, reinforcing the conclusion that the identified proteins are homologs (Methods and SI Appendix, Table S3). The slow convergence of the PSI-BLAST search likely arises from the high sequence divergence between the PATRONUS family members and from their intrinsically disordered character.

Next, we repeated the PSI-BLAST analyses using the O. lucimarinus sequence (XP_001422400.1) as bait and expanded the interrogation to the full eukaryotic tree. After eight iterations, a few proteins of unknown function from bivalves and gastropod species were identified, such as the bivalve mollusk...
Looking at the conservation patterns of the securin proteins (SI Appendix, Fig. S4), in each clade (monocots, dicots, Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Caenorhabditis elegans), the same profile appeared: conserved KEN and D boxes in the N-terminal and an invariant glutamic acid (E) within a relatively conserved patch. This glutamic acid is followed by a glycine residue (G) and a conserved patch. This glutamic acid is followed by a glycine residue (G) and a conserved patch.
Acid has been shown to be pivotal for the inhibition of the separase cleavage site in the mammalian and the two fungus clades (pink stars in Fig. 9) and is also shared with separase substrates (4, 7, 32, 33). In plants, two glutamic acids (E) are well conserved (SI Appendix, Fig. S4, unfilled stars in Fig. 9) and are thus prime candidates important for separase inhibition. One is in the C-terminal third of the protein and is very well-conserved among the entire plant lineage (monocots, dicots, basal plants, and algae). However, it is not conserved in some groups of species (e.g., the Phalaenopsis orchids and several algae such as Micractinium conductix), making this candidate less likely. The other candidate glutamates lie in the middle of the proteins in domains conserved within dicots and within monocots in different regions (E-x-F-L-D/H-N/H and W/A–K/R–D/E–G–V/I–E, respectively; Fig. 9 and SI Appendix, Fig. S4) but of unknown function (14, 20). The monocot glutamate is extremely well-conserved in the Viridiplantae, ranging from monocots to very distant species such as algae (SI Appendix, Fig. S6), supporting its pivotal role. However, standard algorithms did not align the conserved E-containing patch from dicots with the conserved E-containing patch from monocots. We favor the scenario in which these E-containing domains represent the separase inhibition site, but it has diverged too much in dicots to be properly aligned using the current algorithms.

Discussion
Securin is the central regulator of chromosome distribution at both mitosis and meiosis in animals and fungi. Securin inhibits the separase protease, thus preventing untimely release of sister chromatid cohesion. At the onset of anaphase, APC/C degrades securin, releasing separase and thereby allowing chromosome distribution. However, to date, securin counterparts in plants have been elusive, suggesting that an alternative mechanism may be at play. We provide five pieces of strong evidence that patronus are securin homologs in plants. First, at meiosis, depletion of PANS1 leads to the premature release of cohesion (14); conversely, expression of an APC/C-insensitive PANS1 abolishes cohesion release and chromatid...
separation, mimicking the depletion of separase (10). If expressed constitutively, the APC/C-insensitive PANS1 is lethal. The single pans1 mutation provokes some chromosome missegregation and aneuploidy (17, 19). No defects were detected in the pans2 mutant, but the pans1 pans2 double mutation is lethal (14). We propose that PANS1 and PANS2, whose duplication has been retained from the whole genome duplication at the origin of Brassicaceae, have redundant functions, but that expression and/or activity levels of PANS2 are higher than those of PANS1. Supporting that possibility, both genes are expressed in all dividing tissues with PANS2 being globally 6.3 times more expressed than PANS1 (34) (SI Appendix, Table S4). Our data support the conclusion that PATRONUS1 and/or PATRONUS2 prevent cohesion release in both mitosis and meiosis and that degradation of PATRONUS by APC/C is required to lift this inhibition. Second, we showed that PANS1 controls chromosome segregation independently of SGOs, excluding the alternative hypothesis that patronus regulates shugoshin. Third, we demonstrated that the PATRONUS1 and PATRONUS2 proteins interact directly with SEPARASE and APC/C. Fourth, using a forward genetic screen, we identified mutations in SEPARASE that suppress the defects of patronus1 mutants, showing that PATRONUS1 and SEPARASE have antagonistic functions. Lastly, we identified remote sequence similarity between plant PATRONUS/RSS1 proteins and animal securins. Altogether, this strongly supports the conclusion that PATRONUS/RSS1 is the plant securin.

We propose that all plant PATRONUS homologs, including the rice RSS1 (35), represent plant securins. Consistent with this conclusion, RSS1 is expressed in dividing cells and is regulated by the APC/C in a D box-dependent manner (20). Further, expression of RSS1 deleted of its N-terminal domain that contains D and KEN boxes is lethal. RSS1 functions in the regulation of the cell cycle, but the rss1 mutant is viable and fertile (20). However, RSS1 has an uncharacterized paralogue in the rice genome (Oslg0898400) (20) (SI Appendix, Fig. S5), which may act redundantly with RSS1, as PANS1 and PANS2 do. RSS1 has been shown to interact with the PP1 phosphatase (20, 35, 36), suggesting that RSS1 is either regulated by PP1 or has an additional function than inhibiting securin.

It is intriguing that securin proteins have such a poorly conserved sequence although they play such a central role in cell division. The only conserved features are the presence of D and KEN boxes, which are involved in cell-cycle regulation, and a variable conserved patch containing glutamic acid (E), which is pivotal in separase inhibition. In the plant lineage, the phylogenetic analysis of PANS1/RSS1 suggests that, in addition to securin expression, the plants may have evolved and/or experienced a history of gene duplication/gene loss (14). Securin interacts with separase along all its intrinsically unstructured length (7) (SI Appendix, Fig. S9) and acts as a pseudosubstrate. It is likely that securin has no catalytic activity. One possibility is that the securin sequence simply drifts passively due to the absence of any selective pressure on its sequence, leading to relaxed purification selection. Alternatively, securin being a key regulator of cell cycle, pivotal in development and the stress response, may evolve rapidly in response to selective pressures. In support of this idea, the rice rss1 and the Arabidopsis pans1 mutants are hypersensitive to a range of abiotic stresses (14, 19, 20) and intrinsically disordered protein regions are frequent targets of positive selection (37, 38). The rapid divergence of PATRONUS and its securin homologs may thus represent the accumulation of physiological and developmental responses to a constantly changing environment.

Methods

Plant Materials and Growth Conditions. Arabidopsis thaliana plants were grown in greenhouse or growth chambers (16 h day/8 h night, 20 °C, 70% humidity) and Nicotiana benthamiana in greenhouses (13-h day 25 °C/11-h night 17 °C). For in vitro culture, Arabidopsis seeds were surface sterilized for 10 min in 70% ethanol + 0.05% sodium dodecyl sulfate and washed for 10 min in 70% ethanol and grown in Petri dishes with culture medium (Gamaglobure B5 medium-Duchefa supplied with 0.16% bromocresol purple and 0.1% sucrose). The culture medium was supplemented with 80 mM NaCl for the root-growth experiment and with 100 μM kanamycin sulfate (Euro- medex) or 25 μM hygromycin B (Duchefa) for selection of transformants. Arabidopsis transformation was performed using the floral dip method: Agrobacterium tumefaciens (EHA105 strain) was grown at 28–30 °C to saturation, centrifuged, and resuspended in a 1% sucre 0.05% Silwet 77-solution. This solution is used for dipping A. thaliana plants. Plants were maintained under glass overnight to increase humidity (39). The A. tumefaciens (CSBCL strain) was used for N. benthamiana leaf infiltration (40).

The pans1-1 (Salk_070337), Atsgo-1 (SK2556), Atsgo-2 (line 34303), spot1-1–3 (Salk_146172), and esp-2 (Salk_037016) mutants were genotyped by PCR (30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C) and two primer pairs were used (SI Appendix, Table S5). The first pair is specific to the wild-type allele, and the second pair is specific to the left border of the inserted sequence. pans1-1: N570337U and N570337L, N570337L and Lbsalk1, Atsgo-1: SK2556U and SK2556L, SK2556U and pSKTal1, Atsgo-2: SGO2U and SGO2L, SGO2U and GABI, spot1-1-3: N646172U and N646172L, N646172L and Lbsalk2, esp-2: N537016U and N537016L, N537016L and Lbsalk2.

The esp-5066W and esp-P2156S mutants were genotyped using PCR. The PCR products were digested by restriction enzymes. For esp-5066W, the S02- pb fragment (esp-5066U and esp-5066L) was digested with a SfiI (Thermo Fisher Scientific) restriction enzyme at 65 °C for 1 h. The wild-type allele yielded three DNA fragments (441, 49, and 12 pb) and the esp-5066W allele yielded four DNA fragments (300, 141, 49, and 12 pb).

For esp-P2156S, the 1040-pb PCR product (esp2156U and esp2156L) was digested with the HindIII (Thermo Fisher Scientific) restriction enzyme at 37 °C for 1 h. The wild-type allele yielded the DNA fragments of 556 and 474 pb and the esp-P2156S allele yielded one DNA fragment (1,040 pb).

The RFP-tagged tubulin (RFP:TUB4) line and the GFP-tagged REC8 (REC8:GFP) were provided by A.S. Homozygous lines for RFP:TUB4 and REC8:GFP were transformed using floral dip with the pDM1:C1:PatronusD construct. Transformants were selected on Petri dishes containing in vitro culture medium supplemented with kanamycin.

Suppressor Screen. For the suppressor screen, homozygous pans1-1 seeds were incubated for 17 h at room temperature in 5 mL of 0.3% (vol/vol) ethyl methanesulfonate (EMS) (Sigma) with gentle agitation. EMS was neutralized by adding 5 mL of 1 M sodium thiosulfate for 5 min. Then, 3 mL of water was added to make the seeds sink. The supernatant was removed, and seeds were washed three times for 20 min with 15 mL of water. These M1 seeds were grown in the greenhouse and selfed to produce M2 seeds. M2 seeds were sterilized and sown in Petri dishes containing in vitro culture medium supplemented with 80 mM NaCl (~15 M2 per M1). M2 plants with long roots were transferred in the greenhouse and visually scored for root length. Mutants or mutant populations were sequenced using Illumina technology (GET platform https://get.genotoul.fr/en). Mutations were identified using the MutDetect pipeline (41). The esp-5066W line identified in the suppressor screen was back crossed with pans1-1. Seeds were selected in the F2 population for having long roots on an NaCl medium. Bulk genome sequencing of these plants identified the esp-5066W mutation as the most strongly linked (36/41 mutant reads) to the phenotype among the selected plants allowing isolation of the mutation segregating in that line, supporting that it is causal.

Mosaic Chromosome Spreads and Immunolocalization. Meiotic chromosome spreads and immunolocalization were performed following Ross et al (42) with modifications. Inflorescences were harvested in 3:1 fixative (3 vol EtOH:1 vol acetic acid). Fixative was replaced once. For slide preparation, inflorescences were washed twice in water and once in citrate buffer (10 mM trisodium citrate, pH adjusted to 4.5 with HCl). They were digested for 3 h at 37 °C in a moist chamber with a digestion mix (0.3% [w/vol] pectolyase Y-23 [MP Biomedical], 0.3% [w/vol] Drlsease [Sigma] 0.3% [w/vol] cellulase [Onozuka R10] [Duchefa] 0.1% sodium azide in 10 mM citrate buffer). Three 0.5-mm washed buds were transferred on a slide in a drop of water and dilacerated with thin needles to generate a cell suspension. After adding 10 μL of 60% acetic acid, the slide was incubated on a hot block at 45 °C for 1 min and the cell suspension was stirred with a hooked needle. Another 10 μL of 60% acetic acid was added and stirred during one more minute. The cell suspension drop was surrounded by fresh 2.1% fixative, and the slide was rinsed with fixative. Dry slides were ready for DAPI staining and immunolocalization.

For DAPI staining, a drop for DAPI solution (2 Μglycerol:1 Μacetic acid) was added. The slide was covered with a coverslip and air-dried. Images were acquired using a Leica DM 4000 B microscope and Leica Application Suite software. Images were acquired using a Leica DM 4000 B microscope and Leica Application Suite software.
Oli M27 objective, Optotax 12.5X Tubelens. DAPI was excited at λ 335-385 nm and detected at λ between 420 and 470 nm. For immunofluorescence, slides were microwaved in 10 mM citrate buffer pH 6 for 45 s at 850 W and immediately transferred to 0.1% Triton in PBS. Slides were incubated with primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS for 48 h at 4 °C, then washed in 0.1% Triton in PBS three times for 15 min before adding the secondary antibodies in 1% BSA in PBS. After 1 h of incubation at 37 °C slides were washed in 0.1% Triton in PBS three times for 15 min and mounted in Vectashield antifade medium (Vector Laboratories) with 0.2 μg/mL DAPI. Slides were observed using a Zeiss Axio Imager 22 microscope with image software. Images were acquired using a Plan-Apochromat 100x/1.4 Oil M27 objective, Optotax 12.5X Tubelens. DAPI was excited at λ 335–385 nm and detected at λ between 420 and 470 nm. Green fluorescence was excited at λ 484–504 nm and detected at λ between 517 and 537 nm. Red fluorescence was excited at λ 576–596 nm and detected at λ between 612 and 644 nm. The rat anti-ATRB6 polyclonal antibody has been described by ref. 14 and was used at a dilution of 1:250. The secondary antibody Alexa568 goat anti-ration (Thermo Fisher Scientific) was used at a dilution of 1:250.

Live Meanosis Image Acquisition and Analysis. Live cell imaging was performed following Prusicki et al. (21) with modifications. Briefly, flowers buds of 0.4–0.6 mm were isolated on a slide. Buds were carefully dissected to isolate undamaged anthers. Anthers were transferred into a slide toped by a spacer (Invitrogen Molecular ProbesSecure- Seal spacer, eight wells, 9 mm diameter, 0.12 mm deep) filled with 10 μL of water, and covered by a coverslip. Time lapses were acquired using a Leica SP8 confocal laser-scaning and LAS X 3.5.0.18371 software.

Images were acquired using harmonic compound system, plan apochromats, confocal scan2 (HC PL APO CS2) 20×0.75 Imm and HC PL APO CS2 63×1.20 WATER objectives upon illumination of the sample with an argon laser and diode-pumped solid-state laser (516 nm) GFP was excited at λ 488 nm and detected at λ between 494 and 547 nm. RFP was excited at λ 561 nm and detected at λ between 570 and 592 nm. Detection was performed using Leica HyD detectors. Time lapses were acquired as a series of Z-stacks, (between 15 and 20 μm distance). Interval time varied from 1.5 to 3 min depending on sample conditions. Deconvolution was performed using the lightening deconvolution option. Images processing were done with Fiji. Image drift was corrected by the Stack Reg plugin (Rigid Body option). Decrease in fluorescence was corrected using the bleach correction option (simple ratio- background intensity 0- or histogram matching).

Plasmid Construction. To generate the pDMC1::PANS1 construct, the PANS1 genomic fragment was amplified using PCR with PANS1_XhoI and PANS1_SalI primers. The amplification covered PANS1 from the ATG to 477 bp after the stop codon. The PCR product was cloned by restriction digestion with XhoI and SalI into the pPeF048 vector (43) to fuse the PANS1 genomic fragment with the DMTC1 promoter. The DMTC1 promoter covered −2,940 bp before ATG and +205 pb after ATG and has been amplified from the Landsberg erecta accession. The primers −pDMC1::PANS1 DNA fragment was amplified with DMTC1_fwd and SpelB_rev primers using a synthetic droplet tail (Thermo Fisher Scientific) and cloned into pDONR207 to create pENTR; pDMC1::PANS1, on which directed mutagenesis was performed using the Stratagene Quick-Change Site-Directed Mutagenesis Kit. The primer PANS1S1D was used to create the pDMC1::PANS1S1D. For plant transformation, the LR reaction was creating the pDMC1::PANS1S1D. For plant transformation, the LR reaction was performed with the binary vector pGBWI (44).

For Y2H experiments, cDNA was amplified using the corresponding primers (SI Appendix, Table S5). They were cloned using the Gateway cloning system (Thermo Fisher Scientific) into pDONR221 to generate pENTR clones and into pDEST22 (prey plasmid) and pDEST 32 (bait plasmid) Gateway, ProQuest (Thermo Fisher Scientific). To generate the pDONR221-ESP-E446–2180 construct, a 4,474–bp fragment of ESP cDNA was obtained by DNA synthesis (GeneArt-Thermo Fisher Scientific) (sequence available in SI Appendix, Table S5). For BIFC experiments, the pENTR clones described above for Y2H were cloned by LR recombination reaction into the pBIF2 and pBIF3 vectors (40). Yeast two-hybrid assay. DNA product was cloned using Gateway (Invitrogen) into the pDONR221 vector (Invitrogen) to create pENTR. LR reactions were carried out on the pDEST32 (bait) and pDEST22 (prey) vectors (Invitrogen). Plasmids encoding the bait (pDEST32) and prey (pDEST22) were transformed into the yeast strain AH109 and Y187 (Clontech) by the LiAc method following the protocol in the MATCHMAKER GAL4 Two Hybrid System 3 manual (Clontech). The TDM protein self-interaction was used as positive controls (45). Transformed yeast cells were selected on synthetic dropout (SD) plates without Leu (SD-L) for bait or without Trp (SD-W) for prey. Interactions between proteins were assayed using the mating method. The resulting diploid cells were selected on synthetic dropout medium lacking a combination of amino acids, driven by the auxotrophy genes carried by the cloning vectors. Protein interactions were assayed by growing diploid cells on SD-LWH and SD-LWHA.

**BIFC.** Protein interactions were tested in planta using BIFC assays (46) in leaf epidermal cells of *N. benthamiana* plants expressing a nuclear GFP fused to histone 2B (47). N-terminal fusions, using the pENTR clones described above for Y2H, with two yellow fluorescent protein (YFP) complementary regions (YPFP + YFPC) were co-infiltrated in *N. benthamiana* leaves and scored after 3 or 4 d for fluorescence as described in ref. 41. YFPN-DEFICIENS and YFPCC- GLOBOSA, two interacting components of an Anthrinium majus MADS box transcription factor (48), were used as positive control. Each experiment was replicated at least twice, each corresponding to the infiltration of two different plants.

Observations were made using a Leica SP8 confocal laser-scanning microscope. Optical sections were collected with a Leica HCX PL APO CS2 20 x 0.70 IMM UV water objective upon illumination of the sample with a 514-nm argon laser line with an emission band of 520–560 nm for the YFP or with a 458-nm argon laser line with an emission band of 463–490 nm for the CFP. Detection were performed using Leica HyD detectors. The specificity of the YFP signal was systematically checked by determining the fluorescence emission spectrum between 525 and 600 nm with a 10-nm window and under an excitation at 514 nm. Images were processed using Leica LASX and Adobe Photoshop software.

**Pull-Downs.** Three pull-downs on Arabidopsis cell suspension culture expressing N-terminally GS15tagged PANS1 were performed as described (49). On-head digested samples were analyzed on a Q Exactive mass spectrometer (Thermo Fisher Scientific), and copurified proteins were identified using standard procedures (27). After identification, the protein list was filtered using VasaDB, a list of nonspecific proteins: assay products assembled similarly as described (27). True interactors that might have been filtered out due to their presence in the list of nonspecific proteins were selected by means of semiquantitative analysis using the average normalized spectral abundance factors (NSAF) of the identified proteins in the PANS1 pull-downs. Proteins identified with at least two peptides in at least two experiments, showing high (at least 10-fold) and significant [−log10(p value (t test) ≥ 10] enrichment compared with calculated average NSAF values from a large dataset of pull-downs with nonrelated bait proteins, were selected.

**Protein Sequence Analyses.** Searches for homologs of AtPANS1 (PANS1, ARATH) were performed using PSI-BLAST (50) against the nr database using e values thresholds from 1e-3 to 1e-5. Multiple sequence alignments were calculated using MAFFT (51) and represented using Jalview (52). PSI-BLAST searches starting from AtPANS1 established the homologous relationship with OsRSS1 (XP_015627424.1) after four iterations, but further iterations were not successful in identifying plant homologs in clades other than monocots and dicots. Repeating the search from OsRSS1 on plant sequences excluding dicots helped identify homologs in mosses. These sequences were used in turn as inputs to search for the most likely homologs of AtPANS1 in green algae and one sequence in O. lucimarinus was detected (XP_001422400.1) as a potential candidate describing highly homologous sequence in O. lucimarinus protein against the nr sequence database restricted to the Viridiplantae clade recovered OsRSS1 as a significant match after seven iterations and the Arabidopsis PATRONUS1 and 2 after 13 iterations.

PSI-BLAST analyses using (XP_001422400.1) also detected as significant homologs several sequences from bivalves such as *M. yesoensis* (XP_021355525.1) after 10 iterations without apparent divergence of the sequence profile. PSI-BLAST from *M. yesoensis* (XP_021355525.1) against metazoan sequences integrated in a list of nonspecific proteins were selected by means of semiquantitative analysis using the average normalized spectral abundance factors (NSAF) of the identified proteins in the PANS1 pull-downs. Proteins identified with at least two peptides in at least two experiments, showing high (at least 10-fold) and significant [−log10(p value (t test) ≥ 10] enrichment compared with calculated average NSAF values from a large dataset of pull-downs with nonrelated bait proteins, were selected.

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