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.

45 The root cap provides protection for the root apical meristem (RAM) and senses
46 environmental conditions [1]. The RAM encompasses stem cells, surrounding the Quiescent
47 Centre (Fig. 1a), both for the tissues of the root proper, and for the root cap. Although both
48 forms of stem cells continuously produce new cells, the root cap has a defined size, due to
49 homeostatic balance between generation and loss of root cap cells [2]. Many species shed living,
50 single cells, so called border cells, but in *Brassicaceae*, including *Arabidopsis thaliana*, the
51 outermost layer of the root tip is released intact from the underlying cell layer [3]. The
52 *Arabidopsis* root cap distal to (below) the QC, consists of a core of 5-6 tiers of gravity-sensing
53 columella cells (COL) surrounded by layers of lateral root cap (LRC) cells (Fig. 1a). The LRC
54 extends to cover the meristematic zone (MZ), and there the outermost layer regularly
55 undergoes programmed cell death (PCD) whereby it is replaced by a new LRC layer
56 developing underneath the old one [4]. Less is known regarding the genetic and molecular
57 mechanisms governing the detachment of the outer layers of the distal root cap, how the
58 frequency of sloughing is regulated and how the homeostatic balance with stem cells is
59 secured [2].

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

67 Mutations in four genes encoding closely related NAC (NAM, ATAF, CUC)
68 transcription factors (TFs) – FEZ, SOMBRERO (SMB), BEARSKIN1 (BRN1) and BRN2 –
69 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 maturation[6-8], while the double mutant *brn1 brn2* is impaired in cell wall degradation and accumulates additional undetached root cap layers[8].

Downstream of these TFs, genes have been identified that encode enzymes involved cell wall remodelling and synthesis [8]. Similar genes have been discovered in other cell separation processes in flowering plants [9,10]. 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* mutant [16], 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

120 interaction with non-native receptors [20]. With the aim of testing whether IDL1-HSL2 was
121 specifically involved in root cap sloughing, we therefore used the promoter of *IDL1* itself to
122 generate Enhanced IDL1 (EnhIDL1) plants with estradiol (ES)-inducible enhanced expression
123 of both *IDL1* and *GUS* (Fig. 2a-b) [21]. EnhIDL1 plants were crossed to an *hsl2* knockout
124 mutant [16] to test the importance of HSL2 for IDL1 function. Cell-specific and efficient ES-
125 induction was confirmed by strongly enhanced GUS expression in EnhIDL1 roots both in
126 wild type Col-0 and *hsl2* mutant background as compared to the *pIDL1:GUS* expression
127 pattern and by qRT-PCR (Fig. 1b, 2b and Supplemental Fig. 1). These lines did not deviate in
128 root length (Supplementary Fig. 1).
129 Cell elongation, cell division and differentiation take place in the root tip during the
130 first days after germination. To ensure that homeostatic balance between gain and loss of root
131 cap layers had been established, our experiments were performed on roots with full-sized
132 RAM and root cap (Supplementary Fig. 2). Seven days after germination (DAG) roots of Col-
133 0, EnhIDL1 and EnhIDL1 *hsl2* seedlings grown vertically on agar with 10 µM ES, covered by
134 a cover slip, were tracked, using a laser scanning microscope with a vertical stage [22]. Images
135 taken in two experiments every 15 minutes for 64 and 70 hours (h) revealed a stepwise
136 sloughing process (Fig. 2c-e). The first signs of a forthcoming sloughing event were
137 characteristic fractures or gaps in LRC cell files in a region just above the Quiescent Centre
138 (QC) and about 5h later at the level of the youngest starch-filled COL tiers (Fig. 2c, red and
139 yellow arrows). These gaps initiated a gradual loosening of the outermost LRC layer towards
140 the tip. Finally, the COL cells at the centre of the cap separated from the layer underneath
141 followed by detachment of the whole layer as it slid to the side and was lost (Fig. 2c, white
142 arrow and Supplementary Video 1). On average, 0.6 root cap layers were shed per root per
143 day in Col-0 (Fig. 2f), with an average of ~18h for a completed sloughing process, and

144 thereafter another ~18h before a new event of root cap layer separation would be initiated (Fig.
145 2g and Supplementary Table 1).

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

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

157 Live imaging was supplemented with data for roots grown on vertical agar plates. In
158 this set-up, shed layers of the distal cap, recognizable by detached LRC files, did not slide
159 away immediately, but accumulated in front of the growing roots, most likely due to less
160 friction when the roots were not covered by a cover slip. Caps were, however, easily lost
161 when removed from the agar, and sloughed caps were therefore counted directly on the plates
162 at 6, 8 and 10 DAG, with and without 10 µM ES (Fig. 3a-b). Col-0, EnhIDL1 *hsl2*, and
163 EnhIDL1 on non-inducing medium sloughed about 0.6 caps per day (Fig. 3a-b). On ES
164 medium, on the other hand, EnhIDL1, accumulated up to eight sloughed root cap layers
165 (Supplementary Fig. 3) and differed significantly with an average loss of one root cap layer
166 per day (Fig. 3a). Thus, these results were in full accordance with the live imaging results.
167
168

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 [6]. 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* [16] 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

194 and the mutants, a subtle, but significant difference was observed regarding the number of
195 sloughed cap layers at 10 DAG with on average 3.0 caps for Col-0 and 2.4 for the mutants
196 (Fig. 4a). Inspection of the youngest LRC cell files furthermore suggested that the frequencies
197 of generation of new LRC cell layers had slowed down in both mutants. After the initial
198 periclinal division of a EPI/LRC stem cell, the new LRC cell file is extended by anticlinal
199 divisions. The new file will be neighbouring an older and longer LRC cell file on the lower
200 side and an epidermal cell file on the upper side until the next new LRC layer is initiated (Fig.
201 4b-d). In the wild type, one cell of the two youngest cell files neighboured the epidermal cell
202 file, in contrast to 2-3 cells in both the *hsl2* and *idl1* mutants (Fig. 4b-c). This is an expected
203 result if the periclinal divisions initiating a new LRC file take place less frequently relative to
204 the anticlinal divisions of already initiated LRC cell files (Fig. 4d).
205
206 **Mutation in *HSL2* affects genes in both the LRC and the COL cell layers**
207 To gain a better understanding of the function of the signalling initiated by activation of *HSL2,*
208 RNA sequencing was performed on RNA isolated from the RAM of root tips of Col-0 and
209 *hsl2*. In line with the subtle phenotype of *hsl2,* few genes were grossly affected by the
210 mutation. However, using a P adjusted (Padj) cut-off value of 0.1, 156 genes were identified
211 with an expression level < 70 % of the Col-0 level (Supplementary Table 2). In an attempt to
212 identify where the genes affected by HSL2 signalling were expressed, we took advantage of a
213 data set for tissue-specific gene expression in the Arabidopsis RAM [23]. This dataset provides
214 information on differential gene expression in the different root tissue, QC, LRC, COL, EPI,
215 cortex (COR) and endodermis (END), based on cell sorting using marker genes for these
216 tissues [23]. Eighty-one of the *hsl2* down-regulated genes were identified as differentially
217 expressed according to this data set. We defined a gene as characteristic for a specific tissue if
218 it had the highest expression level for this tissue compared all the other tissues. Evaluation

219 according to a binomial test (p=0.1, see Methods for details) found significant enrichment of
220 genes expressed in LRC and COL (Table 1). Genes encoding extracellular proteins were well
221 represented among the LRC expressed genes, while many genes most highly expressed in
222 COL encoded proteins involved in catalytic and hydrolytic activity including starch and cell
223 wall degradation.

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

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

242 The *ROOT CAP POLYGALACTURONASE* (*RCPG*), a direct target of BRN1 [8], was of
243 the most strongly downregulated genes both in the triple mutant *smb brn1 brn2* and *hsl2*

(Supplementary Table 2). In loosened Col-0 root cap layers the construct pRCPG:nYG, with nuclear targeted YFP driven by the *RCPG* promoter [8], was expressed in more cells and with higher intensity than in *hsl2* and *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 *RCGP* expression suggests that IDL1-HSL2, like the BRN TFs, are involved in the final step of the sloughing process – the actual cell separation.

IDL1-HSL2 regulates *BFN1* expression at PCD Site II

We noted that in contrast to *hsl2* and *idl1,* most Col-0 cells expressing *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 [4]. Interestingly, in the *hsl2* transcriptome five out of eight genes strongly associated with dPCD were down-regulated; the *BIFUNCTIONAL NUCLEASE1* (*BFN1*), *METACAPASE9* (*MC9*), *SAPOSIN-LIKE ASPARTYL PROTEASE3* (*PASPA3*), *EXITUS* (*EXI*) and *RIBONUCLEASE3* (*RNS3*) [4,26] (Supplementary Table 2). To investigate the potential involvement of IDL1-HSL2 in dPCD in the root cap, a transcriptional fusion construct with the *BFN1* promoter and a nuclear-targeted GFP (*$BFN1_{pro}$:nGFP*) [27] 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 $BFN1_{pro}$:nGFP expression in EnhIDL1 at these sites was similar to Col-0. In contrast, *idl1* and *hsl2* root tips had significantly fewer cells expressing the $BFN1_{pro}$: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 IDL1-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 [2]. 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 IDA-HSL2 in this process (Fig. 6). Under our conditions, 0.6 distal cap layers were shed per root per day in Col-0. Enhanced expression of *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 IDL1 as a peptide ligand that in nanomolar concentrations binds and activates the HSL2 receptor. Both IDL1-increased sloughing and the balancing increase in stem cell divisions were dependent on HSL2, i.e. IDA-HSL2 signalling controls the dynamics of sloughing at the root tip.

We had expected cell separation phenotypes of *idl1* and *hsl2* similar to the total deficiency in floral organ abscission seen in the *ida* mutant and the double mutant *hae hsl2* [16]. 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* [25,28]. In contrast, the *brn1 brn2* double mutant, and *active quiescent center1-1* (*aqc1-1*), are delayed in separation of root cap layers [8,29]. 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 [4]. 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 [10]. 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.

318 BRN1, BRN2 and SMB are implicated in root cap maturation and root cap detachment
319 [7,8]. pHSL2::Venus-H2B was found to have overlapping expression pattern with the BRN TFs
320 in the LRC, and a subset of genes regulated by these TFs was also found downstream in the
321 HSL2 signalling pathway. Differences in spatial expression patterns compared to Col-0 of
322 transcriptional and translational reporter construct crossed into our lines, indicated faster
323 maturation in EnhIDL1 and slower maturation in the mutants. Together this suggests HSL2 to
324 play a role in maturation and cell layer separation in addition to the role in initiation. $BFN1_{pro}$-
325 controlled maker-gene expression and PI-stained nuclei in LRC cells of root caps undergoing
326 sloughing, indicate that root cap sloughing involves both cell separation and cell death.
327 RNAseq data for *hsl2* combined with publicly available information on differential
328 gene expression in root tissues were used in an attempt to identify the cell types that primarily
329 respond to HSL2 activation. This analysis identified overrepresentation of genes preferably
330 expressed in LRC and COL cells. The major effect of receptor activation will occur in the
331 cells where the receptor is expressed, indicating that HSL2 is present and responsive for
332 activation both in LRC and COL cells. Additionally, this analysis suggests that activation of
333 HSL2 gives different outcomes in different tissues.
334 IDL1-HSL2 communication facilitates dynamic regulation of the homeostatic balance
335 between stem cell division and sloughing activity. Since the root cap is guiding root growth
336 by sensing and responding to external cues, like gravity, water potential or the presence of
337 obstacles in the soil [1,2], it is highly likely that the frequency of root cap shedding is triggered
338 by environmental factors, possibly by influencing the expression level of IDL1 and/or HSL2.
339 At present, it is not known whether or how external condition might feed into the circle of
340 generation and loss of root cap layers. Factors involved in transcriptional regulation of *IDL1*
341 and *HSL2*, peptide processing and export, and interacting partners have not been identified.
342 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 [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.

Methods

Accession numbers of the genes studied in this work: *IDL1 – At3g25655, HSL2 – At5g65710; HAE – At4g28490; BRN1 – At1g33280, BRN2 – At4g10350, BFN1 – AT1G11190 and RCPG– At1g65570.*

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

Gateway cloning and floral dipping was also used generate Enhanced IDL1 (EnhIDL1) plants; the *IDL1* coding region was inserted after the estradiol inducible promoter in the vector pMDC221, and a 1555 bp *IDL1* promoter fragment was cloned in front of the XVE transcription factor in the pLB12 vector [21]. Of six *pIDL1 pLB12* lines checked for GUS expression pattern (after induction) comparable to that of the published *pIDL1:GUS* [10,30], a single-locus line (checked on 25 mg/l Hygromycin (Hyg)), was crossed to a single-locus *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 [33]. 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 [34]. 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 ^{o}C for 72 h. Seedlings were grown at 22 ^{o}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 [21]. 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

391 difference in length was observed between genotypes or with ES in the medium
392 (Supplementary Fig. 1).

393 **Oxidative burst measurements and receptor-ligand binding assay.** *N. benthamiana* leaves
394 transiently expressing HSL2 constructs were used for oxidative burst measurements and
395 peptide binding assays as previously described [12]. EC50 and IC50 curves are representative
396 for N=3 independent experiments, each with three technical replicates.

397 **Microscopy. H**istochemical GUS assays were performed as previously described [16]. Confocal
398 laser scanning microscopy (CLSM) was carried out with Olympus FV1000 and ZEISS
399 LSM880 confocal microscopes at the Oslo NorMIC imaging node. Roots were stained with
400 10 μM propidium iodide (PI).
401 **For live imaging** seeds were surface sterilized by chlorine gas, sown on 1% agar AM-
402 medium (0.5 x MS, pH 5.8, 1% agar), stratified for 3 days at 4°C and cultivated in a growth
403 incubator at 22°C in a 16/8 h day/night cycle with 120–140 μmol/m²/s light.

404 Eight day-old plants were put on blocks (rectangular parallelepipeds) of 1.5% agar AM-
405 medium (0.5 x MS, pH 5.8) supplemented with 10μM 17-β-estradiol. Roots and shoots were
406 put on the blocks' basis and sides, respectively [22]. These samples were put into the chambered
407 cover-glass (Thermo Scientic Nunc, catalogue no: 15536), which were then mounted onto a
408 laser scanning microscope with a vertical stage (roots down, shoots up). Photographs of root
409 tips were taken every 15 minutes, in two experiments over 64 and 70 h, with in total 9 roots
410 for Col-0 and 10 roots for EnhIDL1 and EnhIDL1 *hsl2*.

411 **RNA extraction and RNAseq analysis.** Total RNA was extracted in three replicates from 0.5
412 mm segments of primary root tips of 7-day-old Col-0 and *hsl2* seedlings using
413 Trizol/chloroform extraction follow by purification using the RNeasy Plant Mini Kit
414 (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 $\binom{\cdot}{\cdot}$ 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*.

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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

C.-L.S., T.I., S.S., U.H., M.A.B, M.W., M.K.A. and V.O. generated *Arabidopsis* lines; J.F. and D.v.W. designed, D.v.W. and I.K. performed, and D.v.W., I.K. and R.B.A. analysed live imaging experiments; G.F., M.A., M.A.B designed and M.W. performed IDL1-HSL2 interaction studies; M.C. and R.B.A. designed and A.K., M.C. and R.B.A. analysed RNAseq data; the rest of the experiments were designed by C.-L.S. and R.B.A., performed by C.-L.S. together with U.H., M.W., V.O. and analyzed by R.B.A., C.-L.S., M.W. and U.H.; C.-L.S. drafted the manuscript; R.B.A. wrote the paper with input from other authors.

Competing interests

The authors declare no competing interests.

[12]

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 [8]. 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 EnhIDL1*hsl2*, 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 EnhIDL1*hsl2* [10]. **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-

588 test $P_{idl1} < 10^{-3}$, $P_{hsl2} < 10^{-6}$, N = 10). **b,** Stem cell niche of the indicated genotypes. Asterisks
589 indicate LRC/Epidermis stem cells (pink), and LRC cells directly neighbouring the epidermal
590 cell file (light and dark blue). **c,** Schematic presentation of cellular patterning shown in b.
591 Arrows show the directions of cell divisions. **d,** Number of cells in the first and second LRC
592 cell file neighbouring the epidermal cell file. * - significantly different from Col-0 (Student's
593 t-test P < 10-5). N_{Col-0}=22, N_{hsl2}=17 and N_{idl1} =18. Standard deviations are given as bars.

594

595 **Fig. 5 | IDL1-HSL2 regulates genes involved in cell wall degradation and programmed**
596 **cell death.** Confocal images of PI stained 7 DAG root tips of the indicated genotypes. The
597 white ellipses mark the QCs. **a,** Medial optical sections of roots expressing RCPG, lower
598 panel only the channel showing the PI stain. Note many PI-stained nuclei in Col-0 (cf. also
599 Supplementary Fig. 6). **b,** 3D reconstructions from z-stacks of root tips expressing the PCD
600 marker $BFN1_{pro}$:nGFP. Note reduced expression in the *idl1* and *hsl2* mutants, especially at
601 Site II (see also Supplementary Fig. 6)**. c**, Medial optical section of PI stained 7 DAG root
602 expressing pHSL2::Venus-H2B, with enlargement (blue rectangle) of LRC cell file in the MZ.
603 Arrow heads point to PI stained nuclei.

604

605 **Fig. 6 | IDL1-HSL2 signalling affects the dynamics of root cap sloughing.** IDL1-activated
606 HSL2 regulates the dynamics of sloughing events, through regulation of PCD and cell wall
607 remodelling genes, and triggering of COL and LRC stem cell divisions.

608

609

Table 1. Tissue specificity of HSL2 root tip regulated genes.

Tissue	Marker[1]	# genes[2] with highest expression[3]	P-values	FDR adjusted p-values
LRC	LRC	20	9.0 E-04	3.1E-02
COL	pet111	29	1.16E-06	8.14E-06
QC	AGL42	8	0.908	0.999
Epidermis (hair cells)	COBL9	12	0.494	0.999
Epidermis (non-hair cells)	gl2	1	0.999	0.999
Cortex	CORTEX	10	0.737	0.999
Endodermis	scr5	1	0.999	0.999

[1]Makers used for sorting cells of a particular tissue prior to RNA isolation (Nawy et al. 2005)

[2]Genes down regulated in the *hsl2* mutant relative to Col-0, cf. Supplementary Table 2.

[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.