Floral organ abscission peptide IDA and its HAE/HSL2 receptors control cell separation during lateral root emergence

Robert P. Kumpf[a], Chun-Lin Shi[a], Antoine Larrieu[b], Ida Myhrer Stø[a], Melinka A. Butenko[a], Benjamin Péret[b,1], Even Sannes Riiser[a,2], Malcolm J. Bennett[b], and Reidunn B. Aalen[a,3]

[a]Department of Biosciences, University of Oslo, Blindern, 0316 Oslo, Norway; and [b]Centre for Plant Integrative Biology, University of Nottingham, Nottingham LE12 5RD, United Kingdom

Edited by Philip N. Benfey, Duke University, Durham, NC, and approved February 11, 2013 (received for review June 25, 2012)

Throughout their life cycle, plants produce new organs, such as leaves, flowers, and lateral roots. Organs that have served their purpose may be shed after breakdown of primary cell walls between adjacent cell files at the site of detachment. In *Arabidopsis*, floral organs abscise after pollination, and this cell separation event is controlled by the peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), which signals through the leucine-rich repeat receptor-like kinases HAESA (HAE) and HAESA-LIKE2 (HSL2). Emergence of new lateral root primordia, initiated deep inside the root under the influence of auxin, is similarly dependent on cell wall dissolution between cells in the overlaying endodermal, cortical, and epidermal tissues. Here we show that this process requires *IDA*, *HAE*, and *HSL2*. Mutation in these genes constrains the passage of the growing lateral root primordia through the overlaying layers, resulting in altered shapes of the lateral root primordia and of the overlaying cells. The HAE and HSL2 receptors are redundant in function during floral organ abscission, but during lateral root emergence they are differentially involved in regulating cell wall remodeling genes. In the root, *IDA* is strongly auxin-inducible and dependent on key regulators of lateral root emergence—the auxin influx carrier LIKE AUX1-3 and AUXIN RESPONSE FACTOR7. The expression levels of the receptor genes are only transiently induced by auxin, suggesting they are limiting factors for cell separation. We conclude that elements of the same cell separation signaling module have been adapted to function in different developmental programs.

root development | peptide signaling | pectin degradation | polygalacturonases | propidium iodide

More than 200 genes encoding leucine-rich repeat receptor-like kinases (1) and more than 1,000 genes encoding putative secreted peptides have been identified in *Arabidopsis thaliana* (2), suggesting that peptide ligand–receptor interactions are important for cell-to-cell communication in plants. However, fewer than a dozen signaling modules, including INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)-HAESA (HAE)/HAESA-LIKE2 (HSL2) controlling the separation step of floral organ abscission, have been identified by genetic and/or biochemical methods (3). Both the *ida* mutant and the *hae hsl2* double mutant retain their floral organs indefinitely owing to lack of breakdown of the middle lamella between cell layers of the abscission zone (AZ) at the base of organs to be shed (4–6). *IDA* is expressed in the AZ region of the flowers (4), whereas both *HAE* and *HSL2* expression is confined specifically to the specialized AZ cells (5, 6). Overexpression of *IDA* leads to premature and ectopic abscission, but not in *hae hsl2* mutant background, consistent with IDA being the ligand of these receptors (5, 6).

The abscission process involves initial cell wall loosening by enzymes like xyloglucan endotransglucosylase/hydrolases (XTHs) and expansins (EXPs) (7, 8). The loosening facilitates the access of cell wall degradation enzymes like polygalacturonases (PGs) that hydrolyze pectins, which are major components of plant cell walls (7, 8). Mutational analyses indicate that PGs are of particular importance in cell separation events (8). Microarray data suggest that the IDA-HAE/HSL2 signaling module is involved in the regulation of cell wall remodeling (*CWR*) genes (7). However, *XTHs*, *EXPs*, and *PGs* are expressed not only in the floral AZs but also at other sites of cell wall remodeling and breakdown, including the dehiscence zone (DZ) of the siliques, which undergo cell separation to allow seed shedding, and the cells overlaying emerging lateral roots (7–10). *IDA* and *HAE* are also found expressed in DZs of mature siliques (11). We therefore hypothesized that IDA-HAE/HSL2 signaling might control other cell-separation processes than just floral organ abscission.

Here we demonstrate that the IDA-HAE/HSL2 signaling module is important for lateral root emergence (LRE), a process mainly regulated by auxin accumulating in the central cells of early-stage lateral root primordia (LRP) and later at the tip of the LRP (12). We show that *IDA-HAE/HSL2* is part of the genetic network regulated by auxin, where *IDA* and *HAE* control expression of *PGs* and pectin degradation in the overlaying endodermal (EN), cortical (CO), and epidermal (EP) cells. In single mutants as well as the *hae hsl2* double mutant, morphogenesis of the LRP is altered owing to constraints imposed by the overlaying layers. Hence, the IDA-HAE/HSL2 signaling module has been adapted to function in different root and shoot cell separation processes.

Results

***IDA*, *HAE*, and *HSL2* Are Required for Normal Progression of LRP Through the Overlaying Layers.** The involvement of the IDA peptide and the HAE and HSL2 receptor-like kinases during LRE was initially indicated by significant lower densities of emerged LRs for the *ida* mutant [*ida-2* in Col-0 background (5)] and the *hae hsl2* double mutant compared with WT (ecotype Col-0) (Fig. 1*A* and Fig. S1*A*). The single mutants of the receptor genes show no abscission defect (5, 6), but interestingly both showed a lateral root (LR) phenotype with significantly reduced LR density (Fig. S1 *A* and *B*), suggesting that they influence LR development in different ways.

Author contributions: M.A.B., B.P., M.J.B., and R.B.A. designed research; R.P.K., C.-L.S., A.L., I.M.S., E.S.R., and R.B.A. performed research; R.P.K. contributed new reagents/analytic tools; R.P.K., C.-L.S., A.L., I.M.S., M.J.B., and R.B.A. analyzed data; and R.B.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]Present address: Laboratoire de Biologie du Développement des Plantes, Service de Biologie Végétale et de Microbiologie Environnementales, Institut de Biologie Environnementale et Biotechnologie, Commissariat à l'Energie Atomique et aux Energies Alternatives de Cadarache, 13108 Saint Paul lez Durance, France.

[2]Present address: Bioforsk, 1432 Ås, Norway.

[3]To whom correspondence should be addressed. E-mail: reidunn.aalen@ibv.uio.no.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1210835110/-/DCSupplemental.

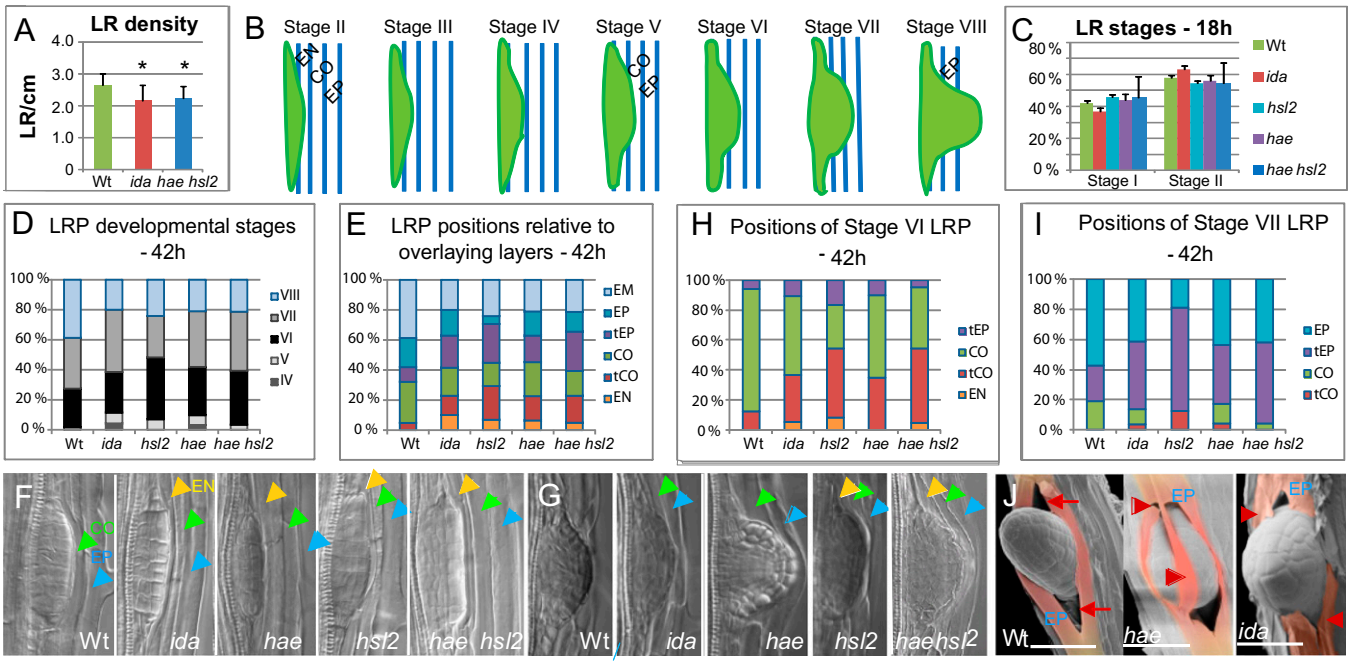

Fig. 1. Mutations in *IDA*, *HAE*, and *HSL2* delay LR emergence. (*A*) LR density (number of LRs per cm root) for *ida* ($n = 21$), *hae hsl2* ($n = 22$), and WT plants ($n = 20$) 12 d after stratification. *Significant deviation from WT based on Student *t* test. (*B*) Schematic presentation of the stages of LR development. (*C*) Percentage of LRP at stages I and II for *ida* ($n = 68$), *hae* ($n = 68$), *hsl2* ($n = 81$), *hae hsl2* ($n = 81$), and WT plants ($n = 69$), 18 h after LR induction. (*D*) Percentage of LRP at stages IV to VIII (emerged) for *ida* ($n = 68$), *hae* ($n = 59$), *hsl2* ($n = 58$), *hae hsl2* ($n = 56$), and WT plants ($n = 62$), 42 h after LR induction. (*E*) Percentage of LRP with tips positioned in the overlaying EN, CO, and EP tissues or touching the CO or EP layer (tCO and tEP, respectively) for WT and mutants, as indicated. EM, emerged LRs. Numbers as in *D*. (*F* and *G*) WT and mutant LRP, as indicated, at stage V (*F*) and stage VI to VII (*G*). Overlaying EN, CO, and EP cells are indicated in orange, green, and blue arrowheads, respectively. (*H*) Percentage of stage VI LRP with tips positioned in the overlaying EN, CO, and EP tissues or touching the CO or EP layer (tCO and tEP, respectively) for WT and mutants, as indicated. (*I*) Percentage of stage VII LRP with tips positioned in the overlaying CO and EP tissues or touching the CO or EP layer (tCO and tEP, respectively) for WT and mutants, as indicated. (*J*) Cryo SEM images of emerged WT, *hae*, and *ida* LRs. The EP cells on each side of the LR have been colored. Arrows, symmetrical openings between EP cells in WT. Arrowheads, rifts in flattened and ruptured EP cells of mutant. (Scale bar: 50 μM.)

To monitor whether reduced LR density reflected differences in rates of LRP initiation and/or emergence, we used an assay exploiting the ability of a root gravitropic response to induce LRP organogenesis (13–17). Before emergence (stage VIII), LRP go through seven developmental stages, defined by the number of cell layers, and have to pass through overlaying layers of EN, CO, and EP cells (Fig. 1*B*) (18). Eighteen hours after gravitropic induction of LR development approximately 40% of LRP were at stage I and the remaining 60% at stage II in both WT and mutants (Fig. 1*C*), indicating that LR initiation was not affected. However, after 42 hours, 40% of the WT but only 20–24% of mutant LRs had emerged, with the highest percentage in *hsl2* (Fig. 1*D*). More stage IV and V LRP were present in mutant lines; stage VII was in particular overrepresented for *ida* and *hae*, stage VI for *hsl2*, and stages VI and VII for *hae hsl2*, compared with WT (Fig. 1*D*). For all of the mutants the distribution of stages deviated significantly from a distribution expected if the mutants equaled WT distribution ($P < 0.03$). As anticipated if IDA and the two receptors were functioning in the same pathway, the *ida* and *hae hsl2* distributions of LRP stages were not significantly different ($P = 0.2$), whereas the *hsl2* distribution deviated significantly from that of *ida* ($P = 0.04$).

The size and number of cells of LRP at a given stage varies in the WT, but the number of cell layers and the shape of the LRP are characteristic for each stage [reported in companion article by Lucas et al. (19)]. The delayed emergence in the mutants suggested that defects in the IDA-HAE/HSL2 module delayed the passage of LRP through EN, CO, and/or EP layers. This could be due to restricted growth of the LRP and/or to constraints imposed by the overlaying tissues. Therefore, we registered the position of each LRP tip relative to the overlaying layers, a relationship that has been described as very consistent for WT LRP (Fig. 1*B*) (18, 20). The distribution of positions of the mutant LRP deviated highly significantly from WT distribution ($P < 10^{-10}$) (Fig. 1*E*). The passage of the LRP from EN to CO involves the breaking of the Casparian strip, a lignified network forming an impermeable barrier between the EN and the outer layers (21, 22), and WT LRP stage IV–V changes from "flat-topped" to "dome-shaped" (compare LRP in Fig. 1 *F* vs. *G*) (19). In the mutants more and older-staged LRP seemed to be trapped in the EN layer, displaying an extraordinary flattened shape (Fig. 1 *E* and *F*). In accordance with previous observations (18), WT stage VI primordia were predominantly (>80%) positioned in the CO, whereas in the mutants a substantial fraction of stage VI LRP were only touching the CO (tCO) and had not yet penetrated the CO layer (Fig. 1*H*). At stage VII the majority of the WT LRP had, as expected, entered the EP layer, whereas most mutant stage VII LRP were only touching this layer (tEP) (Fig. 1*I*), often pushing intact overlaying cell files outwards (Fig. 1*G*). Additionally, mutant LRP with the size and shape of just-emerged WT LRs were observed covered with one or two cell layers of primary root tissues (Fig. S2*A*).

As for the distribution of stages, the *ida* and *hae hsl2* LRP distribution of positions were statistically equivalent. The distribution of positions of *hsl2* stage VI and VII LRP deviated most from the WT and the other mutants (Fig. 1 *H* and *I*). However, the distribution of stages and positions in the *hae hsl2* double mutant was compatible with an additive effect of the two receptor single mutants: the double mutant displayed a percentage of emerged roots similar to that in *hae* and lower than that in *hsl2*

(Fig. 1*D*), and the percentage of stage VI LRP not yet positioned in the CO layer was similar to that in *hsl2* and higher than that for *hae* (Fig. 1*H*).

We concluded that the three genes encoding the IDA-HAE/HSL2 signaling module were required for normal progression of LRP through the overlaying layers. Cryo scanning electron microscopy (SEM) allowed a more detailed inspection of emerged LRP. In the WT, EP cells on each side of emerged LRs were nicely rounded and curved like a pair of lips, with triangular equal sized openings between the separated cells above and below the emerged WT LR (Fig. 1*J*), as previously shown with environmental SEM (23). In contrast, in 9 of 10 *ida* and *hae* mutant roots, these openings, if present, were of unequal size, and the cells surrounding emerged roots appeared as flattened and torn sheets clinging to the LR itself (Fig. 1*J* and Fig. S2*B*). In the most affected cases no normal opening was present at all (Fig. 1*J* and Fig. S2*B*), indicating LRP penetration of cells impeded in cell separation.

***IDA*, *HAE*, and *HSL2* Belong to an Auxin-Dependent Network of Genes Expressed in Cells That Separate During LRE.** The expression patterns of *IDA*, *HAE*, and *HSL2* during LRE were investigated using promoter–reporter gene constructs. In the root, other members of the *IDA* gene family are mainly expressed in the vasculature (Fig. S3*A*). In contrast, expression of the *IDA* promoter β-glucuronidase (GUS) reporter construct *pIDA:GUS* (4) was detected in cells overlaying new LRP (Fig. 2*A* and Fig. S3*B*), initially in single EN cells overlaying stage I–IV LRP. Subsequently, *pIDA:GUS* expression was observed in the CO layer before the entrance of the LRP. Likewise, *pIDA:GUS* was active in the EP from stages IV to V before the LRP had entered this layer. Moreover, the GUS signal persisted in these cells after LRE (stage VIII) (Fig. 2*A*).

Plants transformed with a promoter:YFP construct for *HSL2* (*pHSL2:YFP*) revealed expression in the EN, CO, and EP from stage II to III, and a weak expression in the LRP (Fig. 2*B* and Fig. S4*A*). The expression in the LRP increased from stage IV to stage VI and further in emerged LR (Fig. S4 *B*–*D*). Interestingly, at the same time expression was reduced in separated CO and EP cells surrounding the emerging LRP (Fig. S4 *C* and *D*). After emergence, prominent expression was seen in the tip of the LR, similar to the expression pattern of *pHSL2:YFP* in the primary root tip (Fig. S4 *E* and *F*). Thus, the late-stage LRP expression could be related to a subsequent function in the tip of the emerged root.

HAE expression was investigated using a functional translational YFP fusion construct under the control of the *HAE* promoter (*pHAE:HAE-YFP*). In agreement with the hypothesized involvement of HAE in IDA-dependent cell separation, HAE-YFP was detected in cells overlaying LRP from stage II with stronger expression in the epidermis at later stages (Fig. 2 *C* and *D*). From stage VI an intensified HAE-YFP signal was found at the base of the LR (Fig. 2*D*).

LRP outgrowth and cell separation in overlaying tissues are coordinated by auxin derived from the tip of the primordium reprogramming EN, CO, and EP cells. In the overlaying CO and EP cells, the auxin influx carrier LIKE AUX1-3 (LAX3) is induced, resulting in preferential auxin accumulation (10). Auxin-induced degradation of the SOLITARY ROOT1 (SLR1)/IAA14 repressor releases the AUXIN RESPONSE FACTOR (ARF) 7 and ARF19 transcription factors, leading to the induction of *CWR* gene expression (10, 24). We have investigated the relation between *IDA* and this auxin-controlled gene regulatory network. *pIDA:GUS* expression was first observed at stage IV in *lax3* mutant background and was clearly delayed compared with WT (Fig. S3*B*). Thus, *IDA* expression in the root seems dependent on auxin. Consistent with this, the *pIDA:GUS* transgene was activated in the root by exogenous indole-3-acetic acid (IAA) or naphthaleneacetic acid (NAA) (1 μM) (Fig. S3*C*), and real-time quantitative RT-PCR (RT-qPCR) demonstrated a more than 100-fold increase in *IDA* expression level from 6 to 18 h after addition of IAA (Fig. 2*E*). In the *arf19* background the induction level was significantly reduced, whereas in the *arf7* mutant normal *IDA* induction was blocked. *HAE* and *HSL2* induction by IAA was only transient and weak, especially for *HSL2*; however, the expression peaks after 3 h were strongly dependent on *ARF7* and also significantly reduced in *arf19* background (Fig. 2 *F* and *G*). We conclude that the expression patterns of *IDA*, *HAE*, and *HSL2* and the strong ARF7-dependent *IDA* induction by auxin are consistent with a role for *IDA* signaling through these receptors in the process of LRE.

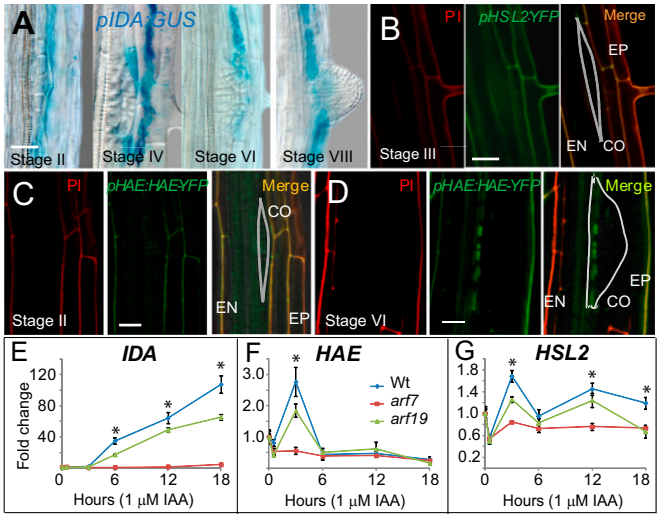

Fig. 2. *IDA*, *HAE*, and *HSL2* are expressed in cells overlaying new LRP and induced by auxin. (*A*) *pIDA:GUS* expression shown for the stages indicated. (*B*) Confocal images of *pHSL2:YFP* expression at stage III. (*Left*) PI-stained root; (*Center*) YFP signal; (*Right*) merge. The outline of the LRP is shown. Note that PI does not penetrate the EN or the LRP and therefore does not stain the inner EN wall and LR cell walls. (*C* and *D*) Confocal images of *pHAE:HAE-YFP* expression in *hae hsl2* mutant (stage II) and WT background (stage IV), respectively. Note that the *pHAE:HAE-YFP* construct rescued the *hae hsl2* mutants phenotypes (Fig. S5 *A* and *B*). Annotation as in *B*. (*E*–*G*) Relative expression levels of *IDA*, *HAE*, and *HSL2* in roots in the presence of 1 μM IAA in WT, *arf7*, and *arf19* background determined by RT-qPCR. The WT expression level before IAA addition was set to 1. SDs are shown. *Significant differences between WT and both mutants ($P < 0.05$).

***IDA* and *HAE* Are Involved in Control of Pectin Degradation.** The pectin-rich middle lamella at the interphase of adjacent plant cells must be broken down for cell separation to occur. We developed an assay for pectin breakdown based on propidium iodide (PI), commonly used to stain root cell walls. PI binds the negatively charged carboxyl and hydroxyl groups on homogalacturonans, which constitute a major component of pectic polysaccharides in the middle lamella, and it was recently demonstrated that cell wall degradation by pectinase reduces PI fluorescence (25). In the WT, reduced PI staining of cell walls between EN and CO cells directly overlaying LRP could be observed already at stage I (Fig. 3 *A* and *B*), consistent with an expected pectin dissolution during cell separation (7). Interestingly, diminished PI fluorescence was also seen in *hsl2* roots (Fig. 3*C*), indicating that the pectin degradation occurred as in the WT. In contrast, the PI intensity of the EN/CO cell wall in front of the primordia in the *ida*, *hae*, and *hae hsl2* mutants was equal to the intensity at positions above and below the LRP (Fig. 3 *D*–*G*), suggesting a defect in pectin degradation in these mutants. Three-dimensional images indicated that the pectin component of the middle lamella between the EN and CO layers overlaying the *hae*, *ida*, and *hae hsl2* LRP was intact (Fig. 3*E* and Fig. S5 *A* and *C*). The *pHAE:HAE-YFP* construct rescued this *hae*

PLANT BIOLOGY

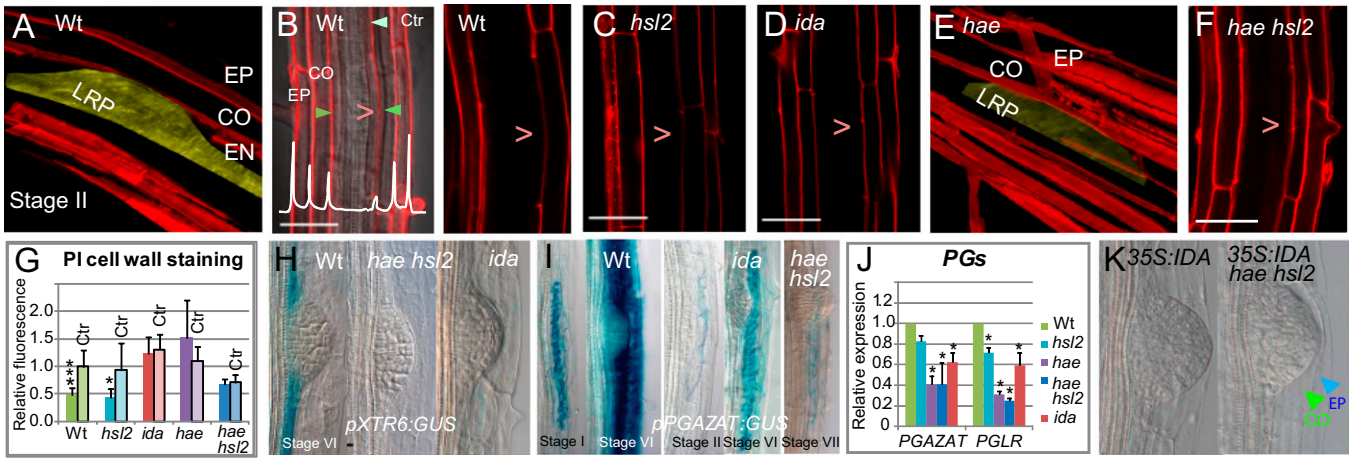

Fig. 3. *IDA*, *HAE*, and *HSL2* influence cell wall remodeling and degradation. (*A*) 3D Z-stack confocal image of PI-stained WT root at the site of a stage I WT LR. The LRP has been colored yellow. (*B*) 2D confocal image showing scan (white graph) of the fluorescence from PI-stained EN, CO, and EP cell walls at the site of WT LRP (green arrowheads). Control scans were taken below and above the LRP (light green arrowhead). >, position of LRP. (*C–F*) Confocal images of PI-stained roots at the site of a stage I LRs for *hsl2* (2D), *ida* (2D), and *hae* (3D, yellow colored LRP) and *hae hsl2* (2D). (*G*) PI fluorescence of LRP-overlaying cell walls between EN and CO relative to the reference point at the opposite side based on scans for six to nine roots. Bars indicate SD. Asterisks denote significant differences from fluorescence at control positions relative to the same reference point (Ctr) (*$P < 0.05$; ***$P < 0.001$, Student *t* test). (*H* and *I*) Expression of *pXTR6:GUS* and *pPGAZAT:GUS* and in WT, *ida*, and *hae hsl2* background at the LR stages indicated. (*J*) Expression levels of *PGAZAT* and *PGLR* in mutant 7-d-old roots relative to WT determined by RT-qPCR. Bars indicate SD. *Significant deviation from WT ($P < 0.05$). (*K*) Stage VII LRP of *35S:IDA* and *35S:IDA hae hsl2*, as indicated. Arrowheads, cell walls of overlaying CO and EP cells. (Scale bars: 30 μM in 3D images, 20 μM in 2D images.)

hsl2 phenotype as well as the abscission defect (Fig. S5 *A* and *B*). Likewise, introduction of the WT *IDA* gene into *ida* background (4) reduced PI staining to WT levels (Fig. S5*C*). Taken together, the results of the PI assay suggest that *IDA* regulates LR emergence by signaling through the *HAE* receptor to control breakdown of cell wall components such as pectin.

***IDA*, *HAE*, and *HSL2* Regulate Expression of *CWR* Genes.** Because the mutant phenotypes indicated deficiencies in cell wall degradation, we investigated the expression levels of representative members of the *XTH*, *EXP*, and *PG* gene families encoding CWR enzymes expressed in cell layers overlaying LRP [i.e., *XTH 23*/*XYLOGLUCAN ENDOTRANSGLYCOSYLASE6* (*XTR6*), *EXP17*, *PG LATERAL ROOT* (*PGLR*), and *PG ABSCISSION ZONE ARABIDOPSIS THALIANA* (*PGAZAT*)] (9, 10, 24, 26). The latter, also called *ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE2* (*ADPG2*), is regulated by *IDA* in the AZ and implicated in cell separation during dehiscence and floral organ abscission (8, 26).

The involvement of IDA, HAE, and HSL2 in control of expression of these *CWR* genes was demonstrated by reduced transcript levels and delayed expression of promoter:GUS reporter constructs in *ida* and *hae hsl2* mutant background (Fig. 3 *H–J* and Fig. S6 *A–C*). In the WT, *pXTR6:GUS* expression was detected in LRP-overlaying tissues (10) and additionally in EN cells neighboring primordia and later at the base of LRP (Fig. 3*G* and Fig. S6*B*), where both *HAE* and *HSL2* are expressed (Fig. 2 *B–D*). RT-qPCR, used to investigate the role of each of the receptor genes, revealed that the receptors were functionally redundant in regulating *XTR6* and also *EXP17*, because substantial reduction in transcript levels was seen in the *hae hsl2* double mutant but not single mutants (Fig. S6*A*). In contrast, the *PGAZAT* and *PGLR* levels were very similar in *hae* and *hae hsl2* background (Fig. 3*J*), indicating that the expression levels of these two genes are influenced mainly by IDA signaling through the HAE receptor. Interestingly, *pXTR6:GUS*, *pPGLR:GUS*, and *pPGAZAT:GUS* expression was controlled by IDA-HAE/HSL2 during floral organ abscission (Fig. S6 *B–D*) (26).

Plant lines overexpressing IDA using the 35SCaMV promoter (*35S:IDA*) in WT and *hae hsl2* background have been used to demonstrate that IDA signals through HAE and HSL2 in aboveground organs (6). These lines were used to substantiate that this is also the case in the root. *35S:IDA* produce few seeds (6) with low germination rate, developmentally arrested seedlings, and restricted root growth and development, including very low production of LRs (Fig. S7*A*). Deficiencies in seed germination and root development were rescued in *35S:IDA hae hsl2* plants (Fig. S7*B*), which displayed the characteristic *hae hsl2* LR phenotype with delayed penetration of the overlaying layers. This phenotype was not seen in the few lateral roots produced by *35S:IDA* plants (Fig. 3*I*).

Discussion

In this work we have demonstrated that the IDA-HAE/HSL2 signaling module is required to facilitate the passage of LRP through overlaying root tissues. Lateral roots in *Arabidopsis* originate exclusively from pericycle cells located deep within the primary root tissues (18). As cells within new LRP divide, they push against overlaying EN, CO, and EP tissues (summarized in Fig. 4). Auxin originating from the tips of new LRP is likely to promote LR emergence by generating different chemical and mechanical signals (19) and reprogram overlaying cells to separate by controlling the expression of aquaporin water channels (16) and cell wall remodeling enzymes (10). We report that the peptide signal IDA and its receptor-like kinases HAE and HSL2 are important components in this auxin-regulated pathway by controlling cell separation. The reduced expression of PG genes, consistent with the persistently high fluorescence level in the PI assay in the *ida*, *hae*, and *hae hsl2* mutants, argues for direct involvement of IDA and HAE in control of pectin degradation in walls of LRP overlaying cells. Moreover, where the receptors have overlapping expression patterns, they may both relay the IDA signal and be functionally redundant in controlling other *CWR* genes.

We propose a model for auxin-IDA-HAE/HSL2 regulated LRP emergence based on the spatiotemporal distribution of these signaling components and their downstream targets (Fig. 4): at stages I and II, auxin derived from the LRP induces *IDA* expression in overlaying EN cells. Next, IDA signals through HAE and HSL2 receptors already present in the membrane of these cells, resulting in up-regulation of *CWR* genes like *XTR6* and *EXP17*. Subsequently,

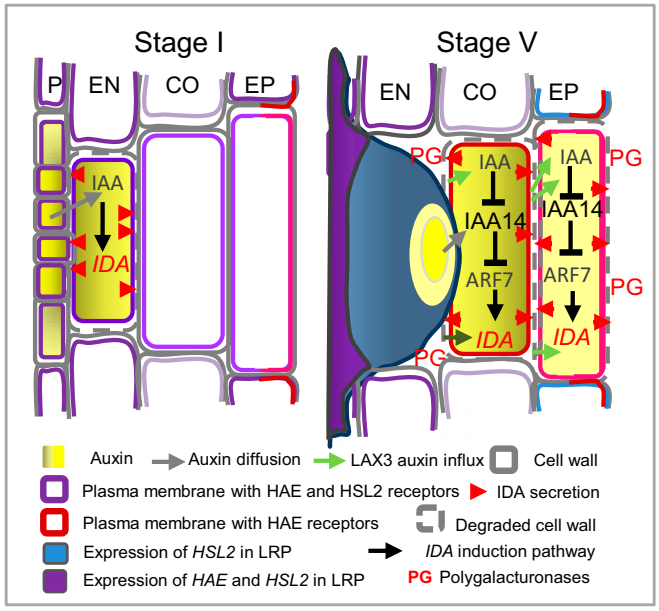

Fig. 4. Model of IDA-HAE/HSL2 signaling in LR emergence. *IDA* expression and signaling to open a gateway for the LRP is hypothesized to occur in a wave-like fashion in the cells directly overlaying the developing LRPs. *IDA* is suggested induced in the EN by auxin diffusing from the young LRP and secreted to interact with HAE and HSL2 receptors already present, to trigger expression of *CWR* genes, leading to loosening and breakdown of the cell wall of the EN cells directly overlaying LRPs. Expression of *IDA* in the CO and EP overlaying the LRP is dependent on auxin influx via LAX3 and the auxin-dependent cascade leading to activation of ARF7, which also transiently induces expression of the receptor genes. *HAE* and *HSL2* function redundantly to up-regulate some *CWR* genes like *XTR6,* for instance at the base of the LRP, whereas IDA signaling, mainly through the HAE receptor, in overlaying tissues will induce downstream genes encoding cell wall degradation enzymes like PGs, leading to cell separation.

LAX3-mediated auxin influx into overlaying CO and thereafter EP cells induces *IDA* expression and transiently increases the expression of the receptors in an *ARF7*-dependent manner. In the cells directly overlaying the LRP, IDA signaling through HAE triggers the expression of CWR enzymes like PGAZAT and PGLR that dissolve cell walls and open a gateway for the developing LR. Thus, the concerted but differential regulation of *IDA*, *HAE*, and *HSL2* in the auxin-governed network of regulatory and enzymatic factors facilitates fine-tuned control of cell wall remodeling and cell wall degradation during LRE.

IDA, *HAE*, and *HSL2* have previously been reported to regulate another cell separation process in *Arabidopsis*, floral organ abscission (4). Our analyses of expression patterns, mutant phenotypes, and genetic relationships allow a comparison of the role of IDA, HSL2, and HAE during both cell separation processes. In both cases, peptide signaling cross-talks with classic hormone signals. Interestingly, auxin has a promoting effect on *IDA* expression and LRE, but exogenous auxin commonly delays organ shedding and counteracts the abscission-promoting effect of ethylene (27, 28). *AUXIN RESPONSE FACTORS*, including *ARF7* and *ARF19*, are involved in the timing of abscission (29), but it is not known whether auxin is involved in the regulation of *IDA* or its receptors in the floral organ AZs. This indicates organ-specific regulation of key components common to different cell separation processes.

Exogenous auxin induced *IDA* expression 100-fold in root tissues, but *HAE* and *HSL2* mRNAs were only up-regulated transiently, suggesting that these receptors, and not the peptide, are the limiting factors controlling cell separation. This may explain why a general separation of cells is not observed at the used auxin concentration. With substantially higher concentrations, abnormal sloughing of root cells has been observed (30, 31). The HAE and HSL2 receptors are also limiting factors for above-ground organ shedding. Overexpression of IDA does not lead to general cell separation, only ectopic organ abscission at sites with vestigial AZ where *HAE* and *HSL2* are found expressed (5, 6, 32, 33). Here we report that overexpression of IDA affects root growth, that this phenotype is dependent on functional HAE and HSL2 receptors, and that overexpression of IDA cannot override the delay in LRE of *hae hsl2*.

In floral organ AZs, *HAE* and *HSL2* exhibit identical expression patterns overlapping with that of *IDA*, and single mutants display no aberrant phenotypes (5, 6). In the root, the patterns are more complex, because both single mutants show a phenotype (i.e., reduced LR density and delayed emergence). Differential expression patterns and mutant stage and position profiles indicate involvement of HAE and HSL2 in different aspects of the emergence process. Strong expression of *pHSL2:YFP* from stage VI and the particular accumulation of stage VI *hsl2* LRP may indicate that *HSL2* directly influences LRP growth. We cannot exclude that *IDL* genes expressed in the LRP, which in part can substitute for IDA function (6), may signal through HSL2.

The modest but significant difference in the distribution of LRP stages between WT and *ida* and the receptor mutants could also suggest that the IDA-HAE/HSL2 signaling module directly affects the development of the LRP. However, both receptors are expressed in the overlaying layers where *IDA* is expressed. Furthermore, the most significant difference between the WT and the mutants was the delayed passage through these layers, evident from the highly significant difference in distribution of LRP positions. This suggests that the primary effect of the mutations is a defect in the passaging of the LRP through these layers. The simplest explanation for this would be that the IDA peptide, expressed in the EN, CO, and EP, signals through the receptors present in the very same layers to induce *CWR* genes executing cell separation, consistent with the documented function of IDA-HAE/HSL2 in cell separation in the floral organ AZ (4–6). The delay in LRP development could be an effect of a feedback to the primordia, similar to mutants with increased or changed deposition of material to the Casparian strip of the endodermis that are delayed at different stages of the LRE process (19).

Materials and Methods

Plant Material and Constructs. The *arf7*, *arf19*, *lax3*, *ida-1*, *ida-2* (SALK_133209), *hae*, *hsl2*, and *hae hsl2* mutants, *pIDA:GUS*, *pXTR6:GUS*, *pPGLR:GUS*, and *pPGAZAT:GUS* lines, and the *35S:IDA* line have been described in refs. 6, 9, 10, and 34). Plants were cultivated in growth chambers at 22 °C for 8 h of dark and 16 h of light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$). For root experiments sterilized seeds were plated on square plates with 0.5× MS medium (35), stratified 2–3 d in the dark at 4 °C, and thereafter placed vertically under the above conditions. For auxin treatment, seedlings were transferred 5 d after germination onto plates containing 1 μM auxin. Emerged LRs were counted 9 or 12 d after stratification. For staging of LR development from initiation to emergence, seedlings were grown for 10 d on vertical plates and then rotated 90° to induce LR formation (13–15). The χ^2 test was used to test whether the distribution of stages and positions were statistically different between mutants and WT and among the mutants, using the WT, *ida*, or *hae hsl2* distribution as alternative null hypotheses.

A single-locus *pIDA:GUS* line in ecotype C24 (11) was introgressed into Col-0 WT and the *lax3* mutant in Col-0 background. The construct *pHSL2:YFP* with 2,300 bp upstream of the start codon in vector *pHGY* (RIKEN Plant Science Center), *pHAE:HAE-YFP* with 1,601 bp upstream region in vector *pMDC111*, were generated using Gateway technology.

RT-qPCR. cDNA was prepared with SuperScript II reverse transcriptase (Invitrogen) from RNA isolated with Spectrum Plant Total RNA Kit or the RNeasy kit (Qiagen), and expression levels for selected genes were determined using LightCycler 480, using four replicates. Expression levels were normalized to At1G04850 and At5g18800 and analyzed for significant differences using REST 2009 Softeware (Qiagen).

Cryo SEM. Five- to seven-day-old *Arabidopsis* seedlings were frozen in liquid nitrogen and analyzed with an accelerating voltage of 5–10 kV using a Hitachi S-4800 SEM microscope. Seven to ten roots were inspected per genotype. The "Gatan-CRYO chamber" sputter coated the nonsupplemented samples with paladium-gold. In Cryo SEM images EP cells neighboring the LRP were colored using Adobe Photoshop.

Staining Procedures. GUS assays were performed and inspected using differential interference contrast optics as described previously (18, 36). Root cell walls were stained with 10 μM PI for YFP analyses and 20–30 μM PI for 30 s for analyses of cell wall degradation. PI fluorescence emission at 550–680 nm with 488-nm excitation was captured using an inverted LSM 710 META confocal microscope (Carl Zeiss) equipped with 40- and 63-fold Plan Apochromat oil immersion objectives (Carl Zeiss). The fluorescence signals at the point of highest fluorescence in front of stage I LRP (LR position), at the opposite side of the LRP (reference position), and below and above the LRP (control positions) were analyzed using Zen2011 (Zeiss) software. PI fluorescence for the overlaying cells and for the control positions was calculated relative to the reference fluorescence using 6–10 roots per genotype.

Primers. The sequences of all primers used can be found in Table S1.

Accession Numbers. Sequence data from the genes investigated can be found in the GenBank/European Molecular Biology Laboratory (EMBL) data libraries under accession numbers AT1G68765 (*IDA*), AT4G28490 (*HAE*), AT5G65710 (*HSL2*), AT5G14650 (*PGLR*), AT2G41850 (*PGAZAT*), AT4G25810 (*XTR6*), and AT4G01630 (*EXP17*).

ACKNOWLEDGMENTS. We thank Roy Falleth and Solveig H. Engebretsen for technical assistance; Tara Holman [Centre for Plant Integrative Biology (CPIB)] for the reference genes for qPCR; Zinnia Gonzalez-Carranza for the *pPGAZAT:GUS* line; Mari Wildhagen for root length measurements; the Electron Microscopy Laboratory at the Department of Biosciences, University of Oslo, where the cryo SEM was performed; and the Confocal Laser Scanning Microscopy Core Facility at Oslo University Hospital (http://core.rr-research.no/index.php?section=141). This work was supported by Research Council of Norway Grants 175238/S10 (to C.-L.S. and R.B.A.) and 178049 (to M.A.B. and R.B.A.); Belgian Scientific Policy Contract (Belgian Arabidopsis Root Network) grant (to A.L. and M.J.B.); and by Biotechnology and Biological Sciences Research Council and Engineering and Physical Sciences Research Council funding to the CPIB (to A.L. and M.J.B.).

1. Shiu SH, Bleecker AB (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proc Natl Acad Sci USA* 98(19):10763–10768.
2. Lease KA, Walker JC (2006) The *Arabidopsis* unannotated secreted peptide database, a resource for plant peptidomics. *Plant Physiol* 142(3):831–838.
3. Butenko MA, Vie AK, Brembu T, Aalen RB, Bones AM (2009) Plant peptides in signalling: Looking for new partners. *Trends Plant Sci* 14(5):255–263.
4. Butenko MA, et al. (2003) *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in *Arabidopsis* and identifies a novel family of putative ligands in plants. *Plant Cell* 15(10):2296–2307.
5. Cho SK, et al. (2008) Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 105(40):15629–15634.
6. Stenvik G-E, et al. (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* 20(7):1805–1817.
7. Cai S, Lashbrook CC (2008) Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: Enhanced retention of floral organs in transgenic plants overexpressing *Arabidopsis* ZINC FINGER PROTEIN2. *Plant Physiol* 146(3):1305–1321.
8. Ogawa M, Kay P, Wilson S, Swain SM (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are Polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. *Plant Cell* 21(1):216–233.
9. González-Carranza ZH, Elliott KA, Roberts JA (2007) Expression of polygalacturonases and evidence to support their role during cell separation processes in *Arabidopsis thaliana*. *J Exp Bot* 58(13):3719–3730.
10. Swarup K, et al. (2008) The auxin influx carrier LAX3 promotes lateral root emergence. *Nat Cell Biol* 10(8):946–954.
11. Butenko MA, et al. (2006) Ethylene dependent and -independent pathways controlling floral abscission are revelaed to converge using promoter:reporter gene constructs in the *ida* abscission mutant. *J Exp Bot* 58:3719–3730.
12. Péret B, et al. (2009) *Arabidopsis* lateral root development: An emerging story. *Trends Plant Sci* 14(7):399–408.
13. De Smet I, et al. (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. *Development* 134(4):681–690.
14. Lucas M, Guédon Y, Jay-Allemand C, Godin C, Laplaze L (2008) An auxin transport-based model of root branching in *Arabidopsis thaliana*. *PLoS ONE* 3(11):e3673.
15. Moreno-Risueno MA, et al. (2010) Oscillating gene expression determines competence for periodic *Arabidopsis* root branching. *Science* 329(5997):1306–1311.
16. Péret B, et al. (2012) Auxin regulates aquaporin function to facilitate lateral root emergence. *Nat Cell Biol* 14(10):991–998.
17. Péret B, et al. (2012) AUX/LAX genes encode a family of auxin influx transporters that perform distinct functions during *Arabidopsis* development. *Plant Cell* 24(7):2874–2885.
18. Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* 124(1):33–44.
19. Lucas M, et al. (2013) Lateral root morphogenesis is dependent on the mechanical properties of the overlaying tissues. *Proc Natl Acad Sci USA* 110:5229–5234.
20. Casimiro I, et al. (2003) Dissecting *Arabidopsis* lateral root development. *Trends Plant Sci* 8(4):165–171.
21. Naseer S, et al. (2012) Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc Natl Acad Sci USA* 109(25):10101–10106.
22. Roppolo D, et al. (2011) A novel protein family mediates Casparian strip formation in the endodermis. *Nature* 473(7347):380–383.
23. Péret B, Larrieu A, Bennett MJ (2009) Lateral root emergence: A difficult birth. *J Exp Bot* 60(13):3637–3643.
24. Laskowski M, Biller S, Stanley K, Kajstura T, Prusty R (2006) Expression profiling of auxin-treated *Arabidopsis* roots: Toward a molecular analysis of lateral root emergence. *Plant Cell Physiol* 47(6):788–792.
25. Rounds CM, Lubeck E, Hepler PK, Winship LJ (2011) Propidium iodide competes with Ca(2+) to label pectin in pollen tubes and *Arabidopsis* root hairs. *Plant Physiol* 157(1):175–187.
26. González-Carranza ZH, et al. (2012) A novel approach to dissect the abscission process in *Arabidopsis*. *Plant Physiol* 160(3):1342–1356.
27. Aalen RB (2011) Flower and floral organ abscission—control, gene expression and hormone interaction. *The Flowering Process and Its Control in Plants: Gene Expression and Hormone Interaction*, ed Yaish MW (Research Signpost/Transworld Research Network, Kerala, India), pp 307–327.
28. Sexton R, Roberts JA (1982) Cell biology of abscission. *Annu Rev Plant Physiol* 33:133–162.
29. Ellis CM, et al. (2005) *AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis thaliana*. *Development* 132(20):4563–4574.
30. Boerjan W, et al. (1995) *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell* 7(9):1405–1419.
31. Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM (1995) Formation of lateral root meristems is a two-stage process. *Development* 121(10):3303–3310.
32. Jinn TL, Stone JM, Walker JC (2000) HAESA, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes Dev* 14(1):108–117.
33. Leslie ME, Lewis MW, Youn JY, Daniels MJ, Liljegren SJ (2010) The EVERSHED receptor-like kinase modulates floral organ shedding in *Arabidopsis*. *Development* 137(3):467–476.
34. Fukaki H, Nakao Y, Okushima Y, Theologis A, Tasaka M (2005) Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in *Arabidopsis*. *Plant J* 44(3):382–395.
35. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497.
36. Beeckman T, Engler G (1994) An easy technique for the clearing of histochemically stained plant tissue. *Plant Mol Biol Rep* 12:37–42.