Low phosphate activates STOP1-ALMT1 to rapidly inhibit root cell elongation

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Environmental cues profoundly modulate cell proliferation and cell elongation to inform and direct plant growth and development. External phosphate (Pi) limitation inhibits primary root growth in many plant species. However, the underlying Pi sensory mechanisms are unknown. Here we genetically uncouple two Pi sensing pathways in the root apex of Arabidopsis thaliana. First, the rapid inhibition of cell elongation in the transition zone is controlled by transcription factor STOP1, by its direct target, ALMT1, encoding a malate channel, and by ferroxidase LPR1, which together mediate Fe and peroxidase-dependent cell wall stiffening. Second, during the subsequent slow inhibition of cell proliferation in the apical meristem, which is mediated by LPR1-dependent, but largely STOP1–ALMT1-independent, Fe and callose accumulate in the stem cell niche, leading to meristem reduction. Our work uncovers STOP1–ALMT1 as a signalling pathway of low Pi availability and exuded malate as an unexpected apoplastic inhibitor of root cell wall expansion.
Phosphate limitation (−Pi), prevailing in around 70% of global cultivated land, is a common abiotic stress that profoundly alters root growth with critical consequences for crop yields. However, the underlying mechanisms of root growth inhibition by low Pi availability remain largely unknown.

To address this problem at the cellular and molecular level, we chose the primary root of Arabidopsis thaliana seedlings as the experimental system because it displays a transparent and simple anatomical structure. Roots longitudinally and indeterminately grow at their apex. Decades of laboratory work have shown that cells proliferate in the root apical meristem (RAM) in concentric layers (epidermis, cortex and endodermis surrounding the stele). These cell types originate in the stem cell niche (SCN), enclosing the quiescent centre (QC), divide as transit-amplifying cells before rapid and extensive cell expansion ensues in the elongation zone (EZ) and divide as transit-amplifying cells before rapid and extensive cell expansion ensues in the elongation zone (EZ) and the root apex, comprising the RAM and early EZ (Supplementary Fig. 1a). A complex set of regulatory networks and developmental genes coordinate cell proliferation and cell elongation required for root extension.

Previous studies with Arabidopsis seedlings showed that acute −Pi stress is locally sensed at the primary root apex in a Fe-dependent manner and inhibits root growth by affecting both cell elongation and cell proliferation. The local response is independent of internal root Pi status and is therefore not caused by a metabolic deficiency syndrome.

To date, several mutants specifically altered in the local root response have been isolated, but only a few corresponding mutated genes have been identified. Much has been learned from LOW PHOSPHATE ROOT 1 (LPR1) and PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2), which genetically interact and are expressed in cell type-specific but overlapping domains of the root apex, comprising the RAM and early EZ. While LPR1 encodes a cell wall (CW) targeted multicopper oxidase with ferroxidase activity, PDR2 codes for ATP5A, the single P5-type ATPase in Arabidopsis, which is thought to repress LPR1 biogenesis or activity. Under −Pi, the genetic PDR2–LPR1 module mediates accumulation of Fe in the SCN and EZ within 20 h, which triggers generation of reactive oxygen species (ROS) as well as deposition of callose and pectin in CWs of the RAM and EZ. Callose deposition in the SCN obstructs cell-to-cell communication as demonstrated by impaired movement of SHORT-ROOT, a key transcription factor of root patterning and cell fate specification, which is followed by reduced stem cell maintenance and RAM activity.

Here we identify and characterize two key players of a new signalling pathway, which is activated under −Pi to rapidly inhibit root growth by targeting cell expansion in the EZ: the transcription factor STOP1 (SENSITIVE TO PROTON TOXICITY1) and the malate efflux channel ALUMINUM-ACTIVATED MALATE TRANSPORTER1 encoded by ALMT1, a direct STOP1 target gene. Surprisingly, mutations in either gene spatially and temporally uncouple the rapid inhibition of cell elongation in the EZ (<2 h) from the slower inhibition of cell proliferation in the SCN and RAM (<4 days), which are both dependent on LPR1 ferroxidase activity. Based on our data, we propose that malate efflux by internal cell files of the root tip facilitates Fe chelation in the apoplast. LPR1-dependent Fe redox cycling stimulates peroxidase-catalysed CW stiffening followed by inhibition of root cell elongation.

**Results**

**STOP1 and ALMT1 repress primary root growth under −Pi.**

To discover new genes important for primary root growth inhibition under −Pi, we screened ∼4 x 10^6 ethylmethane sulfonate-mutagenized WT seedlings (see Methods). We isolated 85 independent mutants with a significantly longer primary root when grown under −Pi. Genetic mapping and molecular analysis identified 26 lpr1 alleles, suggesting that our screen approached saturation for isolating complete or partial loss-of-function mutations conferring a lpr1-like root phenotype. We also identified 24 mutant alleles of the transcription factor STOP1 and 14 alleles of the malate efflux channel ALMT1 (see Methods, Fig. 1a–c and Supplementary Fig. 1b), we describe here a representative subset of both complementation groups. All almt1 and stop1 mutations are recessive, except for Stop1 and Stop1, which are dominant negative mutations displaying the same phenotype as seedlings homozygous for stop1 null alleles (Supplementary Fig. 1c,d). The presumably truncated mutant STOP1 proteins dominantly inhibit ALMT1 expression, possibly by titrating a limiting factor required for ALMT1 activation.

**ALMT1 promotes primary root growth arrest under −Pi.**

Previous studies showed that STOP1 promotes root exudation of malate and citrate by stimulating expression of ALMT1 and MATE efflux transporters, respectively. Root exudation of organic acids such as malate and citrate is thought to improve plant growth by solubilizing Pi anions complexed with metal cations like iron (Fe) and aluminium (Al) in soil. We were therefore intrigued by the observation that stop1 and almt1 mutants develop a longer primary root than the WT in −Pi conditions. We thus analysed in more detail the role of STOP1 and ALMT1 in Pi-dependent root growth inhibition.

First, chemical complementation by exogenous malate application restored in a dose-dependent manner primary root growth inhibition on −Pi medium in almt1 mutants (Fig. 1d); whereas no reduced root growth was observed in +Pi conditions (Supplementary Fig. 2a). This result is consistent with the hypothesis that the ALMT1-dependent malate exudation inhibits WT root growth under −Pi.

We then tested whether expression of ALMT1 and STOP1 is regulated by Pi availability. One hour after transfer from the +Pi to −Pi medium the expression level of ALMT1 increases and reaches a plateau at 24 h (Fig. 1c). However, on both media ALMT1 expression is abrogated in strong stop1 mutants (Supplementary Fig. 2b). MATE is also upregulated on −Pi medium (Fig. 1e) in a STOP1-dependent manner (Supplementary Fig. 2b). However, under −Pi the mateKO mutant does not grow a long primary root, and the main root of the almt1KO;mateKO double mutant is as long as the root of the almt161 single mutant (Supplementary Fig. 2c). The pattern of MATE expression in the primary root is different from that of ALMT1, in particular it is not expressed in the root tip, and this may explain why the mateKO single mutant is not altered in the root growth under −Pi. We thus tested whether expressing MATE under the control of the ALMT1 promoter could restore root growth inhibition by −Pi of the almt1KO;mateKO double mutant. First, we confirmed that this in line the palmt1::MATE construct is functional and complements the A^3+ sensitivity of the almt1KO;mateKO double mutant (Supplementary Fig. 2d). However, expression of palmt1::MATE in almt1KO;mateKO plants did not restore primary root growth inhibition on −Pi medium (Supplementary Fig. 2d). Together, these results indicate that citrate exudation by MATE cannot replace malate exudation by ALMT1 to inhibit primary root growth under −Pi.

A prominent role of ALMT1 as an effector downstream of STOP1 for root growth inhibition under −Pi is supported by the following observations: (i) the root length of stop1 and almt1 mutants are similar (Fig. 1c), and (ii) the constitutive expression of ALMT1 fully rescues both almt1 KO (Supplementary Fig. 2e) and the dominant negative Stop1mutant phenotypes (Supplementary Fig. 2f).
We quantitatively showed in yeast one-hybrid assays that STOP1 recognizes a small region of the ALMT1 promoter including the STOP1-binding site found previously by others (Supplementary Fig. 2g). Interestingly, the low Pi-stimulated expression of ALMT1 is independent of PHR1 and PHL1 (Supplementary Fig. 3a), two master transcription factors controlling many Pi-starvation responses (29).

**Pi enhances expression of ALMT1 in the root apex.** To determine the spatial expression pattern of ALMT1 and STOP1, we established several independent transgenic lines carrying the GUS reporter gene placed under the control of their respective promoters (pALMT1::GUS and pSTOP1::GUS). The pALMT1::GUS lines (WT background) showed ALMT1 expression in the whole root (Supplementary Fig. 3b). This histological marker
likely reports the genuine expression pattern of endogenous
ALMT1, confirming our quantitative PCR with reverse
transcription (qRT – PCR) analysis (Fig. 1e). As shown in Fig. 1f
and Supplementary Fig. 3b, pALMT1::GUS was more highly
expressed in –Pi than in +Pi conditions, but was not detectable
in the Stop1KO mutant background (Supplementary Fig. 3c).

We previously showed that the root growth arrest does not
occur under –Pi at pH > 6.3, or when iron is omitted in the
medium. We therefore assessed whether this observation is due
to the lack of ALMT1 expression. Our results showed that
ALMT1 expression was not detectable in roots on +Pi medium
at pH 7.1 ( Supplementary Fig. 4a), whereas Fe omission did not
impair stimulation of ALMT1 expression by –Pi ( Supplementary
Fig. 4a). On the other hand, WRKY46, a transcriptional factor
whose expression is enhanced in roots by Fe deficiency and
which represses ALMT1 expression, does not seem to modulate
the root growth inhibition under –Pi because the wrky46KO
mutant behaved like the WT control (Supplementary Fig. 5). These results support the view that Fe does not play a
role in the induction of ALMT1 by –Pi.

As shown in longitudinal and transverse sections of the root
meristem (Fig. 1f), ALMT1 is expressed during –Pi limitation in
the SCN, including the QC, and in all cell files, although at a
lower level in the external root cap layer and columnella cells.
We therefore demonstrated here that, in addition to other cues,
–Pi stimulates ALMT1 expression in the root tip.

The psSTOP1::GUS marker revealed that STOP1 is expressed
in the entire root tip (Supplementary Fig. 6a); however, in contrast
to ALMT1, STOP1 expression is not enhanced by –Pi exposure
(Fig. 1e and Supplementary Figs 2b, 3a and 6a). This suggests
that STOP1 is post-transcriptionally regulated to stimulate
ALMT1 expression under –Pi. We thus tested whether nuclear
localization of STOP1 is regulated. We fused the Green
Fluorescent Protein (GFP) to the STOP1 N terminus and the
GFP-STOPI coding region was placed under control of the
pSTOP1 or pUBQ10 promoter. The translational fusions are
functional because they complement the root growth response of the
stop1KO mutant (Supplementary Fig. 6b). Confocal micro-
scopy of root tips revealed nuclear localization of GFP-STOPI
under +Pi (Fig. 1g and Supplementary Fig. 6c), which
interestingly intensified on seedling transfer to –Pi medium
(Fig. 1g and Supplementary Fig. 6c). These results indicate that
STOP1 is post-transcriptionally regulated and suggest that the
stimulation of ALMT1 expression under –Pi is, at least partly,
due to increased accumulation of STOP1 in the nucleus. Overall,
our results show that the STOP1–ALMT1 module is a component of
a new, PHRI-independent regulatory pathway activated by
–Pi stress.

Root growth arrest under –Pi is rapid. To characterize with
higher temporal resolution, the early phase of primary root
growth inhibition under –Pi, we performed a time course
experiment. WT seedlings grown under +Pi were transferred to
either +Pi (control) or –Pi agar before primary root length was
measured at 20 min intervals for 7 h (Fig. 2a). When compared to
the control, we detected root growth inhibition 2 h after transfer
to –Pi medium, a fast morphological response (Fig. 2b). In
contrast, the main root of stop1, almt1 and lpr1;lpr2 mutants did not
respond during the entire 7.5 h time course (Fig. 2c). The short
primary root length of WT under –Pi correlated with a
strong reduction of root epidermal cell length (Fig. 2d). In the
mutants, epidermal cell length was not or only slightly reduced
under –Pi (Fig. 2d). Our data show that STOP1, ALMT1 and the
LPRs mediate a rapid inhibition of root cell elongation that
contributes to root growth inhibition on –Pi medium.

stop1 and almt1 uncouple the –Pi response of the RAM and EZ.
Previous work showed that the root growth arrest under –Pi is a
Fe-dependent response. Inductively coupled plasma mass
spectrometry analysis did not show significant differences in total
Fe accumulation in roots of mutants compared to WT (Supplementary Fig. 7a). However, using histological Perls/DAB
staining, it was reported that labile (non-haem) Fe accumulates in Pi-deprived WT but not lpr1;lpr2 root tips. We thus tested whether the stop1 and almt1 mutants alter the pattern of Fe accumulation. By using
Perls/DAB staining, we confirmed that WT but not lpr1;lpr2 root tips accumulated Fe in the EZ and SCN on –Pi medium
(Fig. 3a and Supplementary Fig. 7b). Interestingly, stop1 and almt1 mutants did not show Fe accumulation in the EZ (Fig. 3a
and Supplementary Fig. 7b), but, to our surprise, both mutant
lines still accumulated Fe in the SCN similar to the WT (Fig. 3a
and Supplementary Fig. 7b).

Callus accumulation in the EZ and SCN is another root
response of the WT, but not of the lpr1;lpr2 double mutant,
when grown under –Pi (Fig. 12; Fig. 3b). Mirroring the
Fe accumulation (Fig. 3a), the stop1 and almt1 mutants still
depicted callosic in the SCN but not in the EZ (Fig. 3b). Note
that exogenous malate application complemented iron and callose
deposition in the EZ of the almt1 root tip (Supplementary Fig. 8).
These unexpected observations indicate that loss of STOP1 or
ALMT1 uncouples the two major regions of Fe and callose
accumulations in the root apex.

Because inhibition of cell elongation under –Pi is a rapid
process (Fig. 2b) and occurs well before the onset of root
eristem size reduction, we hypothesized that primary root

Figure 1 | Phenotype of almt1 and stop1 mutants and expression of ALMT1. (a) Position and nature of the almt1 and stop1 mutations. (b) Appearance of representative WT and mutant seedlings after germination and growth for 6 days on +Pi or –Pi medium. (c) Primary root length of WT (Col-0) and
mutant seedlings grown for 6 days in +Pi or –Pi conditions. Mean ± s.d., n = 9–14 seedlings per line and condition; unpaired two-tailed t-test;
**P < 0.0001. (d) Malate complementation. WT and almt1 seedlings grown under +Pi for 2 days were transferred for 6 days to –Pi medium with or
without the indicated concentrations of malate (mean ± s.e.m., n = 7–14 seedlings per condition; two-way ANOVA, ***P < 0.0001; **P < 0.01;
*P < 0.05; NS, not significant). (e) Gene expression analysis. WT seedlings grown under +Pi for 5 days were transferred to +Pi or –Pi medium
for 1–48 h. Total root RNA was extracted and expression of the indicated genes analysed by qRT-PCR. SPX1 was used as a positive marker for the
–Pi stress. Relative expression levels are normalized to that of the +Pi at 1 h (mean ± s.e.m., n = 6 independent experiments; unpaired two-tailed t-test; **P < 0.01;
*P < 0.05; NS, not significant). (f) Pattern of ALMT1 expression in primary root tip. Transverse (top) and longitudinal (bottom) sections of the root tip of
pALMT1::GUS (WT). Seedlings were grown 5 days under +Pi and transferred to +Pi or –Pi medium for 48 h prior to GUS staining. (g) GFP-fluorescence
of GFP-STOPI in root tips. Four-day-old stop1KO,pSTOP1::GFP-STOPI (# B10A) seedlings grown under +Pi were transferred to +Pi or –Pi plates for 24 h,
and GFP-fluorescence was visualized by confocal microscopy. Left panel: the two roots were mounted side by side on the same microscope slide
(× 10 objective). Middle panel: magnification of the root tips (× 20 objective). See also Supplementary Fig. 6c for additional lines. Right panel: intensity of
the GFP fluorescence (a.u., arbitrary units) in nuclei of the root tip (mean ± s.d., n = 54 and 43 nuclei in +Pi and –Pi, respectively; unpaired two-tailed t-test; ****P < 0.0001). Two (c,d) or three (g) independent experiments were performed with consistent results and one representative experiment is
shown. Scale bars, 1 cm (b), 100 μm (fg).
Tukey whiskers; Mann–Whitney test: ****

the indicated genotype were transferred to shown (mean
WT and mutant seedlings transferred to
mechanical properties of their surrounding CW 34. We reasoned
ism that could alter cell expansion is the modification of the
/C0
Pi stress rapidly stiffens CW in the transition zone. We next
Pi stress to
– Pi. Indeed, when grown for
more than 4 days on
– Pi medium, the root length of
stop1 and
almt1 mutants was shorter when compared to
lpr1; lpr2
roots (Fig. 3c), which correlated with a shorter RAM, a proxy
of rapid inhibition of root growth of
almt1
under
– Pi correlates with reduced accumulation of Fe and callose in the
EZ, whereas the late root growth inhibition correlates with
accumulation of Fe and callose in the SCN and reduced RAM
size. Thus, inactivation of the STOP1–ALMT1 module uncouples
the effect of
– Pi on root cell elongation and proliferation, whereas loss of LPR function impacts both processes (Fig. 3e).

– Pi stress rapidly stiffens CW in the transition zone. We next
asked how
– Pi inhibits cell elongation. In plants, one mechan-
ism that could alter cell expansion is the modification of the
mechanical properties of their surrounding CW 34. We reasoned
that CW stiffness increases early after the onset of
– Pi stress to
prevent expansion of the encased root cell symplast. To test
this hypothesis, we used atomic force microscopy (AFM) with
microindentation probe, which allows measuring stiffness of
small plant cell surfaces in living organs 35,36. We measured
the stiffness of the root surface of the transition zone
(located between the RAM and EZ, Supplementary Fig. 1a),
where cells are predestined to rapidly elongate when
conditions are permissive.

AFM measurements were performed on WT seedlings
on transfer from
+ Pi to
+ Pi or
– Pi for up to 2 h (see Methods).
Interestingly, CW stiffness increased as early as 30 min after
transfer to
– Pi and continued to increase at later time points
(Fig. 4a); by contrast, we observed a reduced stiffness under
+ Pi (Fig. 4a). A higher CW stiffness (see also WT in Fig. 4b)
was not detectable for the
stop1, almt1
and
lpr1;lpr2
lines (Fig. 4b) in
which root cell expansion is not restrained under
– Pi (Fig. 2d), nor in WT grown in
– Pi-Fe condition during
which root growth is not inhibited8,12 (Fig. 4c). Thus, our results
reveal a negative reciprocal relation between CW stiffness in
the root transition zone and the final epidermal cell length.
This suggests a simple causal scenario where
– Pi triggers
CW stiffening of pre-elongated cells to restrict their elongation.

Peroxidase activity increases CW stiffening under
– Pi. Class
III peroxidases are thought to inhibit cell expansion by
catalysing crosslinks between some polysaccharides or proteins,
thereby tightening the CW 34,37. The Arabidopsis genome contains
73 genes of class III peroxidases 38, many of which are expressed

Figure 2 | Rapid primary root growth arrest under
– Pi. (a) Scheme depicting the
Δ primary root length measured in seedlings transferred from a
+ Pi plate to a
+ Pi or
– Pi medium. (b) Time course of the
Δ primary root length of WT seedlings transferred to
+ Pi or
– Pi medium. Inset shows
magnification of the first time points. Three independent experiments were performed with consistent results and one representative experiment is shown (mean ± s.e.m., n = 10 seedlings per condition; two-way ANOVA, multiple comparisons; ****P < 0.0001). (c) Time course of the
Δ primary root length of WT and mutant seedlings transferred to
– Pi. Two independent experiments were performed with consistent results and one representative experiment is shown (mean ± s.e.m., n = 8–10 seedlings per line and condition; two-way ANOVA, multiple comparisons; ****P < 0.0001). (d) Seven-day-old seedlings of the indicated genotype were transferred to
+ Pi or
– Pi medium for 24 h before measuring the final length of root epidermal cells (median ± interquartile; Tukey whiskers; Mann–Whitney test: ****P < 0.0001; n, number of cells).
in root tissues\textsuperscript{39,40}. To test our hypothesis that the $-\Pi$ cue leads to CW stiffening in root tips, we used a pharmacological approach to inhibit peroxidase activity and to overcome a presumed functional redundancy among these enzymes. Salicylhydroxamic acid (SHAM) and methimazole are two peroxidase inhibitors \textsuperscript{41,42} and DAB is a classical peroxidase substrate\textsuperscript{43}, which we used as a competitive inhibitor. These three chemicals restored the WT root growth under $-\Pi$ in a dose-dependent manner (Fig. 5a–d). Consistent with our hypothesis, SHAM strongly decreased CW stiffness in $-\Pi$-challenged WT (Fig. 5e), and SHAM as well as DAB treatment significantly increased root epidermal cell length (Fig. 5f).

These results prompted us to histologically visualize peroxidase activity in the root apex of the WT and mutant lines using...
We detected peroxidase activity in the WT root apex, from the root cap to the early EZ (Fig. 5g). Supporting our hypothesis, peroxidase staining was more intense in Pi-deprived than Pi-replete WT root tips (Fig. 5g). SHAM-treated seedlings displayed reduced staining, thus confirming SHAM-dependent inhibition of peroxidase activity in planta (Fig. 5g and Supplementary Fig. 9a). Remarkably, in the lpr1;lpr2 double mutant, peroxidase activity was strongly reduced in both +Pi and −Pi conditions, whereas the stop1 and almt1 mutants displayed intermediate activities (Fig. 5h). Note, when compared to the WT in both +Pi and −Pi conditions, the stained zone of the stop1 root tips was shorter and particularly weaker in the EZ (Fig. 5h).

To further challenge the idea that high peroxidase activity correlates with reduced root growth under −Pi, we performed peroxidase staining on WT seedlings grown at pH 7.1 or in a Fe-deficient medium, which are two permissive conditions for root growth under −Pi (ref. 8). As shown (Supplementary Fig. 9b,c) in either condition, peroxidase activity of WT root tips was highly reduced and indistinguishable to that of the mutants. Note that at pH 7.1 no ALMT1 expression was detected (Supplementary Fig. 4a). These results convincingly show that under −Pi, LPR, and to lesser extent STOP1 and ALMT1, stimulate peroxidase activity in the root apex, which in turn inhibits root growth. Combined with our AFM and

Figure 4 | − Pi induces cell wall stiffening in the root transition zone. (a) WT (Coler105) seedlings were transferred to − Pi or + Pi medium for the indicated time prior to measuring the stiffness of the cell surface by AFM in the transition zone of the primary root (see Methods). (b) Seedlings of the indicated genotypes were transferred to + Pi or − Pi medium for 30 min prior to measuring the stiffness of the cell surface by AFM in the transition zone of the primary root. (c) WT (Coler105) seedlings were transferred to − Pi or − Pi-Fe medium for 30 min prior to measuring the stiffness of the cell surface by AFM in the transition zone of the primary root (median ± interquartile, Mann-Whitney test. ****P<0.0001; **P<0.01; *P<0.05; NS, not significant (P>0.05)). The experiment was performed twice with consistent results and one representative experiment is shown.

4-chloro-1-naphtol staining. We detected peroxidase activity in the WT root apex, from the root cap to the early EZ (Fig. 5g). Supporting our hypothesis, peroxidase staining was more intense in Pi-deprived than Pi-replete WT root tips (Fig. 5g). SHAM-treated seedlings displayed reduced staining, thus confirming SHAM-dependent inhibition of peroxidase activity in planta (Fig. 5g and Supplementary Fig. 9a). Remarkably, in the lpr1;lpr2 double mutant, peroxidase activity was strongly reduced in both +Pi and −Pi conditions, whereas the stop1 and almt1 mutants displayed intermediate activities (Fig. 5h). Note, when compared to the WT in both +Pi and −Pi conditions, the stained zone of the stop1 root tips was shorter and particularly weaker in the EZ (Fig. 5h).
pharmacological results, these observations support the view that peroxidase activity catalyses crosslinks in some CW components, thereby reducing cell expansion under $-\text{Pi}$.

It is worth noting that the CW stiffness of SHAM-treated WT (Fig. 5e), as well as of WT grown under $-\text{Pi}$-$\text{Fe}$ (Fig. 4c) and in lpr1;lpr2 double mutant grown under $-\text{Pi}$ (Fig. 4b), is lower than in the corresponding controls grown under $+\text{Pi}$. This softening is correlated with reduced peroxidase activity observed in these roots (Fig. 5g, Supplementary Fig. 9b and Fig. 5h). These observations may indicate that in conditions permissive for root growth, the LPR ferroxidases and some peroxidases participate to maintain a basal strength of the CW.

Under $-\text{Pi}$, peroxidase activity also represses cell proliferation and accelerates cell differentiation in the RAM (Fig. 5d, pictures of the root tips). This is reminiscent to the role of UPB1, which regulates the transition from cell proliferation to differentiation in the root tip via the expression of some class III peroxidases. However, primary root growth of the upb1 mutant and an UPB1-overexpressor line is not altered in response to $-\text{Pi}$ exposure (Supplementary Fig. 9d), indicating that UPB1 is not essential in this process.
— Pi promotes ROS accumulation in the EZ. Correlated with the deposition of Fe and callose, the generation of ROS in CWs of the RAM and EZ was observed in WT, but not in the lpr1/lpr2 double mutant, when transferred to — Pi medium for 1 day. We also observed accumulation of ROS (H₂O₂ and peroxidases-mediated oxidation of CM-H₂DCFDA) in the transition and differentiation zones of WT seedlings transferred to — Pi for 24 h (Supplementary Fig. 10). Under — Pi, accumulation of ROS is reduced when iron is omitted or when SHAM is added to the growth medium (Supplementary Fig. 10). This result confirms that — Pi induces accumulation of ROS in the root tip and indicates that peroxidase activity could play a role.

Malate efflux into the RAM inhibits root growth under — Pi.

Specific cell types of the RAM control root growth in Arabidopsis (see for example ref. 45). To test whether STOP1 activity in specific cell files inhibits root growth under — Pi, we used cell-type-specific promoters to control and restore STOP1 expression (and thus ALMT1 expression) in a stop KO mutant (Fig. 6a). As shown in Fig. 6b, we fully rescued root growth inhibition by transformation with a pSCR::STOP1 construct, which drives STOP1 expression specifically in the QC and endodermal cell lineage. The pCo2 promoter restored STOP1 expression essentially in young cortex cells of the RAM and complemented stop KO almost fully. The deposition of Fe in the EZ was also complemented in the pSCR::Co2 lines (Fig. 6c).

In contrast, we observed only partial complementation using the pPEP promoter, which is active in cortex cells of the elongation and maturation zones and slightly in the endodermis, and no complementation with the pCASP1 promoter, which is active in mature endodermal cells (Fig. 6b). Using a pALMT1::GUS reporter, the expected pattern where STOP1 is functional in the root and root tip of all these lines was confirmed (Supplementary Fig. 11a,b). Taken together, our results show that STOP1 expression confined to the endodermis (and QC) or to the early cortex is sufficient for rescuing the stop KO root phenotype.

To test whether these two cell types are necessary for STOP1-dependent growth arrest, we generated WT lines expressing the dominant negative STOP1 gene under the pSCR or pCo2 promoter. Our results show that the two constructs, expressed either alone (Supplementary Fig. 11c) or in combination (Fig. 6d), do not release root growth inhibition under — Pi.

Taken together, these experiments show that expression of STOP1 in endodermis or cortex of root tips is sufficient, but not necessary, for STOP1-dependent root growth inhibition by — Pi. This indicates that ALMT1-exported malate locally diffuses in the apoplastic of root tip cells.

Discussion

Identification of the cellular and molecular mechanisms by which environmental cues affect organ growth is a current challenge for plant biology. Pi starvation is one of the most common limiting factors for plant growth and crop productivity. Previous studies identified Arabidopsis LPRs and PDR2 as part of a pathway controlling primary root growth in response to Pi availability surrounding the root tip, the so-called ‘local response’.

Our work uncovers a novel pathway in local Pi sensing. We identified in a forward genetic screen two functionally interacting components with roles in primary root growth arrest under — Pi: the transcription factor STOP1 and its direct target gene ALMT1. Absence of a reciprocal regulation of pALMT1 and pLPR1 promoter activity (Supplementary Fig. 12a,b) suggests that the STOP1–ALMT1 and LPR1 function in distinct pathways. However, the similar unrestricted root cell elongation in stop1, almt1 and lpr1 mutants (Fig. 2c,d) indicates that the two pathways converge on a common inhibition mechanism. Therefore, the PDR2–LPR1 and STOP1–ALMT1 modules define the first components of two separate pathways targeting the root transition zone to inhibit cell elongation under — Pi (Fig. 7).

To our knowledge, STOP1 is the first plant transcription factor showing a regulation reminiscent of yeast PHO4 (ref. 49), which accumulates more intensely in the nucleus upon Pi starvation. Thus, STOP1 and ALMT1 define a new — Pi regulatory pathway, which is supported by PHR1/PHL1-independent activation of ALMT1 (Supplementary Fig. 3a). In addition to low pH and Al³⁺ (refs 21,22,24,32), low Pi is thus a new environmental stimulus activating the STOP1–ALMT1 pathway in Arabidopsis and most probably in crops. Indeed, the primary root of the Brassica napus, a crop plant known to exude malate under — Pi (ref. 51), is also inhibited under — Pi (Supplementary Fig. 13). This pathway is thus likely of general relevance.

Our work further uncovers that root growth inhibition under — Pi results from rapid inhibition (<2 h) of cell elongation and subsequent inhibition (<4 days) of cell proliferation, previously referred to as meristem exhaustion. Interestingly, we demonstrate that both processes can be genetically uncoupled in stop1 and almt1 mutants. Both inhibitory processes correlate with a higher Fe accumulation, callose deposition, and peroxidase activity in the EZ and SCN, as well as with CW stiffening at least in the EZ. Recently, a comparative

Figure 5 | Cell wall stiffening and inhibition of root cell elongation under — Pi are peroxidase-dependent. (a–c) Dose-response relationship of the Δ primary root length plotted as a function of concentration of peroxidase inhibitors (mean ± s.d.). For each data point, 3-day-old WT (Col0) seedlings were transferred for 7 days to — Pi plates containing different concentrations of inhibitor. The structure of the inhibitors is shown above the corresponding curves. (a) Salicylhydroxamic acid (SHAM). Blue data points: — Pi; yellow data point: + Pi without inhibitor (n = 12–14 seedlings per condition). (b) 3,3'-diaminobenzidine (DAB) (n = 8–12 seedlings per condition). (c) Methimazole (n = 11–13 seedlings per condition). (d) Picture of the effect of peroxidases inhibitors on WT seedlings. Three-day-old WT seedlings were transferred 7 days to + Pi or — Pi plates containing the indicated inhibitors. Top: picture of one representative seedling for each treatment (Meth., methimazole). Arrowheads indicate the primary root tips. Scale bar, 1 cm. Bottom: picture of the root tips corresponding to the seedlings shown above. Note that inhibitors of peroxidases suppress the — Pi-induced early differentiation of root hairs. (e) Three-day-old WT seedlings were transferred for 30 min to + Pi or — Pi medium with or without 15 μM SHAM prior to measuring the stiffness of the cell surface by AFM in the transition zone of the primary root (median ± interquartile, Tukey whiskers, Mann-Whitney test: ***P < 0.0001). (f) Effect of SHAM and DAB on root epidermal cell length. Three-day-old WT seedlings were transferred for 7 days to + Pi or — Pi medium with or without 15 μM SHAM or 100 μM DAB prior to measuring the root epidermal cell length (median ± interquartile; Tukey whiskers, Mann-Whitney test: ***P < 0.0001, n = number of cells). (g) Peroxidase activity revealed by 4-chloro-1-naphthol staining of the WT primary root tip (the colourless dye turns black with peroxidases activity). Four-day-old WT seedlings were transferred for 48 h to + Pi or in — Pi plates with or without 15 μM SHAM (see also Supplementary Fig. 9a). (h) Peroxidase activity revealed by 4-chloro-1-naphthol staining of the primary root tip of the indicated genotypes. Four-day-old seedlings were transferred for 48 h to + Pi or in — Pi plates prior to staining. Single and double-headed arrows indicate the position of the elongation zone. The experiments were performed three times with consistent results and one representative experiment is shown (a–c,g,h). Scale bars, 100 μm (g,h).
transcriptomics/proteomics study revealed that Pi deprivation profoundly alters expression of CW-related genes (>20 h) and leads to accumulation of callose and non-esterified pectins in WT root tips at sites of Fe deposition (EZ, SCN), which are not detected in \textit{lpr1;lpr2} roots. Crosslinking of non-esterified pectins by Ca$^{2+}$ reduces CW extensibility and regulates cell expansion.

It is quite unexpected that malate efflux inhibits elongation of root cells under –Pi, because exudation of small organic acids is thought to promote plant growth by solubilizing scarcely available Pi and Fe nutrients, or by forming non-toxic complexes with Al$^{3+}$ cations. However, malate and Fe form chelates participating in plant Fe homeostasis. Interestingly, when complexed with malate, Fe$^{3+}$ reduction is faster when compared to Fe$^{3+}$–citrate or Fe$^{3+}$–EDTA complexes. Therefore, a low Pi/Fe ratio, as under –Pi, may allow formation of Fe$^{3+}$–malate complexes in CWs of the RAM and EZ, promoting Fe accumulation, LPR1 ferroxidase-dependent Fe redox cycling, ROS generation and callose accumulation (Fig. 7).

The role of Fe might not be limited to ROS production; one might speculate that Fe$^{3+}$ also activates ALMT1 to control malate efflux, as Al$^{3+}$ regulates ALMT1 channels from different.
Figure 7 | Working model depicting the effect of \(-\) Pi on cell proliferation and elongation in the primary root tip. A low Pi/Fe ratio, that is, \(-\) Pi stress\(^{12}\), causes Fe deprivation (yellow star), post-translationally activates STOP1 which induces ALMT1 expression followed by malate exudation into the apoplast. The interaction of malate-altered iron chelation and LPR1 ferroxidase activity inhibits cell elongation by peroxidase-dependent cell wall stiffening. The Pi-type ATPase PDR2 restricts LPR1 function\(^{12}\). The LPR1-PDR2 module interacts with an unknown pathway parallel to STOP1-ALMT1 (question mark) to inhibit cell division in the SCN by a similar mechanism involving accumulation of Fe and callose, peroxidase-activity, and cell wall thickening. Mutations in STOP1 and ALMT1 restore cell elongation under \(-\) Pi but cell divisions are still inhibited.

Methods

Plant growth. Unless specified, plants and seedlings were grown as previously described\(^{16}\). Briefly, surface-sterilized seeds were sown on a horizontal line in plates that were vertically disposed in a growing chamber (16 h photoperiod; 24.5 °C/21 °C day/night, respectively). The growth medium contained 0.15 mM MgSO\(_4\), 2.1 mM NH\(_4\)NO\(_3\), 1.9 mM KNO\(_3\), 0.34 mM CaCl\(_2\), 0.5 mM KI, 10 mM FeCl\(_3\), 10 mM H\(_2\)BO\(_3\), 10 mM MnSO\(_4\), 3.5 mM ZnSO\(_4\), 0.1 mM CuSO\(_4\), 0.1 mM CoCl\(_2\), 0.1 mM Na\(_2\)MoO\(_4\), 0.5 g l\(^{-1}\) sucrose. The agar (0.8 g l\(^{-1}\)) for plates was from Sigma-Aldrich (A1296 #BCBL6182V). The \(+\) Pi and \(-\) Pi agar medium contained 10 and 500 mM Pi, respectively; the media were buffered at pH 5.6–5.8 with 3.4 mM 2-N-morpholino)ethane sulfonic acid.

Growth media at pH 7.1 were made using 2 mM 1,4-piperazinediethanesulfonic acid pH 7.1. Growth media at pH 4.4 were prepared by mixing equal amounts of autoclaved 2 × liquid medium (composition as above, without Pi and Fe, buffered at pH 4.4) with 2 × agar (dissolved in H\(_2\)O); Pi and Fe were subsequently added according to media. For the pH experiment (Supplementary Fig. 4a), Fe was at 15 μM.

Brassica napus seeds (cv Bohême) were sterilized and seedlings grown on the same media, as for Arabidopsis, except that the growth media contained 100 μM FeCl\(_3\). Briefly, seeds were sown on a \(+\) Pi plate, left 24 h at 4 °C and let germinate for 12 h. Germinating seeds (with the primary root just protruding) were then transferred in \(+\) Pi or \(-\) Pi plates for 3 days before measuring the primary root length.

For testing resistance to Al\(^{3+}\), seeds were sown in 2 ml liquid medium (NH\(_4\)NO\(_3\), 4.1 mM, KNO\(_3\), 3.8 mM, CaCl\(_2\), 200 mM, MgSO\(_4\), 0.3 mM, H\(_2\)BO\(_3\), 20 μM, MnSO\(_4\), 20 μM, MnSO\(_4\), 20 μM, KI 1 μM, ZnSO\(_4\), 6 μM, Na\(_2\)MoO\(_4\), 0.2 μM, CoCl\(_2\), 0.2 μM, CuSO\(_4\), 0.2 μM, sucrose 0.5 g l\(^{-1}\), Homopiperazine-1,4-bis(-ethanesulfonic acid) 2 mM, pH 4) and grown for 4 days before measuring the primary root length.

Mutagenesis and mutants screening. For the mutagenesis, we used Col\(^{105}\), a Columbia background (Col-0) with the null allele erecta-105 (ref. 69) that confers a stunted habitus facilitating handling of adult plants in the growth chambers. About 8 × 10\(^4\)Col\(^{105}\) seeds were mutagenized with 0.2% of ethylmethane sulfonate (EMS, Sigma-Aldrich) for 16 h, under gentle agitation. The EMS was inactivated with 100 mM sodium thiosulfate for 10 min followed by washing the seeds with tap water (10 times). Seeds were sown on soil and the M2 generation was collected in 156 pools of ~400–500 M1 plants each. From each pool, about 2,500 seeds were sown on two plates (12 cm) containing \(-\) Pi medium (each plate contained two horizontal lines of about 625 seeds each). After 12 h at 4 °C plates were placed on a 30 °C vertical angle in a growth chamber. After 7 days, plates were scanned for seedlings with a long primary root (in comparison to neighbouring roots), and putative mutants were transplanted into soil. The mutant phenotype was checked in the M3 generation and backcrossed to the WT Col\(^{105}\) line. When several putative mutants appeared in a pool, only one was kept for further study. From a total of about 390,000 M2 seeds sown on \(-\) Pi medium,
we isolated 85 viable putative mutants with a long primary root. The mutant phenotype was confirmed in the M3 generation.

**Mapping almt1** and **Stop1** mutations. Two non-allelic mutants (ems32 and ems48), that do not carry mutations LPR1 (see below), were chosen to identify the corresponding mutant genes by map-based cloning approaches.

The almt132 mutant was crossed with the Sha accession, which has a short root when grown under –Pi (ref. 6). In the F2 population, grown under –Pi, we selected seedlings with a mutant phenotype. DNA of individual lines was extracted, and a combination of high-resolution melting (HRM) and microsatellites molecular markers were tested for genetic linkage analysis. The mutation was mapped between molecular markers MASAT1–2.62 and PMSHa-2.78. For the fine mapping, we developed new microsatellite and HRM markers (Supplementary Table 1), allowing localization of almt132 between MASAT1–2.62 and HRM2745575 markers in 1.12 Mb intervals.

The same procedure was applied to Stop116 except that we selected F2 seedlings with a WT phenotype (because Stop116 is dominant). The locus was mapped between MASAT1–12.22 and CW11 in a 6 Mb interval. Among 1,440 chromosomes, 7 and 107 were recombinant for MASAT1–12.22 and for CW11, respectively.

**Identification of the almt132 and Stop17 mutations.** The recessive ems32 and the dominant ems48 mutations were mapped on the top arm of chromosome 1 in regions containing 2.5 Mb, containing 31 and 2 Mb regions containing 31 and 60 high-quality SNPs, respectively.

To identify the corresponding mutant genes, we followed a double gene candidate approach reasoning that the ems32 and ems48 mutations could be located in functionally related genes. By looking at the predicted genes in the 120 kb of chromosome interval containing ems32 we found ALMT1 (At1g08430) and in the 2 Mb region containing ems48 we found STOP1 (At1g43770), encoding a putative transcription factor regulating the expression of ALMT1 (ref. 22). Sequencing of ALMT1 in the ems32 mutant and STOP1 in the ems48 mutant confirmed our conjecture. Homozygous T-DNA and Ds insertion mutant alleles of these genes conferred the same long root mutant phenotype as the EMS alleles (Fig. 1c) thus demonstrating that almt1 and stop1 mutants have a long root under –Pi.

**Allele screening.** To identify the potential lpr1 alleles in our collection of EMS-mutagenized mutants, we used a molecular test based on the restriction enzyme ENDO1 that cuts all coding regions of a DNA mismatch. The ENDO1 restriction enzyme was used according to instruction manual (SERIAL GENETICS). Briefly, the EMS-mutagenized mutants, we used a molecular test based on the restriction enzyme ENDO1. The 480 bp fragment of ALMT1 amplified with primers E1 (containing 2.5 Kb of the 5’ end of the gene) and E2 (containing the stop codon and the 3’ end) was cut by ENDO1. To identify the corresponding mutant genes, we followed a double gene candidate approach reasoning that the ems32 and ems48 mutations could be located in functionally related genes. By looking at the predicted genes in the 120 kb of chromosome interval containing ems32 we found ALMT1 (At1g08430) and in the 2 Mb region containing ems48 we found STOP1 (At1g43770), encoding a putative transcription factor regulating the expression of ALMT1 (ref. 22). Sequencing of ALMT1 in the ems32 mutant and STOP1 in the ems48 mutant confirmed our conjecture. Homozygous T-DNA and Ds insertion mutant alleles of these genes conferred the same long root mutant phenotype as the EMS alleles (Fig. 1c) thus demonstrating that almt1 and stop1 mutants have a long root under –Pi.

**Chemical stock solutions.** SHAM (Sigma-Aldrich, France) was dissolved at 10 mM in absolute ethanol, DAB (Sigma-Aldrich, France) was dissolved at 100 mM in dimethyl sulfoxide (Sigma-Aldrich, France) and mehimazole (Sigma-Aldrich, France) was dissolved at 100 mM in H2O.

**Quantitative RT-PCR.** Total RNA was extracted from whole roots using the RNeasy Plant Mini Kit (Qiagen, France) and treated with the RNase-free DNase Set (Qiagen, France) according to the manufacturer’s instructions. Reverse transcription was performed on 400 ng total RNA using the SuperScript VILO DNA Synthesis Kit (Invitrogen). Quantitative PCR (qRT–PCR) was performed on a Light Cycler thermocycler (Roche) using the manufacturer’s instructions with Lightcycler 480 Sybr green I master (Roche) and with primers listed in Supplementary Table 1. We used ROC3 (AT2G16600) as a reference gene for normalization.

**Transgene constructs.** To study promoter activity, promoter–GUS constructs were prepared for pALMT1 and pSTOP1. The 1.7 and 2.1 kb upstream region of the start codon of the ALMT1 and STOP1 gene were amplified respectively from Ws genomic DNA. The PCR products were cloned into the pDONR P4–P14 vector by a BP clonase reaction (LifeTechnologies). GUS was cloned into a pENTR vector and final constructs were obtained by multisite gateway reaction with pB7m34GW.3 destination vector as described elsewhere39.

**GFP-reporter lines using promoter UBQ (UBQQUITIN10) were constructed for ALMT1 32 and STOP1.** For localization of STOP1 additionally GFP-reporter lines with the native promoter were constructed, pEN1142 and pEN 2.2 PGE-LP3 were used for GFP-fusion constructs by multisite gateway cloning. The 640 bp upstream region of the start codon of UBQ10 and 2.1 kb upstream region of the STOP1 gene were amplified from genomic DNA and cloned into the pDONR P4–P14. The full-length coding region of ALMT1 and STOP1 were amplified by PCR and inserted into the pDONR P2R–P3. The final gateway reaction was accomplished with pB7m34GW as destination vector, resulting in pUBQ10::GFP–STOP1, pUBQ10::ALMT1–GFP and p STOP1::GFP–STOP1. The resulting vectors were transformed into Arabidopsis by floral dip method37.

**Promoters used for vector construction are listed in Supplementary Table 1.** Several independent lines segregating in 3:1 (in the T2 generation) for the resistance to the antibiotic carried by transgene were selected for all our constructs.

To introgress pALMT1::GUSstop1 in the T124 background, pollen of Stop116 was used to cross the pALMT1::GUSstop1 WT line and the cross was checked in F1 grown under +Pi. F2 seedlings with a long primary root under –Pi were selected and transferred in phosphoshibrichin (PPT) plates. Several PPT-resistant plants were transferred in soil for selecting the double homogenous (pALMT1::GUSstop1::pALMT1::GUSstop1) by checking the root phenotype under –Pi and the resistance to PPT.

**Cell type-specific complementation.** For cell type-specific complementation, we used pCO2, pPFP, pPASPI and pSCR promoters as described46,47. Constructs were generated by multisite gateway reaction with pB7m24GW.3 as destination vector for STOP145 and Stop148. Vectors were then used to transform the stop1KO or the Col-046 (containing pALMT1::GUSstop1) background. F2 seedlings with PPT or kanamycin resistance were selected and transferred to soil.

**Measurement of the primary root length.** Pictures of the plates were taken with a camera and root lengths measured using the plugin Neuron37 for the ImageJ software4. For the early kinetic analysis, seedlings were grown 7 days under +Pi and then transferred onto +Pi or –Pi medium and pictures of the root tip were taken every 20 min for 7.5 h with an Axiozoom-V16 macroscope (Zeiss). A root lengths were measured with ImageJ software2 and a special M macro developed on purpose (l’Eréme Mutterer, IBMP, Strasbourg).

**Measurement of root epidermal cell and RAM lengths.** To stain the CWs of epidermal and cortex cells, seedlings were dipped, respectively, 3 min in 15 mM propidium iodide (PI) or 5 min in 30 mM PI, at room temperature, and rinsed twice with H2O.

To measure RAM length, based on the files of cortex cells (from the QC to the first elongated cell), images were collected on a Zeiss LSM780 confocal microscope. For localization of STOP1 additionally GFP-reporter lines with the native promoter were constructed, pEN1142 and pEN 2.2 PGE-LP3 were used for GFP-fusion constructs by multisite gateway cloning. The 640 bp upstream region of the start codon of UBQ10 and 2.1 kb upstream region of the STOP1 gene were amplified from genomic DNA and cloned into the pDONR P4–P14. The full-length coding region of ALMT1 and STOP1 were amplified by PCR and inserted into the pDONR P2R–P3. The final gateway reaction was accomplished with pB7m34GW as destination vector, resulting in pUBQ10::GFP–STOP1, pUBQ10::ALMT1–GFP and p STOP1::GFP–STOP1. The resulting vectors were transformed into Arabidopsis by floral dip method37.

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Inductively coupled plasma mass spectrometry analysis. Seeds were sown in line on a sterile nylon mesh (1.5 × 12 cm, 100 μm). They were grown for 5 days under + Pi prior to transfer for 24 h under + Pi or – Pi thanks to nylon mesh. 60 root tips per genotype (± 5 mm length) were harvested with Teflon razor blades and tweezers into cryovial tubes. Samples were mineralized at 80 °C for 3 h using concentrated HNO3/H2O2 75/25 (v/v). Subsequently, they were diluted 10 times by ultrapure water and injected via a peristaltic pump at 400 μl min−1 flow rate in the 7.200× inductively coupled plasma mass spectrometry analysis (ICP/MS) (Agilent). ICP conditions were the following: nebulization gas flow rate 1 ml min−1, plasma gas flow rate 151 ml min−1, auxiliary gas flow rate 11 ml min−1. Power plasma was set to 1.550 W. The cooled plasma was the interferer ion interference filter higher at 4 ml min−1 flow rate. Other conditions were adjusted to both maximize analyte signals and minimize oxide and doubly charged ion formation. Quantification of Fe was performed at m/z = 54 and 56 using external standards. Between analyses, the system was rinsed for 3 min by HNO3 5% a blank was injected to control the absence of any memory effect. Acquisition was performed for 3 s in triplicate.

Yeast one-hybrid assay. The Saccharomyces cerevisiae strains used were YPH501 (diploid, ura3–52, ade2–101, leu2–3,112, trp1–101, his3–1,200, leu2–3,1,12), and YPH499 (MATa, ura3–52, lys2–801, ade2–101, trp1–1, leu2–3,1,12, his3–1,200, leu2–3,1). For yeast transformation, cells were grown in the YPD (yeast extract–peptone–dextrose) medium, and for the yeast one-hybrid assay, cells were grown in raffinose synthetic complete medium minus uracil and tryptophan, supplemented with 1% of galactose synthetic complete medium minus uracil and tryptophan at 30 °C. DNA constructions of bait:reporter fusions were made as follows: the ALMT1 promoter (1.7 kb and sub-fragments) was PCR-amplified (DNA primers in Supplementary Table 1). The amplicons inserted in front of the lacZ reporter gene by homologous recombination within the Xmal-linearized pSH18–34 vector (Invitrogen). To make the effector construct, the E. coli b42 activation domain was fused by homologous recombination to the PCR-amplified (Supplementary Table 1) AStOPI C-terminal coding sequence into the pB42AD (Clontech) vector; expression of this AStOPI-B42 fusion was driven under the pGAL inducible promoter. All constructs were checked by DNA sequencing. The primers used for plasmid construction are shown in Supplementary Table 1.

To stain root CWs, seedlings were dipped 5 min in 30 μM Pi solution at room temperature and rinsed twice with H2O. Images were collected on a Zeiss LSM780 confocal microscope (Carl Zeiss, France) using a Plan Apochromat 10/0.45 objective and a Plan-Apochromat 20/0.8 objective. GFP and Pi were excited sequentially with the blue argon ion laser (488 nm) and the DPF5 laser (561 nm), respectively. Emitted light was collected from 491 to 535 nm for GFP, and from 600 to 675 nm for Pi, using the MBS 488/561 filter. Images were processed using Zen black software (Zeiss).

Quantification of GFP fluorescence. To quantify GFP fluorescence in nuclei, images were obtained with a LSM780 confocal microscope (Carl Zeiss, France) using a ×20 dry objective (Plan Apo NA 0.80). GFP was excited with the Argon laser (488 nm), and emitted light was collected between 493 and 538 nm with a pinhole setting of 1.51 μm. All nuclei were imaged using the same conditions of gain, offset and resolution (with zoom set to 3), at a constant distance (350 μm) from the root tip. A Z-stack of 15 images (separated by 1 μm distance) imaged representative nuclei on the surface of the plant. Images were acquired in 16 bits using Zen black software (Zen black 2012 SP2, Version 11.0.1), then converted to a maximal projection image. Average nuclei fluorescence intensities were quantified using the Zen blue software (Zen 2 blue edition, version 2.0.0.0), by drawing identical ROI (region of interest) over 3–10 visible (in focus) nuclei for each maximal projection image.

OS staining. Os staining was performed as described previously with minor modifications. Bovine serum albumin (BSA) (5 mg ml−1) and 0.3% (w/v) Triton X-100 were added to the staining solution and incubated for 1 h at 37 °C. Plants were observed and documented with a DM6000B upright microscope (Leica; ×10 objective) after clearing with chloral hydrate solution as described.

Visualization of peroxidase activity. 4-Chloro-1-naphthol staining was used to visualize peroxidase activity in roots. A volume of 1 μl of 3% 4-chloro-1-naphthol stock solution (30 mg ml−1 in absolute ethanol) and 1 μl of a 3% H2O2 stock solution were added to 10 ml of 250 mM Tris buffer pH 7.4 resulting in the final staining solution. Plants were stained for 60 s. Staining solution was exchanged with + Pi or – Pi buffer, depending on the sample, and washed three times. Plants were observed and documented with the Leica 6000D (×10 objective) for all samples in the same conditions.

ROS and callose staining. ROS and callose staining were accomplished as described previously. ROS were stained using CM-H2DCFDA (6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate; this compound is oxidized by peroxidases in the presence of H2O2 with minor modifications (for example, the phosphate-buffer was replaced by 20 mM 4-(2-hydroxyethyl)pyperazine-1-ethanesulfonic acid (HEPES) buffer, pH 6.0). Images were collected on Zeiss LSM780 confocal microscope (Carl Zeiss, France).

AFM analysis. Mounting root samples: AFM data were acquired on the primary root from whole seedlings mounted under the microscope. Seedlings were grown 6 days under + Pi prior to transfer under + Pi or – Pi during 0.5–2 h. Seedlings were then glued on a microscope slide (one seedling per slide) as follow: a layer of silicone (MED1–1,356, NuSil Technology LLC, USA), with the thickness of a coverslip slide (130–170 μm), was spread on the slide; silicon was let to dry out for 1 min prior to laid down the seedling on it. To securely anchor the root, a silicone band covering <100 μm of the root tip was stretched using a syringe needle. Then, a droplet of liquid medium was used to apply water to the (minus minus agar) in the (Agarose) layer. A droplet of liquid medium was used to apply water to the root tip. Seedlings were added to the droplet and covered by a coverslip. The thickness of the coverslip was checked by the Z-stack. Seedlings were then observed using the Leica microscope in the (Carl Zeiss, France).

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Statistics and reproducibility of experiments. Statistical analyses were performed using Prism 6.0 (GraphPad). Sample size was not predetermined using statistical methods, but took into account the variability of the traits measured, assessed by the s.d.; no samples were excluded from the analyses. Data sets with normal distributions were analysed using one-way or two-way ANOVA, or using two-sided, unpaired Student’s t-tests and presented as the means ± s.d. except for Figs 1e and 2b,c and Supplementary Fig. 3a where they are presented as the means ± s.e.m. Data sets with non-normal distribution (for example, AFM measurements) were analysed with Mann–Whitney tests and presented as the median ± interquartile. Data sets with dichotomous distributions (Supplementary Fig. 1d) were analysed with a binomial test. Tukey’s whiskers extend to the extreme values included in the interval calculated as ± 1.5 IQR (interquartile range), where the IQR is calculated as the third quartile minus the first quartile. The results for statistical significance tests are included in the legend of each figure; n values represent the number of independent experiments performed or the number of seedlings, cells or meristems per condition. The samples were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment. All micrographs are representative of at least two independent experiments.

Data availability. All data supporting the findings of this study are available from the corresponding author on request.

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**Author contributions**

All experiments contributed to the design of individual experiments and analysis of results, and participated to parts of manuscript preparation. C.Ba. designed and carried out short-term kinetic experiments, contributed to callose and iron-staining experiments, cell length measurements, ICP-MS and AFM experiments. T.Da. prepared plasmids and transgenic lines, performed qRT-PCR and contributed to confocal microscopy. C.B. designed and performed AFM experiments, designed and performed the malate complementation experiment, designed and supervised yeast experiments and carried out the pH experiment with GUS staining. E.L. characterized the EMS mutants and identified most almt1 and stop1 mutations, prepared plasmids and performed the yeast experiments. C.M. designed and performed some GUS staining, the ROS and peroxidase histology and meristem length experiments, participated to confocal microscopy, J.-M.T. designed and performed AFM experiments. A.C. performed mapping of almt112 and screened EMS mutants for lpr1 alleles. M.B. made plasmids and transgenic plants, and assisted the yeast experiments and contributed to ICP-MS experiment. C.Br. carried out iron staining experiments, contributed to measurements of roots and cell lengths and genotyping, contributed to confocal microscopy. A.H. performed the ICP-MS analysis. J.M. contributed to iron and callose staining experiments and to malate complementation experiments. S.C. assisted with GUS histology and qRT-PCR. H.J. assisted and supervised confocal microscopy imaging and quantification. N.L.-B. contributed to genotyping. P.D. contributed to genotyping. B.P. provided materials and tips related to this study. E.D. assisted with HRM analysis and mapping, identified almt12 and Stop116, performed experiments on drugs, rapeseed, root length and GUS staining, generated all the figures and tables; wrote the manuscript with feedback from S.A., C.G., H.J., C.M., C.Ba., T.Da., J.-L.P., B.P., L.N., M.-C.T. and E.D.
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