The dynamics of root cap sloughing in Arabidopsis is regulated by peptide signalling

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ABSTRACT

The root cap protects the stem cell niche of Angiosperm roots from damage. In *Arabidopsis* lateral root cap (LRC) cell files covering the meristematic zone are regularly abolished through programmed cell death, while the outermost layer of the root cap covering the tip is regularly sloughed. Efficient coordination with stem cells producing new layers is needed to maintain a constant size of the cap. We present a signalling pair, the peptide IDA-LIKE1 (IDL1) and its receptor HAESA-LIKE2 (HSL2), mediating such communication. Live imaging over several days characterized the sloughing process from initial fractures in LRC cell files to full separation of a layer. Enhanced expression of IDL1 in the separating root cap layers resulted in increased frequency of sloughing, balanced with generation of new layers in a HSL2-dependent manner. Mutations in either *IDL1* or *HSL2* slowed down cell division, maturation and separation, and suggested involvement of programmed cell death in the sloughing event. Transcriptome analyses linked IDL1-HSL2 signalling to the transcription factors BEARSKIN1/2.
The root cap provides protection for the root apical meristem (RAM) and senses environmental conditions\(^1\). The RAM encompasses stem cells, surrounding the Quiescent Centre (Fig. 1a), both for the tissues of the root proper, and for the root cap. Although both forms of stem cells continuously produce new cells, the root cap has a defined size, due to homeostatic balance between generation and loss of root cap cells\(^2\). Many species shed living, single cells, so called border cells, but in Brassicaceae, including *Arabidopsis thaliana*, the outermost layer of the root tip is released intact from the underlying cell layer\(^3\). The *Arabidopsis* root cap distal to (below) the QC, consists of a core of 5-6 tiers of gravity-sensing columella cells (COL) surrounded by layers of lateral root cap (LRC) cells (Fig. 1a). The LRC extends to cover the meristematic zone (MZ), and there the outermost layer regularly undergoes programmed cell death (PCD) whereby it is replaced by a new LRC layer developing underneath the old one\(^4\). Less is known regarding the genetic and molecular mechanisms governing the detachment of the outer layers of the distal root cap, how the frequency of sloughing is regulated and how the homeostatic balance with stem cells is secured\(^2\).

Root cap sloughing requires breakdown of cell walls and is in that respect similar to other cell separation processes such as flower or fruit abscission\(^5\), however, sloughing is a recurrent event in the same organ. Hence, the challenge is to compensate for each lost root cap layer by generation of a new, and to detach the oldest layer when a new layer has been formed. In the distal root cap, this requires communication between old separating layers and the stem cells of COL and LRC, as well as a sequential coordination of generation, maturation and separation of root cap cells.

Mutations in four genes encoding closely related NAC (NAM, ATAF, CUC) transcription factors (TFs) – FEZ, SOMBRERO (SMB), BEARSkin1 (BRN1) and BRN2 – have provided insights into the development and differentiation of the root cap\(^4,6-8\). FEZ is
implicated in the formative division of the common stem cells for the LRC and the epidermal layer (EPI). SMB regulates differentiation and maturation⁶⁻⁸, while the double mutant brn1 brn2 is impaired in cell wall degradation and accumulates additional undetached root cap layers⁸.

Downstream of these TFs, genes have been identified that encode enzymes involved cell wall remodelling and synthesis⁸. Similar genes have been discovered in other cell separation processes in flowering plants⁹,¹⁰. Floral organ abscission and emergence of the lateral root though the overlying tissues in *Arabidopsis*, are regulated by the small peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) that signals through two closely related leucine-rich repeat receptor-like kinases (LRR-RLKs), HAESA (HAE) and HAESA-LIKE2 (HSL2)¹¹⁻¹⁵. *IDA* belongs to a family of *IDA-LIKE (IDL)* genes, encoding preproproteins with a common C-terminal motif (Supplementary Fig. 1), hypothesized to have a function similar to *IDA*. Here, we address the function in root cap sloughing of IDL1, expressed in the root tip, and identify HSL2 as its receptor. Rather than specifically controlling the cell separation step of the sloughing process, this signalling pair regulates the dynamics of root cap detachment and generation of new root cap layers.

RESULTS

Identification of HSL2 as the receptor of IDL1

IDL1 is prominently expressed in COL cells at the centre of the two adjacent root cap layers of the primary root that will undergo separation (Fig. 1a-b). IDL1 can rescue the abscission deficiency of the *ida* mutant¹⁶, indicating that IDL1 peptide can interact with the receptors of IDA. Using promoter-β-glucuronidase (GUS) reporter lines for *HAE* and *HSL2* we found that only *pHSL2:GUS* had an expression pattern in the primary root tip, with the strongest expression in the youngest LRC cell files (Supplementary Fig. 1). This was confirmed when
using a construct with the HSL2 promoter coupled to a nuclear targeted florescent protein (pHSL2::Venus-H2B) (Fig. 1c). The N-terminal signal sequence of the IDL1 prepropeptide (Supplementary Fig. 1) indicates that the mature peptide is exported and can move in the apoplastic space to interact with the extracellular domain of the preferred receptor \(^{17,18}\). Thus, the expression patterns were compatible with a hypothesized function of IDL1 and HSL2 as a ligand-receptor pair involved in communication between cells in the root cap.

To investigate whether IDL1 could activate and bind the receptor, we took advantage of methods previously used for IDA and HSL2 \(^{12}\). Addition of a synthetic IDA peptide of 12 amino acids, with hydroxylation of the central proline residue (mIDA), to *Nicotiana benthamiana* leaf pieces expressing the HSL2, results in an immediate receptor activation measurable as oxidative burst. The mIDA peptide (Fig. 1d) activates HSL2 down to a half-maximal efficient concentration (EC50) of 1 nM \(^{12}\). The corresponding hydroxylated dodeca IDL1 peptide (mIDL1, Fig. 1d), tested in the same assay, functioned also in a dose-dependent manner with a similar efficiency (EC50 ~3 nM, Fig. 1e).

Competition assays with acridinium-labelled and unlabelled IDA-derived peptides have demonstrated specific and reversible binding of the ectodomain of HSL2 expressed in *N. benthamiana* leaves \(^{12}\). The same experimental set-up showed that unlabelled mIDL1 competed for binding of the HSL2 ectodomain with an efficiency similar to the unlabelled IDA peptide, with a half-maximal inhibitory concentration (IC50) of ~30 nM (Fig. 1f).

Root cap expression, together with binding and activation of HSL2 by IDL1, suggested that this peptide and receptor might serve as a root cap signalling module.

**IDL1 expression increases the frequency of sloughing in a HSL2-dependent manner**

Since a number of LRR-RLKs with high similarity in their ectodomains are expressed in the root \(^{19}\), ectopic ligand expression or exogenously applied synthetic peptides might lead to...
interaction with non-native receptors. With the aim of testing whether IDL1-HSL2 was specifically involved in root cap sloughing, we therefore used the promoter of IDL1 itself to generate Enhanced IDL1 (EnhIDL1) plants with estradiol (ES)-inducible enhanced expression of both IDL1 and GUS (Fig. 2a-b). EnhIDL1 plants were crossed to an hsl2 knockout mutant to test the importance of HSL2 for IDL1 function. Cell-specific and efficient ES-induction was confirmed by strongly enhanced GUS expression in EnhIDL1 roots both in wild type Col-0 and hsl2 mutant background as compared to the pIDL1:GUS expression pattern and by qRT-PCR (Fig. 1b, 2b and Supplemental Fig. 1). These lines did not deviate in root length (Supplementary Fig. 1).

Cell elongation, cell division and differentiation take place in the root tip during the first days after germination. To ensure that homeostatic balance between gain and loss of root cap layers had been established, our experiments were performed on roots with full-sized RAM and root cap (Supplementary Fig. 2). Seven days after germination (DAG) roots of Col-0, EnhIDL1 and EnhIDL1 hsl2 seedlings grown vertically on agar with 10 µM ES, covered by a cover slip, were tracked, using a laser scanning microscope with a vertical stage. Images taken in two experiments every 15 minutes for 64 and 70 hours (h) revealed a stepwise sloughing process (Fig. 2c-e). The first signs of a forthcoming sloughing event were characteristic fractures or gaps in LRC cell files in a region just above the Quiescent Centre (QC) and about 5h later at the level of the youngest starch-filled COL tiers (Fig. 2c, red and yellow arrows). These gaps initiated a gradual loosening of the outermost LRC layer towards the tip. Finally, the COL cells at the centre of the cap separated from the layer underneath followed by detachment of the whole layer as it slid to the side and was lost (Fig. 2c, white arrow and Supplementary Video 1). On average, 0.6 root cap layers were shed per root per day in Col-0 (Fig. 2f), with an average of ~18h for a completed sloughing process, and
thereafter another ~18h before a new event of root cap layer separation would be initiated (Fig. 2g and Supplementary Table 1).

The sloughing frequency in EnhIDL1 was 50 % higher than for Col-0, i.e. 0.9 events per root per day (Fig. 2f). This increase seemed to be caused by a shorter interval between the completion of one event and the initiation of the next (Fig. 2g and Supplementary Table 1), and overlapping events with fractures in the second outermost layer, before the previous event was fully completed (Fig. 2d, green arrow, 2h, and Supplementary Video 2).

In hsl2 background the increased sloughing frequency of EnhIDL1 was rescued and did not deviate from that of Col-0 (Fig. 2f). However, the sloughing process developed most often differently in that the outermost layer was split open close to the tip at the border between the COL and LRC cells (Figure 2e and h). The split cap layer, sometimes leaving the central COL region behind, seemed to be still attached to the LRC sheet surrounding the meristematic region (Fig. 2e, white arrows, and Supplementary Video 3).

Live imaging was supplemented with data for roots grown on vertical agar plates. In this set-up, shed layers of the distal cap, recognizable by detached LRC files, did not slide away immediately, but accumulated in front of the growing roots, most likely due to less friction when the roots were not covered by a cover slip. Caps were, however, easily lost when removed from the agar, and sloughed caps were therefore counted directly on the plates at 6, 8 and 10 DAG, with and without 10 µM ES (Fig. 3a-b). Col-0, EnhIDL1 hsl2, and EnhIDL1 on non-inducing medium sloughed about 0.6 caps per day (Fig. 3a-b). On ES medium, on the other hand, EnhIDL1, accumulated up to eight sloughed root cap layers (Supplementary Fig. 3) and differed significantly with an average loss of one root cap layer per day (Fig. 3a). Thus, these results were in full accordance with the live imaging results.
Higher frequency of IDL1-induced sloughing is compensated by more stem cell divisions

We questioned whether a higher frequency of sloughing reduced the number of attached layers, however, all three lines maintained stably ~6 attached COL tiers (including the stem cell layer) (Supplementary Fig. 3), and did not differ significantly from each other (Student’s t-test $P \geq 0.1$), irrespective of ES in the growth medium (Fig. 3c).

More frequent shedding, but at the same time constant cell number and size of the root cap, imply an increased frequency of new initiations of COL tiers and LRC cell files to compensate for the lost caps. Recent divisions of COL stem cells, positioned just below the QC (Fig. 1a), can be recognized by the smaller cell size in the new 1st COL tier and their weaker cell wall staining with propidium iodide (PI) compared to older COL cells. The COL stem cells are surrounded by the shared stem cells for LRC and epidermal cell files, and in medial optical sections of the root tip, they are seen as four COL stem cells with one EPI/LRC stem cell on each side (Fig. 1a). New epidermal cells are generated by anticlinal division, while periclinal divisions represent initiation of a new LRC cell file. On inductive medium, cell wall patterns indicating recent cell divisions were found for COL in 80% and for LRC in 60% of EnhIDL1 PI-stained root tips (Fig. 3d-e). This was in contrast to Col-0 and even more so for EnhIDL1 $hsl2$, where < 15 % of the root tips showed signs of recent stem cell divisions (Fig. 3d-e).

These findings imply that increased expression level of $IDL1$ not only leads to increased sloughing, but also an increased frequency of initiation of new root cap layers.

Mutations in $IDL1$ and $HSL2$ slow down the sloughing process

To further explore the function of the IDL1-HSL2 signalling in the root cap, we investigated the sloughing behaviour of $hsl2$ and an $idl1$ CRISPR/Cas9 mutant line (Supplementary Fig. 4) compared to Col-0. While the number of attached layers remained close to 6 for both Col-0
and the mutants, a subtle, but significant difference was observed regarding the number of sloughed cap layers at 10 DAG with an average 3.0 caps for Col-0 and 2.4 for the mutants (Fig. 4a). Inspection of the youngest LRC cell files furthermore suggested that the frequencies of generation of new LRC cell layers had slowed down in both mutants. After the initial periclinal division of an EPI/LRC stem cell, the new LRC cell file is extended by anticlinal divisions. The new file will be neighbouring an older and longer LRC cell file on the lower side and an epidermal cell file on the upper side until the next new LRC layer is initiated (Fig. 4b-d). In the wild type, one cell of the two youngest cell files neighboured the epidermal cell file, in contrast to 2-3 cells in both the hsl2 and idl1 mutants (Fig. 4b-c). This is an expected result if the periclinal divisions initiating a new LRC file take place less frequently relative to the anticlinal divisions of already initiated LRC cell files (Fig. 4d).

**Mutation in HSL2 affects genes in both the LRC and the COL cell layers**

To gain a better understanding of the function of the signalling initiated by activation of *HSL2*, RNA sequencing was performed on RNA isolated from the RAM of root tips of Col-0 and hsl2. In line with the subtle phenotype of hsl2, few genes were grossly affected by the mutation. However, using a P adjusted (Padj) cut-off value of 0.1, 156 genes were identified with an expression level < 70% of the Col-0 level (Supplementary Table 2). In an attempt to identify where the genes affected by HSL2 signalling were expressed, we took advantage of a data set for tissue-specific gene expression in the Arabidopsis RAM. This dataset provides information on differential gene expression in the different root tissue, QC, LRC, COL, EPI, cortex (COR) and endodermis (END), based on cell sorting using marker genes for these tissues. Eighty-one of the hsl2 down-regulated genes were identified as differentially expressed according to this data set. We defined a gene as characteristic for a specific tissue if it had the highest expression level for this tissue compared all the other tissues. Evaluation
according to a binomial test (p=0.1, see Methods for details) found significant enrichment of genes expressed in LRC and COL (Table 1). Genes encoding extracellular proteins were well represented among the LRC expressed genes, while many genes most highly expressed in COL encoded proteins involved in catalytic and hydrolytic activity including starch and cell wall degradation.

Activation of RLK receptors is transduced via phosphorylation cascades and activation of transcription factors, which at present are unknown for HSL2 in the root tip. However, we found an overlap between the *hsl2* down-regulated genes and genes identified in a recent transcriptome analysis of NAC TFs \(^8\). Of sixty genes reported to be positively regulated by overexpression of SMB and downregulated in the triple mutant *smb brn1 brn2*, twenty-five genes, a highly significant number (Fischer’s exact test P< 10\(^{-10}\)), was also down regulated in *hsl2* mutant root tips (Supplementary Table 2). There was an overrepresentation of genes encoding extracellular proteins and enzymes, hereunder enzymes involved in cell wall remodelling and starch metabolism (Supplementary Table 2) \(^8,24,25\).

The three NAC TFs were not significantly down regulated in *hsl2* according to our RNAseq analyses, however, to investigate whether IDL1 signalling could affect expression patterns, lines with translational fusion constructs for BRN1 and BRN2 were crossed into the EnhIDL1 line and the *idl1* mutant. Both *BRN1* and *BRN2* are expressed in the outer LRC cell files \(^8\). In EnhIDL1, the expression pattern of *pBRN1:BRN1-GFP* was very similar to Col-0 grown with ES in the medium, while *pBRN2:BRN2-GFP* consistently showed an additional layer of expressing cells at the tip (Supplementary Fig. 5). In the *idl1* mutant, on the other hand, *pBRN2:BRN2-GFP* expression was similar to Col-0, but fewer cells expressed *pBRN1:BRN1-GFP* in the LRC cells (Supplementary Fig. 5).

The *ROOT CAP POLYGALACTURONASE (RCPG)*, a direct target of BRN1 \(^8\), was of the most strongly downregulated genes both in the triple mutant *smb brn1 brn2* and *hsl2*
In loosened Col-0 root cap layers the construct pRCPG:nYG, with nuclear targeted YFP driven by the \textit{RCPG} promoter, was expressed in more cells and with higher intensity than in \textit{hsl2} and \textit{idl1} background (Fig. 5a). Moreover, pRCPG:nYG was expressed in undetached LRC cells in the mutants (Fig. 5a), suggesting delayed cell separation. pRCPG:nYG expression was also seen in undetached root cap layers in the EnhIDL1 line, in this case suggesting premature expression (Supplementary Fig. 5).

This influence on \textit{RCPG} expression suggests that IDL1-HSL2, like the BRN TFs, are involved in the final step of the sloughing process – the actual cell separation.

\textbf{IDL1-HSL2 regulates \textit{BFN1} expression at PCD Site II}

We noted that in contrast to \textit{hsl2} and \textit{idl1}, most Col-0 cells expressing \textit{RCPG} had initiated the sloughing process and their nuclei stained with PI (Fig. 5a and Supplementary Fig. 5), which is indicative of plasma-membrane permeabilization. Such PI entrance has been used as a hallmark of initiation of developmental programmed cell death (dPCD) associated with the recurrent turnover of the LRC covering the MZ. Interestingly, in the \textit{hsl2} transcriptome five out of eight genes strongly associated with dPCD were down-regulated; the \textit{BIFUNCTIONAL NUCLEASE1 (BFN1)}, \textit{METACAPASE9 (MC9)}, \textit{SAPOSIN-LIKE ASPARTYL PROTEASE3 (PASPA3)}, \textit{EXITUS (EXI)} and \textit{RIBONUCLEASE3 (RNS3)} (Supplementary Table 2). To investigate the potential involvement of IDL1-HSL2 in dPCD in the root cap, a transcriptional fusion construct with the \textit{BFN1} promoter and a nuclear-targeted GFP (\textit{BFN1}_{\text{pro}}:nGFP) was crossed into EnhIDL1 and the mutants. In Col-0, two known sites of dPCD, at the upper end of the MZ (Site I) and close to the QC (Site II), were identified as expected (Fig. 5b).

Interestingly, in addition to the strong expression of pHSL2::Venus-H2B in young LRC cell files, HSL2-driven nuclear-targeted Venus was found in LRC cell files surrounding the MZ and stretching past Site II (Fig. 5c). A third PCD site was found in loosened LRC cell files...
consistent with the high number of nuclei there staining with PI (Fig. 5a-b), and suggestive of PCD in the sloughed cells. The pattern of $\text{BFN1}_\text{pro}:\text{nGFP}$ expression in $\text{EnhIDL1}$ at these sites was similar to $\text{Col-0}$. In contrast, $\text{idl1}$ and $\text{hsl2}$ root tips had significantly fewer cells expressing the $\text{BFN1}_\text{pro}:\text{nGFP}$ construct, in particular at Site II (Fig. 5b and Supplementary Fig. 6). Interestingly, Site II coincides with the position of the fractures initiating the detachment of the distal root cap (Fig. 5b), which suggestively may provide a molecular link between the $\text{IDL1}$-$\text{HSL2}$ signalling pair and initiation of the sloughing process.

DISCUSSION

Dynamics of root cap sloughing and mechanisms securing homeostatic balance between loss and gain of layers of the distal root cap have been difficult to study because of the duration of the process, the easily lost, small cap layers, and few available mutants. Continuous live imaging over three days, analyses of transcriptome data and cellular expression patterns have enabled us to detail the different phases of root cap sloughing and the involvement of $\text{IDA-HSL2}$ in this process (Fig. 6). Under our conditions, 0.6 distal cap layers were shed per root per day in $\text{Col-0}$. Enhanced expression of $\text{IDL1}$ increased this frequency by shortening the interval from one event to the next, or by detaching two layers at the same time. We have identified $\text{IDL1}$ as a peptide ligand that in nanomolar concentrations binds and activates the $\text{HSL2}$ receptor. Both $\text{IDL1}$-increased sloughing and the balancing increase in stem cell divisions were dependent on $\text{HSL2}$, i.e. $\text{IDA-HSL2}$ signalling controls the dynamics of sloughing at the root tip.

We had expected cell separation phenotypes of $\text{idl1}$ and $\text{hsl2}$ similar to the total deficiency in floral organ abscission seen in the $\text{ida}$ mutant and the double mutant $\text{hae hsl2}$. However, homeostasis appears maintained in the two mutants, albeit at a much lower frequency. Cell division, maturation and separation were slowed down, but not abolished. It is
likely that other factors, including other peptides and receptors, are involved in the separation step. A few mutants have been identified that release single cells instead of entire root cap layers, like *nin-like protein 7 (nlp7), quasimodo1-1 (qua1-1)* and *qua2-1*. In contrast, the *brn1 brn2* double mutant, and *active quiescent center1-1 (aqc1-1)*, are delayed in separation of root cap layers. The latter mutant is defect in the gene encoding tyrosylprotein sulftransferase (TPST), an enzyme responsible for sulfation of a number of peptide ligands.

The disorganized root cap and stunted growth in this mutant is likely to be a consequence of several dysfunctional peptides lacking sulfation.

Live imaging of roots and marker-gene expression in the EnhIDL1 line and the *idl1* and *hsl2* mutants suggest that IDL1-HSL2 signalling is controlling initiation of the sloughing process. Intriguingly, the region where the first formative fractures or gaps in the LRC cell files are found, seems to coincide with dPCD Site II, and a speculative suggestion is that these fractures are created by PCD. dPCD starting from Site I is involved in the recurrent removal of the LRC surrounding the MZ. Differential control of dPCD Sites I and II would make it possible for the plant to operate with different frequencies of renewal of the LRC surrounding the MZ and of sloughed layers at the tip.

IDL1-HSL2 control of the position of initiation may also explain the split caps still connected to the LRC surrounding the MZ observed for EnhIDL1 in *hsl2* background. With fewer fractures, MZ cell division and COL cell elongation is likely to exert mechanical stress that tears apart cells at other, weaker sites. Similarly, in *ida* and *hae* mutants, separation of cells in the cortex and epidermis overlying developing lateral root primordia (LRPs) is impaired, but the LRPs eventually emerge forcibly through these tissues. These two examples suggest that regulation of growth of the LRP and the primary root is not tightly coupled to the respective cell separation events.
BRN1, BRN2 and SMB are implicated in root cap maturation and root cap detachment\textsuperscript{7,8}. pHSL2::Venus-H2B was found to have overlapping expression pattern with the BRN TFs in the LRC, and a subset of genes regulated by these TFs was also found downstream in the HSL2 signalling pathway. Differences in spatial expression patterns compared to Col-0 of transcriptional and translational reporter construct crossed into our lines, indicated faster maturation in EnhIDL1 and slower maturation in the mutants. Together this suggests HSL2 to play a role in maturation and cell layer separation in addition to the role in initiation. \textit{BFN1\textsubscript{pro}}-controlled maker-gene expression and PI-stained nuclei in LRC cells of root caps undergoing sloughing, indicate that root cap sloughing involves both cell separation and cell death.

RNAseq data for \textit{hsl2} combined with publicly available information on differential gene expression in root tissues were used in an attempt to identify the cell types that primarily respond to HSL2 activation. This analysis identified overrepresentation of genes preferably expressed in LRC and COL cells. The major effect of receptor activation will occur in the cells where the receptor is expressed, indicating that HSL2 is present and responsive for activation both in LRC and COL cells. Additionally, this analysis suggests that activation of HSL2 gives different outcomes in different tissues.

IDL1-HSL2 communication facilitates dynamic regulation of the homeostatic balance between stem cell division and sloughing activity. Since the root cap is guiding root growth by sensing and responding to external cues, like gravity, water potential or the presence of obstacles in the soil\textsuperscript{1,2}, it is highly likely that the frequency of root cap shedding is triggered by environmental factors, possibly by influencing the expression level of IDL1 and/or HSL2. At present, it is not known whether or how external condition might feed into the circle of generation and loss of root cap layers. Factors involved in transcriptional regulation of \textit{IDL1} and \textit{HSL2}, peptide processing and export, and interacting partners have not been identified. Additionally, several parallel pathways partake in regulation of stem cell activity, the NAC
transcription factors FEZ and SMB, the RETINOBLASTOMA-RELATED protein, the
WOX5 transcription factor, and the AUXIN RESPONSE FACTORS ARF10 and ARF16\textsuperscript{30,32},
but their relationship to the IDL1-HSL2 signalling pathway have not yet been unravelled.

It will be exciting to take advantage of root tip tracking by live imaging and to explore
these relations in the future.

\textbf{Methods}

\textbf{Accession numbers} of the genes studied in this work: \textit{IDL1} – At3g25655, \textit{HSL2} –
At5g65710; \textit{HAE} – At4g28490; \textit{BRN1} – At1g33280, \textit{BRN2} – At4g10350, \textit{BFN1} – At1g11190
\textit{and RCPG} – At1g65570.

\textbf{Constructs and plant lines.} The \textit{pIDL1:GUS, pHAE:GUS, pHSL2:GUS, BFN1\textsubscript{pro}:nGFP,}
\textit{pRCPG:nYG, pBRN1:BRN1-GFP and pBRN2:BRN2-GFP} lines have been described
previously\textsuperscript{8,16,27}. A 2300 bp long fragment upstream of the start codon of \textit{HSL2}\textsuperscript{31} was cloned
into destination vector \textit{promoter::Venus-H2B}\textsuperscript{32}, using the Invitrogen Gateway\textsuperscript{TM} system, and
introduced into \textit{Arabidopsis} Col-0 plants by floral dipping. A single-locus homozygote
\textit{pHS2::Venus-H2B} line was identified.

Gateway cloning and floral dipping was also used generate Enhanced IDL1
(EnhIDL1) plants; the \textit{IDL1} coding region was inserted after the estradiol inducible promoter
in the vector pMDC221, and a 1555 bp \textit{IDL1} promoter fragment was cloned in front of the
XVE transcription factor in the pLB12 vector\textsuperscript{21}. Of six \textit{pIDL1 pLB12} lines checked for GUS
expression pattern (after induction) comparable to that of the published \textit{pIDL1:GUS}\textsuperscript{10,30}, a
single-locus line (checked on 25 mg/l Hygromycin (Hyg)), was crossed to a single-locus \textit{IDL1}
pMDC221 line (checked on 50 mg/l Kanamycin (Km)). EnhIDL1 plants homozygous for both constructs were identified by selection on Hyg and Km and checked non-segregating GUS expression. Of two lines with comparable sloughing behaviour, GUS expression level and pattern, one was chosen for the experiments shown here. This line was furthermore used for all crosses to mutants and marker-lines.

Two idl1 mutants were made with CRISPR/Cas9 technology using an IDL1-targeting vector construct based on the pDe-Cas9 systems provided by Holger Puchta. A 20 base pair gRNA target site, unique for IDL1 including BslI recognition motif, was selected for the mutagenesis. Vector construction and generation of mutants were performed as described previously. Through selections with BASTA and CAPS analyses, mutants containing 2 bps deletion (idl1-cr1) and 4 bps deletion (idl1-cr2) in the open reading frame of IDL1 were isolated. Both mutations result in frame shifts generating premature stop codons in the central variable region of IDL1 prepropeptide and the C-terminal peptide motif is thereby deleted (Supplementary Fig. 4). Phenotypic differences between the alleles were not observed (Supplementary Fig. 4). Data for idl1-cr1 (named idl1 here) is presented.

Primers for genotyping and generation of constructs are listed in Supplementary Table 3.

Growth conditions and registration of number of root cap layers. Sterilized seeds were stratified at 4 °C for 72 h. Seedlings were grown at 22 °C, 8 h dark and 16 h light (100 µE/m² light intensity) on square vertical plates with sucrose-free 0.5 x MS medium adjusted to pH 6.0, with 10μM 17-β-estradiol for induction of enhanced expression of IDL1 in EnhIDL1 lines. Sloughed root cap layers were counted and imaged directly on the agar plates (using a Zeiss Axioplan2 imaging microscope) as they easily fall off when roots are moved. Attached caps were imaged and counted after immersion in Lugol solution (Sigma 6265) for 1 minute for detection of the COL cell tiers with amyloplasts (Supplementary Fig. 3). No significant
difference in length was observed between genotypes or with ES in the medium (Supplementary Fig. 1).

**Oxidative burst measurements and receptor-ligand binding assay.** *N. benthamiana* leaves transiently expressing HSL2 constructs were used for oxidative burst measurements and peptide binding assays as previously described. EC50 and IC50 curves are representative for N=3 independent experiments, each with three technical replicates.

**Microscopy.** Histochemical GUS assays were performed as previously described. Confocal laser scanning microscopy (CLSM) was carried out with Olympus FV1000 and ZEISS LSM880 confocal microscopes at the Oslo NorMIC imaging node. Roots were stained with 10 µM propidium iodide (PI).

**For live imaging** seeds were surface sterilized by chlorine gas, sown on 1% agar AM-medium (0.5 x MS, pH 5.8, 1% agar), stratified for 3 days at 4°C and cultivated in a growth incubator at 22°C in a 16/8 h day/night cycle with 120–140 µmol/m²/s light.

Eight day-old plants were put on blocks (rectangular parallelepipeds) of 1.5% agar AM-medium (0.5 x MS, pH 5.8) supplemented with 10µM 17-β-estradiol. Roots and shoots were put on the blocks’ basis and sides, respectively. These samples were put into the chambered cover-glass (Thermo Scientific Nunc, catalogue no: 15536), which were then mounted onto a laser scanning microscope with a vertical stage (roots down, shoots up). Photographs of root tips were taken every 15 minutes, in two experiments over 64 and 70 h, with in total 9 roots for Col-0 and 10 roots for EnhIDL1 and EnhIDL1 hsl2.

**RNA extraction and RNAseq analysis.** Total RNA was extracted in three replicates from 0.5 mm segments of primary root tips of 7-day-old Col-0 and hsl2 seedlings using Trizol/chloroform extraction follow by purification using the RNeasy Plant Mini Kit (Qiagen). The quality of the RNA was ascertained using the Agilent 2100 Bioanalyzer.
RNAseq was performed at the Functional Genomics Center Zurich, Switzerland. DEseq2 with standard parameters was used to analyse expression levels (https://bioconductor.org/packages/3.7/bioc/vignettes/DESeq2inst/doc/DESeq2.html).

**Statistical methods** Student’s t-test (2-tailed) was used to analyse whether root cap phenotype and marker-gene expression in different lines deviated significantly from Col-0 cf. Fig. 2, 3 and 4, and Supplementary Fig. 1, and 6. If not otherwise stated in the figure legends ≥10 roots were used for each genotype, treatment and age. Two to five biological independent repeats were used. In all graphs, standard deviations are shown as bars.

Binomial test was applied to test for enrichment of maximal expression level for a specific tissue across all other tissues. The probability to find \( k \) maximal expressions out of \( N \) genes in total is given by

\[
P(X \geq k) = \sum_{i=k}^{N} \binom{N}{i} p^i (1-p)^{N-i}
\]

whereby \( p \) is the per gene probability to be maximal and \( \binom{N}{i} \) is the binomial coefficient.

Fisher’s exact test was used to calculated the likelihood of overlap of regulated genes by SMBox /smb brn1 brn2 (NAC TFs) and hsl2.
References


Bennett, T., den Toorn, A., Willemsen, V. & Scheres, B. Precise control of plant stem cell activity through parallel regulatory inputs. Develop.141, 4055-4064 (2014).


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Material requests

Constructs or seeds, as well as the raw data underlying the RNAseq data in Supplementary Table 2 are available from R.B.A. upon request, e-mail: reidunn.aalen@ibv.uio.no.

Contributions


Competing interests

The authors declare no competing interests.
**Figure legends:**

**Fig. 1 | IDL1 peptide interacts with and activates HSL2.** a, The tissues of the *Arabidopsis* root tip. LRC – lateral root cap. b, pIDL1:GUS after overnight incubation with X-gluc. c, Confocal medial optical section of 7 DAG PI-stained root tip expressing pHSL2::Venus-H2B. The QC is indicated by an ellipse. d, Alignment of mIDA and mIDL1 peptides. * - hydroxylated proline. e, mIDL1 activates HSL2 transiently expressed in *N. benthamiana* in a dose-dependent manner. Activation resulted in oxidative burst which was monitored as relative light units (RLU) using a luminol-based assay. EC50 - half-maximal efficient concentration. f, Unlabelled mIDL1 compete efficiently for binding of HSL2. The mIDA peptide with an N-terminal Valine residue labelled with acridinium (acri-V-IDA) bound to *N. benthamiana*-expressed HSL2, was treated with increasing concentrations of unlabelled mIDL1 or V-IDA. 100% binding – assay without competitors; negative control (background signal) – leaf tissue not expressing HSL2 incubated with 10 nM acri-V-IDA peptide and increasing concentrations of unlabelled V-IDA. IC50 - half-maximal inhibitory concentration.

**Fig. 2 | Live imaging details the sloughing process in Col-0, the effect of enhanced expression of IDL1 and dependency on HSL2.** a, The two-component constructs for inducible enhanced expression of IDL1 and GUS. The IDL1 promoter controls expression of XVE, which in the presence of estradiol (ES) binds the OlexA-TATA box. b, ES-induced GUS expression in Col-0 and *hsl2* mutant background after 1 hour (h) incubation with X-Gluc. Controls were grown without ES. c, Representative stages of the standard ~ 18h sloughing process of Col-0, captured by live imaging, i.e. initial fracture in LRC cell files, first above, after several h just below the QC (red and yellow arrows), and finally separation of a whole cap layer (white arrow). d, Overlapping sloughing events in EnhIDL1 root tips, i.e. a new
initiating gap (green arrow) occurring before completion of the ongoing event. e, Split root caps of EnhIDL1hsl2, i.e. gaps at the LRC-COL border (blue arrow) followed by detachment of a split cap (white arrows) still attached to the LRC covering the meristematic zone. f, Number of initiations and detachments per root per 24h. Bars indicate standard deviation. * - significantly different from Col-0 (Student’s t-test P< 0.05). Number of roots - Col-0 9, EnhIDL1 and EnhIDL1hsl2. g, Average time from initiation to detachment and from detachment to initiation of a new event. N – number of events per genotype. Standard deviations are given as bars. * - significantly different from Col-0 (Student’s t-test P< 0.04). h, Total number of sloughed caps and their phenotypes (cf. c-e) documented by live imaging over 64 to 70 hours.

**Fig. 3** | **Higher frequency of sloughing is compensated with more stem cell divisions.** a, Number of detached root cap layers 6, 8 and 10 days after germination (DAG). * - significantly different from other lines (grey), and without ES (blue) (Student’s t-test P< 0.005). N=10-12 roots per genotype per treatment and per time point. Standard deviations are given as bars. b, Root tips after 10 days on medium with 10 µM ES, and control without ES. c, Number of attached COL tiers at 8 and 10 DAG. d, Percentage of root caps with new 1st tier COL cells, and new LRC cell generated after division of the respective stem cells. * - significantly different from Col-0 (Student’s t-test P< 0.01). e, Medial optical sections of 7 DAG PI-stained stem cell niches of roots grown with ES, with schematic drawing below. Dots mark novel cell walls in EnhIDL1.

**Fig. 4** | **idl1 and hsl2 mutants influence sloughing frequency and LRC division patterns.** a, Number of sloughed and attached layers. * - significantly different from Col-0 (Student’s t-
test $P_{idl1} < 10^{-3}$, $P_{hsl2} < 10^{-6}$, $N = 10$). b, Stem cell niche of the indicated genotypes. Asterisks indicate LRC/Epidermis stem cells (pink), and LRC cells directly neighbouring the epidermal cell file (light and dark blue). c, Schematic presentation of cellular patterning shown in b. Arrows show the directions of cell divisions. d, Number of cells in the first and second LRC cell file neighbouring the epidermal cell file. * - significantly different from Col-0 (Student’s t-test $P < 10^{-5}$). $N_{Col-0} = 22$, $N_{hsl2} = 17$ and $N_{idl1} = 18$. Standard deviations are given as bars.

**Fig. 5 | IDL1-HSL2 regulates genes involved in cell wall degradation and programmed cell death.** Confocal images of PI stained 7 DAG root tips of the indicated genotypes. The white ellipses mark the QCs. a, Medial optical sections of roots expressing RCPG, lower panel only the channel showing the PI stain. Note many PI-stained nuclei in Col-0 (cf. also Supplementary Fig. 6). b, 3D reconstructions from z-stacks of root tips expressing the PCD marker BFN1pro:nGFP. Note reduced expression in the idl1 and hsl2 mutants, especially at Site II (see also Supplementary Fig. 6). c, Medial optical section of PI stained 7 DAG root expressing pHSL2::Venus-H2B, with enlargement (blue rectangle) of LRC cell file in the MZ. Arrow heads point to PI stained nuclei.

**Fig. 6 | IDL1-HSL2 signalling affects the dynamics of root cap sloughing.** IDL1-activated HSL2 regulates the dynamics of sloughing events, through regulation of PCD and cell wall remodelling genes, and triggering of COL and LRC stem cell divisions.
Table 1. Tissue specificity of HSL2 root tip regulated genes.

<table>
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</tr>
</tbody>
</table>

^\text{1}Makers used for sorting cells of a particular tissue prior to RNA isolation (Nawy et al. 2005)
^\text{2}Genes down regulated in the \text{hsl2} mutant relative to \text{Col-0}, cf. Supplementary Table 2.
^\text{3}Based on expression data in radial tissue types (Nawy et al. 2005). Genes were assigned to the tissue with the highest expression level across all tissues.