A dual function of the IDA peptide in regulating cell separation and modulating plant immunity at the molecular level

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Abstract:

The abscission of floral organs and emergence of lateral roots in Arabidopsis is regulated by the peptide ligand INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and the receptor protein kinases HAESA (HAE) and HAESA-LIKE 2 (HSL2). During these cell separation processes, the plant induces defense-associated genes to protect against pathogen invasion. However, the molecular coordination between abscission and immunity has not been thoroughly explored. Here we show that IDA induces a release of cytosolic calcium ions (Ca^{2+}) and apoplastic production of reactive oxygen species, which are signatures of early defense responses. In addition, we find that IDA promotes late defense responses by the transcriptional upregulation of genes known to be involved in immunity. When comparing the IDA induced early immune responses to known immune responses, such as those elicited by flagellin22 treatment, we observe both similarities and differences. We propose a molecular mechanism by which IDA promotes signatures of an immune response in cells destined for separation to guard them from pathogen attack.

Introduction

All multicellular organisms require tightly regulated cell-to-cell communication during development and adaptive responses. In addition to hormones, plants use small-secreted peptide ligands to regulate these highly coordinated events of growth, development and responses to abiotic and biotic stress (Matsubayashi, 2011; Olsson et al., 2018). In plants, organ separation or abscission, involves cell separation between specialized abscission zone (AZ) cells and enables the removal of unwanted or diseased organs in response to endogenous developmental signals or environmental stimuli. *Arabidopsis thaliana* (Arabidopsis) abscise floral organs (stamen, petals and sepals) after pollination, and this system has been used as a model to study the regulation of cell separation and abscission (Bleecker & Patterson, 1997). Precise cell separation also occurs during the emergence of new lateral root (LR) primordia, root cap sloughing, formation of stomata and radicle emergence during germination (Roberts et al., 2000). In Arabidopsis, the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide mediated signaling system ensures the correct spatial and temporal abscission of sepals, petals, and stamens. The IDA protein shares a conserved C-terminal domain with the other 7 IDA-LIKE (IDL) members (Butenko et al., 2003, Vie et al., 2015) which is processed to a functional 12 amino acid hydroxylated peptide (Butenko et al., 2014). The abscission process is regulated by the production and release of IDA, which binds the genetically redundant plasma membrane (PM) localized receptor kinases (RKs), HAESA (HAE) and HAESA-LIKE 2 (HSL2). IDA binding to HAE and HSL2 promotes receptor association with members of the co-receptor SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family and further downstream signaling leading to cell separation events. (Cho et al., 2008; Meng et al., 2016; Santiago et al., 2016; Stenvik et al., 2008). Recently, it has been shown that IDA and IDL family members also bind and activate HSL1 in the regulation of leaf epidermal pattering, indicating subfunctionalisation within this clade of receptors (Roman et al., 2022) and opens for the possibility that HAE or HSL2 could have additional functions than regulating cell separation events.

A deficiency in the IDA signaling pathway prevents the expression of genes encoding secreted cell wall remodeling and hydrolase enzymes thus hindering floral organs to abscise (Butenko et al., 2003; Cho et al., 2008; Kumpf et al., 2013; Xiangzong Meng et al., 2016; Niederhuth et al., 2013; Stenvik et al., 2008; Isaiah Taylor & John C. Walker, 2018). Interestingly, components of the IDA signaling pathway control different cell separation events during Arabidopsis development. IDA signaling through HAE and HSL2 regulates cell wall remodeling genes in the endodermal, cortical, and epidermal tissues overlaying the LR primordia during LR emergence (Kumpf et al., 2013). In addition, IDL1 signals through HSL2 to regulate cell separation during root cap sloughing (Shi et al., 2018).

Plant cell walls act as physical barriers against pathogenic invaders. The cell-wall processing and remodeling that occurs during abscission leads to exposure of previously protected tissue. This provides an entry point for phytopathogens thereby increasing the need for an effective defense system in these

66 cells (Agustí et al., 2009). Indeed, it has been shown that AZ cells undergoing cell separation express
67 defense genes and it has been proposed that they function in protecting the AZ from infection after
68 abscission has occurred (Cai & Lashbrook, 2008; Niederhuth et al., 2013). IDA elicits the production of
69 Reactive Oxygen Species (ROS) in *Nicotiana benthamiana* (*N.benthamiana*) leaves transiently expressing
70 the HAE or HSL2 receptor (Butenko et al., 2014). As a consequence of defense responses, ROS occurs in
71 the apoplast and is involved in regulating the cell wall structure by producing cross-linking of cell wall
72 components in the form of hydrogen peroxide (H_2O_2) or act as cell wall loosening agents in the form of
73 hydroxyl radical ($^{\cdot}OH$)radicals (Kärkönen & Kuchitsu, 2015). ROS may also serve as a direct defense
74 response by their highly reactive and toxic properties (Kärkönen & Kuchitsu, 2015). In addition, ROS may
75 act as a secondary signaling molecule enhancing intracellular defense responses as well as contributing
76 to the induction of defense related genes (Reviewed in (Castro et al., 2021)). In Arabidopsis, apoplastic
77 ROS is mainly produced by a family of NADPH oxidases located in the plasma membrane known as
78 RESPIRATORY BURST OXIDASE PROTEINs (RBOHs) (Torres & Dangl, 2005). The production of ROS is often
79 found closely linked to an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), where the interplay
80 between these signaling molecules is observed in immunity across kingdoms (Kadota et al., 2015;
81 Steinhorst & Kudla, 2013). Interestingly, ROS and $[Ca^{2+}]_{cyt}$ are known signaling molecules acting
82 downstream of several peptide ligand-receptor pairs. This includes the endogenous PAMPs ELICITOR
83 PEPTIDE(PEP)1, PEP2 and PEP3 which function as elicitors of systemic responses against pathogen attack
84 and herbivory (Huffaker et al., 2006; Ma et al., 2012; Qi et al., 2010), as well as the pathogen derived
85 peptide ligands flagellin22 (flg22) and elongation factor thermo unstable(Ef-Tu) which bind and activate
86 defense related RKs in the PM of the plant cell (Felix et al., 1999; Gómez-Gómez et al., 1999; Ranf et al.,
87 2011).

88 The interplay between ROS and $[Ca^{2+}]_{cyt}$ has been well studied in the flg22 induced signaling system. The
89 flg22 peptide interacts with the extracellular domain of the FLAGELLIN SENSING2 (FLS2) receptor kinase,
90 which rapidly leads to an increase in $[Ca^{2+}]_{cyt}$ (Ranf et al., 2011). In a resting state, the co-receptor
91 BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE1/SERK3) (BAK1) and the cytoplasmic
92 kinase BOTRYTIS-INDUCED KINASE1 (BIK1) form a complex with FLS2. Upon binding of flg22 to the FLS2
93 receptor, BIK1 is released from the receptor complex and activates intracellular signaling molecules
94 through phosphorylation, among other, the PM localized RBOHD (Kadota et al., 2014; Li et al., 2014).
95 The flg22 induced apoplastic ROS produced binds to PM localized Ca^{2+} channels on neighboring cells,
96 activating Ca^{2+} influx into the cytosol. The cytosolic Ca^{2+} may bind to CALCIUM DEPENDENT PROTEIN
97 KINASE5 (CDPKs) regulating RBOHD activity through phosphorylation. The Ca^{2+} dependent
98 phosphorylation of RBOH enhances ROS production, which in turn leads to a propagation of the
99 ROS/Ca^{2+} signal through the plant tissue (Dubiella et al., 2013)

100 A rapid increase in $[Ca^{2+}]_{cyt}$ is not only observed in peptide-ligand induced signaling. Similar $[Ca^{2+}]_{cyt}$
101 changes are observed as responses to developmental signals, such as extracellular auxin and
102 environmental signals such as high salt conditions and drought(reviewed in (Kudla et al., 2018)). Also,
103 $[Ca^{2+}]_{cyt}$ signals are observed in single cells during pollen tube and root hair growth (Monshausen et al.,
104 2008; Sanders et al., 1999). How cells decipher the $[Ca^{2+}]_{cyt}$ changes into a specific cellular response is
105 still largely unknown, however the cell contains a large toolkit of Ca^{2+} sensor proteins that may affect
106 cellular function through changes in protein phosphorylation and gene expression. Each Ca^{2+} binding
107 protein has specific affinities for Ca^{2+} allowing them to respond to different Ca^{2+} concentrations
108 providing a specific cellular response (Geiger et al., 2010; Scherzer et al., 2012).
109
110 Given our previous studies showing IDA induced apoplastic ROS production (Butenko et al., 2014), and
111 the known link between ROS and $[Ca^{2+}]_{cyt}$ response observed for other ligands such as flg22 (Dubiella et
112 al., 2013), we sought to investigate if IDA induces a release of $[Ca^{2+}]_{cyt}$. Here we report that IDA is able
113 to induce an intracellular release of Ca^{2+} in Arabidopsis root tips as well as in the AZ. Pursuing the tight
114 connection between ROS and Ca^{2+} in other defense signaling systems we explored if the the observed
115 production of ROS and Ca^{2+} in the IDA-HAE/HSL2 signaling pathway cold be linked to the involvement of
116 IDA in regulating plant defense (Patharkar et al., 2017). We show that a range of biotic and abiotic
117 signals can induce *IDA* expression and we demonstrate that the IDA signal modulates responses of plant
118 immune signaling. We propose that, in addition to regulating cell separation, IDA plays a role in
119 enhancing a defense response in tissues undergoing cell separation thus providing the essential need
120 for enhanced defense responses in tissue during the cell separation event.
121
122

123 ## Results:

124 ### IDA induces apoplastic production of ROS and elevation in cytosolic calcium-ion concentration

125 We have previously shown that a 12 amino acids functional IDA peptide, hereby referred to as mIDA
126 (Sup Table 1), elicits the production of a ROS burst in *N.benthamiana* leaves transiently expressing *HAE*
127 or *HSL2* (Butenko et al., 2014). To investigate whether mIDA could elicit a ROS burst in Arabidopsis, a
128 luminol-dependent ROS assay on *hae hsl2* mutant rosette leaves expressing the full length HAE receptor
129 under a constitutive promoter (*35S:HAE-YFP*) was performed. *hae hsl2 35S:HAE-YFP* leaf discs treated
130 with mIDA emitted extracellular ROS, whereas no response was observed in wild-type (WT) leaves (Sup
131 Fig.1a-c). We investigated the activity of the *HAE* and *HSL2* promoter in 22 days old Arabidopsis rosette
132 leaves by cloning the promoters fused to the nuclear localized YFP-derived fluorophore Venus protein

133 (*pHAE:Venus-H2B*, *pHSL2:Venus-H2B*) and could observe a decrease in promoter activity in the oldest
134 rosette leaves giving a possible explanation to the lack of mIDA induced ROS in true leaves (Sup Fig.
135 1d,e). Since rapid production of ROS is often linked to an elevation in $[Ca^{2+}]_{cyt}$ (Steinhorst & Kudla, 2013),
136 we aimed to investigate if mIDA could induce a $[Ca^{2+}]_{cyt}$ response. We imaged Ca^{2+} in 10 days-old roots
137 expressing the cytosolic localized fluorescent Ca^{2+} sensor R-GECO1 (Keinath et al., 2015). As a positive
138 control for $[Ca^{2+}]_{cyt}$ release we added 1 mM extracellular ATP (eATP), which leads to a $[Ca^{2+}]_{cyt}$ elevation
139 in the root tip within a minute after application (Fig. 1a) (Breiden et al., 2021). Following application of
140 1 µM mIDA, we detected an increase in $[Ca^{2+}]_{cyt}$ (Fig. 1a and Movie 1). R-GECO1 fluorescence intensities
141 normalized to background intensities (ΔF/F) were measured from a region of interest (ROI) covering the
142 meristematic and elongation zone of the root and revealed that the response starts 4-5 minutes after
143 application of the mIDA peptide and lasts for 7-8 minutes (Fig. 1a). The signal initiated in the root
144 meristematic zone from where it spread toward the elongation zone and root tip, a second wave was
145 observed in the meristematic zone continuing with Ca^{2+} spikes. The signal amplitude was at a maximum
146 within the elongation zone and decreased as the signal spread (Fig. 1a).

147 The $[Ca^{2+}]_{cyt}$ response in roots to the bacterial elicitor flg22 has previously been studied using the R-
148 GECO1 sensor (Keinath et al., 2015). To better understand the specificity of the mIDA induced Ca^{2+}
149 response, we aimed to compare the Ca^{2+} dynamics in mIDA treated roots to those treated with flg22.
150 We observed striking differences in the onset and distribution of the Ca^{2+} signals. Analysis of roots
151 treated with 1 µM flg22 showed that the Ca^{2+} signal initiated in the root elongation zone from where it
152 spread toward the meristematic zone as a single wave and that the signal amplitude was at a maximum
153 within the elongation zone and decreased as the signal spread (Fig. 1b, Movie 2). These observations
154 indicate differences in tissue specificity of Ca^{2+} responses between mIDA and flg22, which we
155 hypothesize depend on the cellular distribution of their cognate receptors. Indeed, when investigating
156 the promoter activity of the *HAE*, *HSL2* and *FLS2* receptors by the use of nuclear localized transcriptional
157 reporter lines we observed a different pattern of fluorescent nuclei in the roots (Fig. 1c,d,e).
158 *pHAE:Venus-H2B* lines had fluorescent expression in the epidermis and stele of the elongation zone (Fig.
159 1c) and *pHSL2:Venus-H2B* lines in the lateral root cap, root tip and root meristem (Fig. 1d); while
160 fluorescent nuclei were observed in the stele of the elongation zone in *pFLS2:Venus-H2B lines* (Fig. 1e).
161 The different patterns of fluorescent nuclei observed for the three different receptor constructs could
162 indeed explain the differences in the Ca^{2+} signatures triggered by mIDA and flg22. However, the
163 receptors promoter activity show a broader expression pattern compared to the respective ligand
164 induced $[Ca^{2+}]_{cyt}$ responses, indicating additional regulating factors for the observed $[Ca^{2+}]_{cyt}$ response.

165 To further investigate the mIDA induced Ca^{2+} response, we used a cytosolic localized Aequorin-based
166 luminescence Ca^{2+} sensor (Aeq) (Knight et al., 1991). The Aeq sensor was chosen due to the well-

167 established use of this sensor in studies investigating peptide induced Ca^{2+} responses (Ranf et al., 2011)
168 Removing the last Asparagine of the mIDA peptide (IDAΔN69; Sup Table 1) renders it inactive (Butenko
169 et al., 2014). 7 days old *Aeq*-Seedlings treated with 1 µM IDAΔN69 did not show any increase in $[Ca^{2+}]_{cyt}$
170 (Sup Fig. 4a). To investigate if the $[Ca^{2+}]_{cyt}$ increase triggered by mIDA was dependent on extracellular
171 Ca^{2+}, we pre-treated seedling with $LaCl_3$ and Ethylene glycol-bis (2-aminoethylether)- N, N, N′,N′-
172 tetraacetic acid (EGTA). $LaCl_3$ and EGTA are inhibitors that block plasma membrane localized cation
173 channels and chelate Ca^{2+} in the extracellular space, respectively (Knight et al., 1997). The mIDA induced
174 response was abolished in *Aeq*-seedlings pre-incubated in 2 mM EGTA or 1 mM $LaCL_3$ (Sup Fig. 4b),
175 indicating that the mIDA dependent $[Ca^{2+}]_{cyt}$ response depends on Ca^{2+} from the extracellular space.

176 Next we set out to investigate whether mIDA would trigger an increase in $[Ca^{2+}]_{cyt}$ in floral AZ cells. We
177 utilized the Aeq expressing line and monitored $[Ca^{2+}]_{cyt}$ changes in flowers at different developmental
178 stages (see Sup Fig. 5a for developmental stages [floral positions]) to 1 µM mIDA. Interestingly, only
179 flowers at a the stage where there is an initial weakening of the AZ cell walls showed an increase in
180 $[Ca^{2+}]_{cyt}$ (Sup Fig. 5). Flowers treated with mIDA prior to cell wall loosening showed no increase in
181 luminescence (Sup Fig. 5), indicating that the mIDA triggered Ca^{2+} release in flowers correlates with the
182 onset of the abscission process and the increase in *HAE* and *HSL2* expression at the AZ (Cai & Lashbrook,
183 2008; Patharkar & Walker, 2015). Using plants expressing the R-GECO1 sensor we performed a detailed
184 investigation of the mIDA induced $[Ca^{2+}]_{cyt}$ response in flowers at position 6 which is the position where
185 initial cell wall loosening occurs. The AZ region was analyzed for signal intensity values and revealed that
186 the Ca^{2+} signal was composed of one wave (Fig. 2, Movie 3). The signal initiated close to the nectaries
187 and spread throughout the AZ and further into the floral receptacle. Flowers treated with 1 µM IDAΔN69
188 did not show any increase in $[Ca^{2+}]_{cyt}$ in the AZ or receptacle, while a clear Ca^{2+} wave could be detected
189 in the whole AZ, receptacle and proximal pedicel after treatment with eATP (Sup Fig.7, Sup Movie 2).
190 Detailed investigation of *HAE* and *HSL2* promoter activity in flowers at position 6 shows restricted
191 promoter activity to the AZ cells (Fig. 2b). The observed promoter activity correlates with the observed
192 mIDA induced $[Ca^{2+}]_{cyt}$ response

193 **The ROS producing enzymes, RBOHD and RBOHF, are not involved in the developmental process of**
194 **abscission.**

195 ROS and Ca^{2+} are secondary messengers that can play a role in developmental processes involving cell
196 wall remodeling, but are also important players in plant immunity (Kärkönen & Kuchitsu, 2015). We
197 therefore set out to investigate if the IDA induced production of ROS and increase in $[Ca^{2+}]_{cyt}$ function in
198 the developmental process of abscission or if these signaling molecules form part of IDA modulated
199 plant immunity.

200 The IDA induced Ca^{2+} response depends on Ca^{2+} from extracellular space (Sup Fig. 4b). Ca^{2+} transport
201 over the plasma membrane is enabled by a variety of Ca^{2+} permeable channels, including the CYCLIC
202 NUCLEOTIDE GATED CHANNELs (CNGCs). Various CNGCs are known to be involved in peptide ligand
203 signaling in plants, including the involvement of CNGC6 and CNGC9 in CLAVATA3/EMBRYO
204 SURROUNDING REGION40 signaling in roots (Breiden et al., 2021), and CNGC17 In phytosulfokine
205 signaling (Ladwig et al., 2015). Using publicly available expression data we identified multiple CNGCs
206 expressed in the AZ of Arabidopsis (Cai & Lashbrook, 2008) (Sup Fig. 8a) and investigated if plants
207 carrying mutations in the *CNGSs* genes expressed in AZs showed a defect in floral organ abscission. We
208 observed no deficiency in floral abscission in the *cngc* mutants (Sup Fig. 8b), in contrast to what is
209 observed in the *hae hsl2* mutant where all floral organs are retained throughout the inflorescence. Ca^{2+}
210 partially activates members of the NOX family of nicotinamide adenine dinucleotide phosphate (NADPH)
211 oxidases (RBOHs), key producers of ROS (Kadota et al., 2015). Two members of this family, *RBOHD* and
212 *RBOHF* have high transcriptional levels in the floral AZs (Cai & Lashbrook, 2008) and have been reported
213 to be important in the cell separation event during floral abscission in Arabidopsis (Lee et al., 2018). We
214 therefore set out to investigate if ROS production in AZ cells was dependent on RBOHD and RBOHF and
215 to quantify to which degree these NADPH oxidases were necessary for organ separation. We treated
216 AZs with the ROS indicator, H2DCF-DA, which upon contact with ROS has fluorescent properties, and
217 observed ROS in both WT and to a lower extent in *rbohd rbohdf* flowers. In addition, we observed normal
218 floral abscission in the *rbohd rbohf* double mutant flowers, indicating that the developmental
219 progression of cell separation is not dependent on RBOHD and RBOHF (Sup Fig. 8c,d). These results are
220 in stark contrast to what has previously been reported, where cell separation during floral abscission
221 was shown to be dependent on RBOHD and RBOHF (Lee et al., 2018). We used a stress transducer to
222 quantify the force needed to remove petals, the petal breakstrength (pBS) (Stenvik et al., 2008), from
223 the receptacle of gradually older flowers along the inflorescence of WT and *rbohd rbohf* flowers.
224 Measurements showed a significant lower pBS value for the *rbohd rbohf* mutant compared to WT at the
225 developmental stage where cell loosening normally occurs, indicating premature cell wall loosening
226 (Sup Fig. 8e). Furthermore, *rbohd rbohf* petals abscised one position earlier than WT (Sup Fig. 8e).
227 Similar to Crick et al 2022,, we found no RBOHD and RBOHF dependent delay or absence of cell
228 separation during floral abscission (Crick et al., 2022). Due to a known function of IDA in inducing ROS
229 production (Sup Fig. 1), these results points towards a role for IDA in modulating additional responses
230 to the process of cell separation.

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233 *IDA* is upregulated by biotic and abiotic factors

234 In Arabidopsis, infection with the pathogen *P.syringae* induces cauline leaf abscission. Interestingly,
235 stress induced cauline leaf abscission was reduced in plants with mutations in components of the IDA-
236 HAE/HSL2 signaling pathway (Patharkar et al., 2017; Patharkar & Walker, 2016). In addition,
237 upregulation of defense genes in floral AZ cells during the abscission process is altered in the *hae hsl2*
238 mutant (Cai & Lashbrook, 2008; Niederhuth et al., 2013), indicating that the IDA-HAE/HSL2 signaling
239 system may be involved in regulating defense responses in cells undergoing cell separation. We further
240 investigated if *IDA* is upregulated by biotic and abiotic elicitors. Transgenic plants containing the *β-*
241 *glucuronidase (GUS*) gene under the control of the *IDA* promoter (*pIDA:GUS*) have previously been
242 reported to exhibit *GUS* expression in cells overlaying newly formed LR primordia (Butenko et al., 2003;
243 Kumpf et al., 2013). To investigate the expression of the *IDA* gene in response to biotic stress, *pIDA:GUS*
244 seedlings were exposed to the bacterial elicitor flg22 and fungal chitin. Compared to the control
245 seedlings, the seedlings exposed to flg22 and chitin showed a significant increase in *GUS* expression in
246 LR primordia (Fig. 3a). Also, when comparing untreated root tips to those exposed to flg22 and chitin
247 we observed a GUS signal in the primary root of flg22 and chitin treated roots (Fig. 3b). We used the
248 fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (4-MUG) which is hydrolysed to the
249 fluorochrome 4-methyl umbelliferone (4-MU) to quantify GUS-activity (Blazquez, 2007). A significant
250 increase in fluorescence was observed for flg22 and chitin treated samples compared to untreated
251 controls, (Fig. 3b), which was not observed when exposing seedlings to IDAΔN69 indicating that
252 increased *IDA* expression is not triggered simply by the application of a peptide (Fig. 3). Spatial *pIDA:GUS*
253 expression was also monitored upon abiotic stress. When *pIDA:GUS* seedling were treated with the
254 osmotic agent mannitol or exposed to salt stress by NaCl, both treatments simulating dry soil, a similar
255 increment in GUS signal was observed surrounding the LR primordia (Fig. 3). However, no *pIDA:GUS*
256 expression was detected in the primary root (Fig. 3b). Taken together these results indicate an
257 upregulation of *IDA* in tissue involved in cell separation processes such as the LR primordia and,
258 interestingly, the root cap upon biotic stress. The process of cell separation is a fast and time-restricted
259 response where cells previously protected by outer cell layers are rapidly exposed to the environment.
260 These cells need strong and rapid protection against the environment, and we therefore aimed to
261 investigate if IDA is involved in modulating immune responses in addition to inducing ROS and $[Ca^{2+}]_{cyt}$
262 responses.
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266 mIDA can induce production of callose as a long term defense response

267 We aimed to investigate a possible role of IDA in regulating long-term defense responses in the plant,
268 such as the production of callose. Callose deposition is a resulting hallmark of the late plant immunity
269 responses that together with other events such as stomatal closure and production of ethylene leads to
270 inhibition of pathogen multiplication and containment of disease (reviewed in (Wang et al., 2021)).
271 Callose deposition is highly increased in response to flg22 (Gómez-Gómez et al., 1999). Promoter activity
272 studies of *pHAE* and *pHSL2* indicates a weak activity of the promoters in the cotelydons, (Sup Fig. 1d)
273 and we thus treated WT seedlings with 1 µM mIDA and measured the callose depositions per area in
274 the cotyledons. We detected no difference to water treated controls indicating that either callose
275 deposition is not a cellular outcome for mIDA treatment (Fig. 4a,b), or that receptor availability in the
276 cotyledons is too low to observe a response We further tested if mIDA could induce callose depositions
277 in cotyledons expressing the full length HAE receptor under a constitutive promoter (*35S:HAE-YFP*). An
278 increase in the callose depositions per area upon mIDA treatment compared to water treated controls
279 was observed in these lines (Fig. 4a,b). As a control, flg22 treated WT seedlings showed increased
280 callose deposition, which was not observed in flg22 treated *fls2* seedlings (Fig. 4a,b). These results
281 indicate that mIDA, is capable of promoting a HAE dependent deposition of callose as a long-term
282 defense response.

283

284 mIDA triggers expression of defense-associated marker genes associated with innate immunity.

285 A -well-known part of a plants immune response is the enhanced transcription of genes involved in
286 immunity. Transcriptional reprogramming mostly mediated by WRKY transcription factors takes place
287 during microbe-associated molecular pattern (MAMP)- triggered immunity and is essential to mount an
288 appropriate host defense response (Birkenbihl et al., 2017). We selected well-established defense-
289 associated marker genes: *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*), a specific and early immune-
290 responsive gene activated by multiple MAMPs (Asai et al., 2002; He et al., 2006), *MYB51*, a WRKY target
291 gene, previously shown to increase in response to the MAMP flg22 (Frerigmann et al., 2016) resulting
292 in biosynthesis of the secondary metabolite indolic glucosinolates; and an endogenous danger peptide,
293 ELICITOR PEPTIDE 3 (PEP3) (Huffaker et al., 2006). Seven days-old WT seedlings treated with 1 µM mIDA
294 were monitored for changes in transcription of the aforementioned genes by RT-qPCR compared to
295 untreated controls at two time points, 1 h and 12 h after treatment. All genes showed a significant
296 transcriptional elevation 1 h after mIDA treatment (Fig. 5a). This in accordance with previous reports,
297 where the expression of *FRK1* and *PEP3* was monitored in response to bacterial elicitors (He et al., 2006;
298 Huffaker et al., 2006). After 12 h, *FRK1* and *PEP3* transcription in mIDA treated tissue was not

299 significantly different from untreated tissue; while transcription of *MYB51* in mIDA treated tissue
300 remained elevated after 12 h but was significantly lower compared to 1 h after mIDA treatment (Fig.
301 5a). Interestingly, the observed increase in transcription of the defense genes *MYB51* and *PEP3*, was
302 only partially reduced in the *hae hsl2* mutant (Sup Fig. 9). In contrast, *FRK1* showed higher
303 transcriptional levels in the *hae hsl2* mutant compared to WT (Sup Fig. 9). These results indicate that
304 also other receptors than HAE and HSL2 are involved in the mIDA-induced enhancement of the defense
305 genes *FRK1*, *MYB51* and *PEP3*.

306 We then analyzed a plant line expressing nuclear localized YFP from the MYB51 promoter
307 (*pMYB51:YFP*^{N}) (Poncini et al., 2017) treated with mIDA. Enhanced expression of p*MYB51:YFP* was
308 predominantly detected in the meristematic zone of the root after 7 h of mIDA treatment, compared to
309 a non-treated control (Fig. 5b). Moreover, comparison of mIDA induction with the elicitor-triggered
310 response of *pMYB51:YFP*^{N} to flg22 showed similar temporal expression in the root (Sup Fig. 10) .
311 Together, these data show that IDA can trigger a rapid increase in the expression of key genes involved
312 in immunity. We next investigated if the mIDA induced transcription of defense related genes was
313 similar to that induced by flg22. Seven days-old Col-0 WT seedlings were treated for 1h with mIDA, flg22
314 or a combination of mIDA and flg22 to explore potential additive effects. The relative increase in
315 transcription of the genes tested is similar when seedlings are treated with mIDA and flg22 (Fig. 6a).
316 However, when treating seedlings with both peptides the relative transcription of the genes of interest
317 increased substantially. To our surprise, the combined enhanced transcription when seedling where co-
318 treated with both flg22 and mIDA exceeds a simple additive effect of the two peptides. To investigate if
319 the observed increase is specific to a combination of mIDA and flg22, we investigated if co-treatment
320 with mIDA and PIP1, an endogenous DAMP peptide known to amplify the immune response triggered
321 by flg22, (Hou et al., 2014) gave a similar effect. Seedlings were treated for 1h with mIDA, PIP1 or a
322 combination of the peptides to explore additive effects. In contrast to what we observed with flg22, no
323 enhanced transcription of *FRK1*, *MYB51* and *PEP3* were observed after co-treatment with PIP1 and mIDA
324 (Sup Fig. 11). These results suggest a role of IDA in enhancing the defense response triggered by flg22.
325 To explore whether cells capable of undergoing cell separation in response to IDA and IDL peptides
326 could be expressing *HAE* or *HSL2* in combination with *FLS2* we made transcriptional reporter lines
327 expressing each of the receptors in fusion with either the nuclear localized YFP-derived fluorophore
328 Venus protein or the RFP-derived Tomato protein (*pRECEPTOR:Venus/Tomato-H2B*) and crossed the
329 lines to each other. Plants expressing both constructs were inspected for fluorescent nuclei in 7 day-old
330 roots as well as in floral organs of floral position 7. Fluorescent nuclei with overlapping Venus and
331 Tomato expression were observed both in the vascular tissue of the root, in cells surrounding LRs as
332 well as in cells of the AZs of plants expressing *pFLS2-Venus-H2B* and *pHAE-Tomat-H2B* (Fig. 6b). Similar

333 observations were done in the AZ of lines expressing *pFLS2-Venus-H2B* and *pHSL2-Tomat-H2B lines*,
334 however in these lines overlapping fluorescent nuclei in roots were mainly observed in the epidermal
335 cells surrounding the LR and in the root tip, as well as in the root cap (Fig. 6c). Based on these results
336 we propose a role for IDA in enhancing immune responses in tissue undergoing cell separation, possibly
337 by enhancing cellular responses activated by immune receptors, such as FLS2.

338

339 <u>Discussion:</u>

340 Several plant species can abscise infected organs to limit colonization of pathogenic microorganisms
341 thereby adding an additional layer of defense to the innate immune system (Kissoudis et al., 2016;
342 Patharkar et al., 2017). Also, in a developmental context, there is an induction of defense associated genes
343 in AZ cells during cell separation and prior to the formation of the suberized layer that functions as a barrier
344 rendering protection to pathogen attacks (Cai & Lashbrook, 2008; Niederhuth et al., 2013; Roberts et al.,
345 2000; I. Taylor & J. C. Walker, 2018). Interestingly, the induction of defense genes during floral organ
346 abscission is altered in *hae hsl2* plants (Niederhuth et al., 2013). It is largely unknown how molecular
347 components regulating cell separation and how the IDA-HAE/HSL2 signaling pathway, contributes to
348 modulation of plant immune responses. Here we show that the mature IDA peptide, mIDA, is involved in
349 activating known defense responses in Arabidopsis, including the production of ROS, the intracellular
350 release of Ca^{2+}, the production of callose and the transcription of genes known to be involved in defense
351 (Sup Fig. 1, Fig. 1, Fig. 2, Fig. 5). We suggest a role for IDA in ensuring optimal cellular responses in tissue
352 undergoing cell separation, regulating both development and defense.
353

354 The intracellular event occurring downstream of IDA is still partially unknown. Here we report the
355 release of cytosolic Ca^{2+} as a new signaling component. Roots and AZs expressing the Ca^{2+} sensor R-
356 GECO1, showed a $[Ca^{2+}]_{cyt}$ in response to mIDA. Moreover, the increased $[Ca^{2+}]_{cyt}$ in response to mIDA in
357 flowers occurred only at stages where cell separation was taking place, linking the observed $[Ca^{2+}]_{cyt}$
358 response to the temporal point of abscission and the need for an induced local defense response. During
359 floral development the expression of the *HAE* and *HSL2* receptors increase prior to the onset of
360 abscission (Cai & Lashbrook, 2008) and may account for the temporal mIDA induced $[Ca^{2+}]_{cyt}$ response
361 observed.

362 Based on these results, it is likely that the expression of the receptors is the limiting factor for a cells ability
363 to respond to the mIDA peptide with a $[Ca^{2+}]_{cyt}$ response. However, promoter activity of the receptors are
364 observed in a broader area of the root than the observed $[Ca^{2+}]_{cyt}$ response. As an example, *pHSL2* is found

365 to have a localized activation in the root tip, and when HSL2 is activated by IDL1 be involved in the
366 regulation of cell separation during root cap sloughing (Shi et al., 2018). IDL1 and IDA have a high degree
367 of similarity in the protein sequence, and *IDL1* expressed under the *IDA* promoter can fully rescue the
368 abscission phenotype observed in the *ida* mutant (Stenvik et al., 2008). We can assume that IDL1 activates
369 similar signaling components in the root cap as those used by IDA. Despite the expression of HSL2 and the
370 involvement of the receptor in root cap sloughing, no $[Ca^{2+}]_{cyt}$ response is observed in this region when
371 plants are treated with the IDA peptide. The absence of an observed $[Ca^{2+}]_{cyt}$ response in the root tip
372 indicates that $[Ca^{2+}]_{cyt}$ is most likely not involved in the cell separation process during root cap sloughing, or
373 that cells involved in this process only are able to induce a $[Ca^{2+}]_{cyt}$ response at a specific developmental
374 time point. Thus, in addition to receptor availability, other factors must be involved in regulating the $[Ca^{2+}]_{cyt}$
375 response. It is intriguing that the mIDA cellular output is similar to that of flg22 (Kadota et al., 2015; Kadota
376 et al., 2014) yet shows some distinct differences. The spatial distribution of the Ca^{2+} wave originated by
377 mIDA differs from that of flg22. The *rbohd rbohf* mutant shows ROS production in the AZ, indicating that
378 other NADPH oxidases or cell wall peroxidases expressed in the AZ may be involved in ROS production, in
379 contrast to the importance of RBOHD RBOHF in flg22 signaling (Kadota et al., 2015). This suggests that while
380 IDA and flg22 share many components for their signal transduction, such as the BAK1 co-receptor
381 (Chinchilla et al., 2007; X. Meng et al., 2016) and MAPK cascade (Asai et al., 2002; Cho et al., 2008), there
382 are other specific components that are likely important to provide differences in signaling. Despite the
383 observation of the numerous defense related responses to mIDA, we cannot exclude that the mIDA induced
384 $[Ca^{2+}]_{cyt}$ and rises in ROS levels may also have an intrinsic developmental role such as modulating cell wall
385 properties and cell expansion similar to what is observed during FERONIA (FER) signaling (Dünser et al.,
386 2019; Wei Feng et al., 2018). To further decipher the importance of the mIDA induced Ca^{2+} and ROS in
387 defense it will be important to identify the specific signaling components responsible for this cellular output
388 in addition to investigating the susceptibility of mutants to pathogen exposure.
389
390 Interestingly, FLS2 arrangement into nanoscale domains at the PM is dependent on FER. FLS2 becomes
391 more disperse and mobile in *fer* mutants, implying a role for FER in regulating FLS2 activity (Gronnier et
392 al., 2022). Also altering of the cell wall affects FLS2 nanoscale organization (McKenna et al., 2019).
393 Formation of nanoscale domains containing the HAE and HSL2 receptors may be an important signaling
394 step in the IDA-HAE/HSL2 signaling pathway. From our promoter analysis we clearly see overlapping
395 activity of the *HAE* and *HSL2* promoters with the *FLS2* promoter *in planta*, providing the possibility that
396 the receptors are localized at the PM of the same cells and can have coordinated signaling events. An
397 intriguing thought is a possible co-localization of HAE and HSL2 with FLS2 in nanoscale domains, making
398 a signaling unit possible to involve multiple different receptors responsible for the signaling outcome
399 observed. In this paper, we show that co-treatment with mIDA and flg22 induces an enhanced

400 transcription of defense related genes. How FLS2, HAE and HSL2 communicate at the plasma membrane
401 of the responsive cells may give the answers to the molecular mechanisms behind the augmented
402 transcriptional regulation observed when co-treatment of mIDA and flg22 is performed (Fig. 6). We can
403 investigate possible interaction partners of HAE and HSL2 looking at available data from a sensitized
404 high-throughput interaction assay between extracellular domains (ECDs) of 200 Leucine Rich Repeat
405 (LRR) (Smakowska-Luzan et al., 2018). Interestingly, LRR-RLKs known to play a function in biotic or
406 abiotic stress responses, such as RECEPTOR-LIKE KINASE7 (RLK7), STRUBBELIG-RECEPTOR FAMILY3 ,
407 RECEPTOR-LIKE PROTEIN KINASE 1 and FRK1, are found to interact mainly with HSL2, whereas HAE
408 shows interaction with LRRs mainly involved in development (Sup Fig. 12). RLK7 has recently been
409 identified as an additional receptor for CEP4, involved in regulating cell surface immunity and response
410 to nitrogen starvation, indication a role of RLK7 as an additional receptor regulating defense responses
411 in known peptide-receptor complexes (Rzemieniewski et al., 2022). It would be interesting to investigate
412 a possible role of RLK7 in IDA-HAE/HSL2 signaling as a signaling partner in the observed IDA-induced
413 defense responses.

414 In this paper, we propose a model where the IDA-HAE/HSL2 signaling pathway modulates defense
415 responses in tissues undergoing cell separation. It is beneficial for the plant to ensure an upregulation
416 of defense responses in tissues undergoing cell separation as a precaution against pathogen attack. This
417 tissue is a major entry route for pathogens, and by combining the need of both the IDA peptide and the
418 pathogen molecular pattern, such as flg22, for the induction of a strong defense response, the plant
419 ensures maximum immunity in the most prone cells without spending energy inducing this in all cells
420 (Fig. 7). Such a molecular mechanism, where the presence of mIDA in tissue exposed to stress leads to
421 maximum activation of the immune responses, will ensure maximal protection of infected cells during
422 cell separation, cells which are major potential entry routes for invading pathogens.

423 Movies

424 **Movie 1:** $[Ca^{2+}]_{cyt}$ dynamics in R-GECO1 expressing root tip in response to 1 µM mIDA. 10 days-old roots
425 were treated with 1 µM mIDA and changes in $[Ca^{2+}]_{cyt}$ was recorded over time. Response shown as
426 normalized fluorescence intensities (ΔF/F). Images were recorded with a frame rate of 5 seconds. Movie
427 corresponds to measurements shown in Fig. 1a. Representative response from 10 roots. Time shown as,
428 hh:mm:ss.

429

430 **Movie 2:** $[Ca^{2+}]_{cyt}$ dynamics in R-GECO1 expressing root tip in response to 1 µM flg22 10 days-old roots were
431 treated with 1 µM flg22 and changes in $[Ca^{2+}]_{cyt}$ was recorded over time. Response shown as normalized
432 fluorescence intensities (ΔF/F). Images were recorded with a frame rate of 5 seconds. Movie corresponds
433 to measurements shown in Fig. 1b. Representative response from 7 roots. Time shown as, hh:mm:ss.

434

435 **Movie 3:** $[Ca^{2+}]_{cyt}$ dynamics in R-GECO1 expressing AZ in response to 1 µM mIDA. Flowers at position 6 were
436 treated with 1 µM mIDA and changes in $[Ca^{2+}]_{cyt}$ was recorded over time. Response shown as normalized
437 fluorescence intensities (ΔF/F). Images were recorded with a frame rate of 5 seconds. Movie corresponds
438 to measurements shown in Fig. 2a. Representative response from 8 flowers. Time shown as, hh:mm:ss.

439

440 **Sup Movie 1:** $[Ca^{2+}]_{cyt}$ dynamics in R-GECO1 expressing root tip in response to 1 mM eATP. 1 mM eATP will
441 induce a strong increase in $[Ca^{2+}]_{cyt}$ and was added as a last treatment to all roots acting as a positive control.
442 Response shown as normalized fluorescence intensities (ΔF/F). Images were recorded with a frame rate of
443 5 seconds. Movie corresponds to measurements shown in Fig. 1a. Representative response from 10 roots.
444 Time shown as, hh:mm:ss.

445

446 **Sup Movie 2:** $[Ca^{2+}]_{cyt}$ dynamics in R-GECO1 expressing AZ in response to to 1 µM of the inactive IDA peptide,
447 $IDA^{\Delta N69}$. Flowers at position 6 were treated with 1 µM $IDA^{\Delta N69}$ and changes in $[Ca^{2+}]_{cyt}$ was recorded over
448 time. Response shown as normalized fluorescence intensities (ΔF/F). Images were recorded with a frame
449 rate of 5 seconds. Movie corresponds to measurements shown in Sup Fig. 6. Representative response from
450 7 flowers. Time shown as, hh:mm:ss.

451

452

453

454 ***Methods***

455 Accession numbers of genes studied in this work:

456 HSL2 At5g65710, IDA At1g68765, FLS2 At5g46330, HAE At4g28490, MYB51 At1g18570, RBOHD
457 At5g47910, RBOHF At1g64060, PEP3 At5g64905, WRKY33 At2g38470, FRK1 At2g19190. Plant lines used
458 in this work: Ecotype Colombia-0 (Col-0) was used as wild type (WT). Mutant line: *hae* (SALK_021905),
459 *hsl2*, (SALK_030520), *fls2* (SALK_062054), *rbohd* (SALK_070610), *rbohf* (SALK_059888), *cngc1*
460 (SAIL_443), *cngc2* (SALK_066908), *cngc4* (SALK_081369), *cngc5* (SALK_149893), *cngc6* (SALK_064702),
461 *cngc9* (SAIL_736), *cngc12* (SALK_093622). SALK lines were provided from Nottingham Arabidopsis Stock
462 Centre (NASC).

463 Plant lines

464 The pIDA:GUS, pHSL2:Venus-H2B and pMYB51:YFPN lines have been described previously (Kumpf et al.,
465 2013; Poncini et al., 2017; Shi et al., 2018).The promoters of HAE (1601 bp (Kumpf et al., 2013) and HSL2

466 (2300 bp (Sto et al., 2015))were available in the pDONRZeo vector (Thermo Fischer Scientific).
467 Sequences corresponding to the FLS2 promoter (988 bp (Robatzek et al., 2006)) were amplified from
468 WT DNA (primers are listed in table 2) and cloned into the pDONRZeo vector (Thermo Fischer Scientific).
469 All promoter constructs were further recombined into the promotor:Venus (YFP)-H2B and Tomato-H2B
470 destination vectors (Somssich et al., 2016) using the Invitrogen Gateway cloning system (Thermo Fischer
471 Scientific). Constructs were transformed into *Agrobacterium tumefaciens* (A.tumefaciens) C58 and the
472 floral dip method (Clough & Bent, 1998) was used to generate transgenic lines. Single-copy homozygous
473 plant lines were selected and used in this study. The CDS of HAE was cloned into the pEarleyGate101
474 destination vector (Earley et al., 2006) using the Invitrogen Gateway cloning system and transformed
475 into A.tumefaciens C58 and further used to generate the *35S:HAE:YFP* lines.

476 Growth conditions

477 Plants were grown in long day conditions (8 h dark and 16 h light) at 22 °C. Seeds were surface sterilized
478 and plated out on MS-2 plates, stratified for 24 h at 4 °C and grown on plates for 7 days before
479 transferred to soil.

480 Peptide sequences

481 Peptides used in this study were ordered from BIOMATIK. Peptide sequences are listed in
482 Supplementary Table 1.

483 Primers

484 Primers for genotyping and generation of constructs were generated using VectorNTI. Gene specific
485 primers for RT-qPCR were generated using Roche Probe Library Primer Design. All primers are listed in
486 Supplementary Table 2.

487 Histochemical GUS assay

488 Seven days-old seedlings were pre-incubated for 12 h in liquid MS-2 medium containing stimuli of
489 interest; 1 µM peptide (table 1), 60 mM Mannitol (M4125 – Sigma), 20 µg/mL Chitin (C9752 – Sigma),
490 50 mM NaCl and then stained for GUS activity following the protocol previously described (Stenvik et
491 al., 2008). Roots were pictured using a Zeiss Axioplan2 microscope with an AxioCam HRc, 20x air
492 objective. The assay was performed on 10 individual roots and the experiment was repeated 3 times.

493 Fluorescent GUS assay

494 Seven days-old seedlings were pre-incubated for 12 h in liquid MS-2 medium with or without stimuli of
495 interest; 1 µM peptide (table 1), 60 mM Mannitol (M4125 – Sigma), 20 µg/mL Chitin (C9752 – Sigma),

496 50 mM NaCl. After treatment, 10 seedlings were incubated in wells containing 1 mL reaction mix
497 described in (Blazquez, 2007) containing: 10 mM EDTA (pH 8.0), 0,1 % SDS, 50 mM Sodium Phosphate
498 (pH 7.0), 0,1 % Triton X-100, 1 mM 4-MUG (, M9130-Sigma) and incubated at 37 °C for 6 h. Six 100 µl
499 aliquots from each well were transferred to individual wells in a microtiter plate and the reaction was
500 stopped by adding 50 µl of stop reagent (1 M Sodium Carbonate) to each well. Fluorescence was
501 detected by the use of a Wallac 1420 VICTOR2 microplate luminometer (PerkinElmer) using an
502 excitation wavelength of 365 nm and a filter wavelength of 430 nm. Each experiment was repeated 3
503 times.

504 Confocal laser microscopy of roots and flowers expressing promoter:Venus/YFP-H2B and promoter:
505 Tomato-H2B construct

506 Imaging of 7 days-old roots was performed on a LSM 880 Airyscan confocal microscope equipped with
507 two flanking PMTs and a central 32 array GaAsP detector. Images were acquired with Zeiss Plan-
508 Apochromat 20x/0.8 WD=0.55 M27 objective and excited with laser light of 405 nm, 488 nm and 561
509 nm. Roots were stained by 1 µM propidium iodide for 10 min and washed in dH_2O before imaging.
510 Imaging of flowers was performed on an Andor Dragonfly spinning disk confocal using an EMCCD iXon
511 Ultra detector. Images were acquired with a 10x plan Apo NA 0,45 dry objective and excited with laser
512 light of 405 nm, 488 nm and 561 nm. Maximum intensity projections of z-stacks were acquired with
513 step size of 1,47 µm. Image processing was performed in FIJI 51. These steps are: background
514 subtraction, gaussian blur/smooth, brightness/contrast. Imaging was performed at the NorMIC Imaging
515 platform.

516

517 Calcium imaging using the R-GECO1 sensor

518 $[Ca^{2+}]_{cyt}$ in roots were detected using WT plants expressing the cytosolic localized single-fluorophore
519 based Ca^{2+} sensor, R-GECO1 in Col-0 background (Keinath et al., 2015). Measurements were performed
520 using a confocal laser scanning microscopy Leica TCS SP8 STED 3X using a 20x multi NA 0.75 objective.
521 Images were recorded with a frame rate of 5 seconds at 400 Hz. Seedling mounting was performed as
522 described in (Krebs & Schumacher, 2013). The plant tissues were incubated overnight in half strength
523 MS, 21°C and continuous light conditions before the day of imaging. $[Ca^{2+}]_{cyt}$ in the abscission zone were
524 detected using WT plants expressing R-GECO1 (Keinath et al., 2015). Abscission zones of position 7 were
525 used. Mounting of the abscission zones were performed using the same device as for seedlings (Krebs
526 & Schumacher, 2013). The abscission zones were incubated in half strength MS medium for 1 h before
527 imaging. Measurements were performed with a Zeiss LSM880 Airyscan using a Plan-Apochromat 10×

528 air NA 0.30 objective. Images were recorded with a frame rate of 10 seconds. R-GECO1 was excited
529 with a white light laser at 561 nm and its emission was detected at 590 nm to 670 nm using a HyD
530 detector. Laser power and gain settings were chosen for each experiment to maintain comparable
531 intensity values. For mIDA and flg22 two-fold concentrations were prepared in half strength MS. mIDA
532 or flg22 were added in a 1:1 volume ratio to the imaging chamber (final concentration 1 µM). ATP was
533 prepared in a 100-fold concentration in half strength MS and added as a last treatment in a 1:100
534 volume ratio (final concentration 1 mM) to the imaging chamber as a positive control for activity of the
535 R-GECO1 sensor (Movie 3). Image processing was performed in FIJI. These steps are: background
536 subtraction, gaussian blur, MultiStackReg v1.45 (http://bradbusse.net/sciencedownloads.html), 32-bit
537 conversion, threshold. Royal was used as a look up table. Fluorescence intensities of indicated ROIs were
538 obtained from the 32-bit images (Krebs & Schumacher, 2013). Normalization was done using the
539 following formula $\Delta F/F = (F-F0)/F0$ where F0 represents the mean of at least 1 min measurement
540 without any treatment. R-GECO1 measurements were performed at the Center for Advanced imaging
541 (CAi) at HHU and at NorMIC Imaging platform at the University of Oslo.

542 Calcium measurements using the Aequorin (pMAQ2) sensor

543 $[Ca^{2+}]_{cyt}$ in seedlings and flowers were detected using WT plants expressing p35S-apoaequorin (pMAQ2)
544 located to cytosol (Aeq) (Knight et al., 1991; Ranf et al., 2011). Aequorin luminescence was measured
545 as previously described (Ranf et al., 2012). Emitted light was detected by the use of a Wallac 1420
546 VICTOR2 microplate luminometer (PerkinElmer). Differences in Aeq expression levels due to seedling
547 size and expression of sensor were corrected by using luminescence at specific time point (L)/Max
548 Luminescence (Lmax). Lmax was measured after peptide treatment by individually adding 100 µL 2 M
549 $CaCl_2$ to each well and measuring luminescence constantly for 180 seconds (Ranf et al., 2012). 2 M $CaCl_2$
550 disrupts the cells and releases the Aeq sensor into the solution where it will react with Ca^{2+} and release
551 the total possible response in the sample (Lmax) in form of a luminescent peak. A final concentration of
552 1 µM mIDA was added to each wells at the start of measurements. For inhibitor treatments, Aeq-
553 seedlings were incubated in in 2 mM EGTA (Sigma-Aldrich) or 1 mM $LaCL_3$ (Sigma-Aldrich) O/N before
554 measurements. For seedlings 3 independent experiments were performed with 12 replications in each
555 experiment. For flowers 3 independent experiments were performed with 4-6 replications in each
556 experiment.

557 Measurements of reactive oxygen species (ROS)

558 ROS production was monitored by the use of a luminol-dependent assay as previously described
559 (Butenko et al., 2014) using a Wallac 1420 VICTOR2 microplate luminometer (PerkinElmer). Arabidopsis
560 leaves expressing *35S:HAE:YFP* were cut into leaf discs and incubated in water overnight before

561 measurements. A final concentration of 1 µM mIDA was added to each well at the start of
562 measurements. All measurements were performed on 6 leaf discs and each experiment was repeated
563 3 times.

564 ROS stain (H2DCF-DA)

565 Flowers at position 6 were gently incubated in staining solution (25 µM (2',7'-dichlorodihydrofluorescein
566 diacetate) (H2DCF-DA) (Sigma-Aldrich, D6883), 50 mM KCL, 10 mM MES) for 10 min and further washed
567 3 times in wash solution (50 mM KCL, 10 mM MES). For the *hae hsl2* mutant the floral organs were
568 forcibly removed immediately before imaging. Imaging was done using a Dragonfly Airy scan spinning
569 disk confocal microscope, excited by a 488 nm laser. A total of 9 flowers per genotype were imaged.
570 The experiment was repeated 2 independent times.

571 Callose deposition staining and quantification

572 Callose deposition experiments were conducted as per (Luna et al., 2011) with modifications. Seeds
573 from WT and *fls2* were vapor-phase sterilized for 4 h. Seeds were transferred to 12-well plates
574 containing 1 mL of filter-sterilized MS medium and 0. 5 % MES with a final pH of 5.7, and stratified in
575 the dark at 4 °C for 2 days. Plates were consecutively transferred to a growth chamber with 16 h of light
576 (150µE m-2 s-1)/8h darkness cycle at 22°C. On the seventh day, growth medium was refreshed under
577 sterile conditions. On the eight day the treatments were applied: addition of 10 µL of water (mock); 10
578 µL of 100 µM flg22; or 10 µL of 100 µM mIDA (final peptide concentrations of 1 µM). After 24 h the
579 growth medium was replaced with 9 5% ethanol and incubated overnight. Ethanol was replaced with
580 an 8 M NaOH softening solution and incubated for 2 h. Seedlings were washed 3 times in water and
581 immediately transferred to the aniline blue staining solution (100 mM KPO4-KOH, pH 11; 0.01 % aniline
582 blue). After 2 h of staining, the seedlings were mounted on 50 % glycerol and imaged under a ZEISS
583 epifluorescence microscope with UV filter (BP 365/12 nm; FT 395 nm; LP 397 nm). Image analysis was
584 performed on FIJI (Schindelin et al., 2012). In brief, cotyledons were cropped and measured the leaf
585 area. Images were thresholded to remove autofluorescence and area of depositions measured to
586 calculate the ratio of callose deposition area per cotyledon area unit. Between 9-12 seedlings were
587 analyzed per genotype x treatment combination.

588 Real time quantitative PCR (RT-qPCR)

589 Seven days-old Arabidopsis seedlings (WT, *hae hsl2*) grown vertically on ½ sucrose MS-2 plates were
590 transferred to liquid ½ MS-2 medium (non-treated) and liquid ½ MS-2 medium containing 1 µM peptide
591 (Supplementary Table 1) and incubated in growth chambers for 1 h or 12 h. Seedlings were flash-frozen
592 in liquid nitrogen before total RNA was extracted using SpectrumTM Plant Total RNA Kit (SIGMA Aldrich).

593 cDNA synthesis was performed as previously described (Grini et al., 2009). RT-qPCR was performed
594 according to protocols provided by the manufacturer using FastStart Essential DNA Green Master
595 (Roche) and LightCycler96 (Roche) instrument. ACTIN2 was used to normalize mRNA levels as described
596 in (Grini et al., 2009). Two-three biological replicates and 4 technical replicates including standard curves
597 were performed for each sample.

598 Petal Break strength (pBS)

599 The force required to remove a petal at a given position on the inflorescence was measured in gram
600 equivalents using a load transducer as previously described (Stenvik et al., 2008). Plants were grown
601 until they had at least 15 positions on the inflorescence. A minimum of 15 petals per position were
602 measured. pBS measurements were performed on WT, *rbohd rbohf* (SALK_070610 SALK_059888) and
603 *hae hsl2* (SALK_021905 SALK_030520) plants.

604 Statistical methods

605 Two tailed students t-test ($p < 0.05$) was used to identify significant differences in the fluorescent GUS
606 assay by comparing treated samples to untreated samples of the same plant line. Statistical analysis of
607 the RT-qPCR results was performed on all replicas using ONE-WAY or TWO-WAY ANOVA (as stated in
608 the figure text) and post-hoc Tukey's test ($p < 0.05$). Two tailed students t-test with ($p < 0.05$) was used
609 in the petal break strength (pBS) measurements to identify significant differences from WT at a given
610 position on the inflorescence.

611

612 Acknowledgements

613 We thank M.K. Anker, I.M. Stø, V. Iversen and R. Falleth for technical assistance in the laboratory and
614 phytotrone. We thank the NorMic Imaging platform for the use and technical support. This work was
615 supported by the Research Council of Norway (grant 230849) to V.O.Lalun and M.A.Butenko Work by R.
616 Simon and and M.Breiden was supported through CEPLAS.

617

618 Author contributions

619 V.O.L generated Arabidopsis lines and constructs, tested IDA expression to biotic and abiotic stress,
620 performed gene expression studies, phenotypic analysis of mutants and ROS measurements. V.O.L and
621 M.B. performed Ca^{2+} measurements. V.O.L, S. G-T., M.B., R.S and M.A.B designed experiments, analyzed
622 data, and drafted the manuscript. V.O.L and M.A.B wrote the paper with input from all authors.

623

624 Competing interests

625 The authors have no competing interests.

626 Materials and correspondence

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628

629

630 Supplementary Tables

631 Supplementary Table 1: Peptide sequences

Peptide	Amino acid sequence
mIDA	PIPPSAoSKRHN
flg22	QRLSTGSRINSAKDDAAGLQIA
IDA$^{\Delta N69}$	PIPPSAoSKRH
PIP1	RFVKHSGoSPSGPGH

632 (o = hydroxyproline)

633

634 Supplementary Table 2: Primers sequences and function

Function	Primer name	Primer sequence 5'-3'
Cloning promoter FLS2	PromFLS2 -988bp Attb1	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTA GAAGTTGTGAATTGTGAT'3
Cloning promoter FLS2	PromFLS2 -988bp Attb2	5'GGGGACCACTTTGTACAAGAAAGCTGGGTA GGTTTAGACTTTAGAAGA'3
Genotype hsl2	Hsl2 LP	5'CGTCTTGAGCTAGCCAACAAC'3
Genotype hsl2	Hsl2 RP	5'GTCCAATCAAGTGGAGAAACG'3

Genotype hae	Haesa LP	5’CACCTTCCTTCTCTCCATTCC’3
Genotype hae	Haesa RP	5’GTTCGAGAAGTGACAAGCGAG’3
Genotype SALK-lines	Lbb1-	5’GCGTGGACCGCTTGCTGCAACT’3
Genotype rbohd SALK_070610C	LP_SALK_070610C	5’TTTCAACGCCTTTTGGTACAC’3
Genotype rbohd SALK_070610C	RP_SALK_070610C	5’GTTACCTATTCTTTTGCCGGG’3
Genotype rbohf SALK_059888	LP_SALK_059888	5’CAAAGAGCTCTTCGTGGTTTG’3
Genotype rbohf SALK_059888	RP_SALK_059888	5’TCTCTATTGTATCTTGTGTCACCG’3
Genotype fls2 SALK_062054	FLS2_SALK_062054 Fw	5’GGTTCGATTCCTTCTGGAATC’3
Genotype fls2 SALK_062054	FLS2_SALK_062054 Rv	5’CCTGAGTTTTTGAAGCTTCCC’3
qPCR	33.FRK1 Fw	5’AACTTAGGAGACTATTTGGCAGGTAA’3
qPCR	33.FRK1 Rv	5’TGCATCTAATGATATCTTCAACCTCT’3
qPCR	63.PEP3 Fw	5’GCGAGGAAGATGAGAGTATCG’3
qPCR	63.PEP3 Rv	5’TCAATGGTCATGCCATCTTCT’3
qPCR	91.MYB51 Fw	5’GGCCAATTATCTTAGACCTGACA’3
qPCR	91.MYB51 Rv	5’CCACGAGCTATAGCAGACCATT’3
qPCR reference gene	act2int2 sense	5’CCCTGAGGAGCACCCAGTTCTACTC’3
qPCR reference gene	act2int2 antisense	5’CCGCAAGATCAAGACGAAGGATAGC’3

635

636

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Main figures - A dual function of the IDA peptide in regulating cell separation and modulating plant immunity at the molecular level

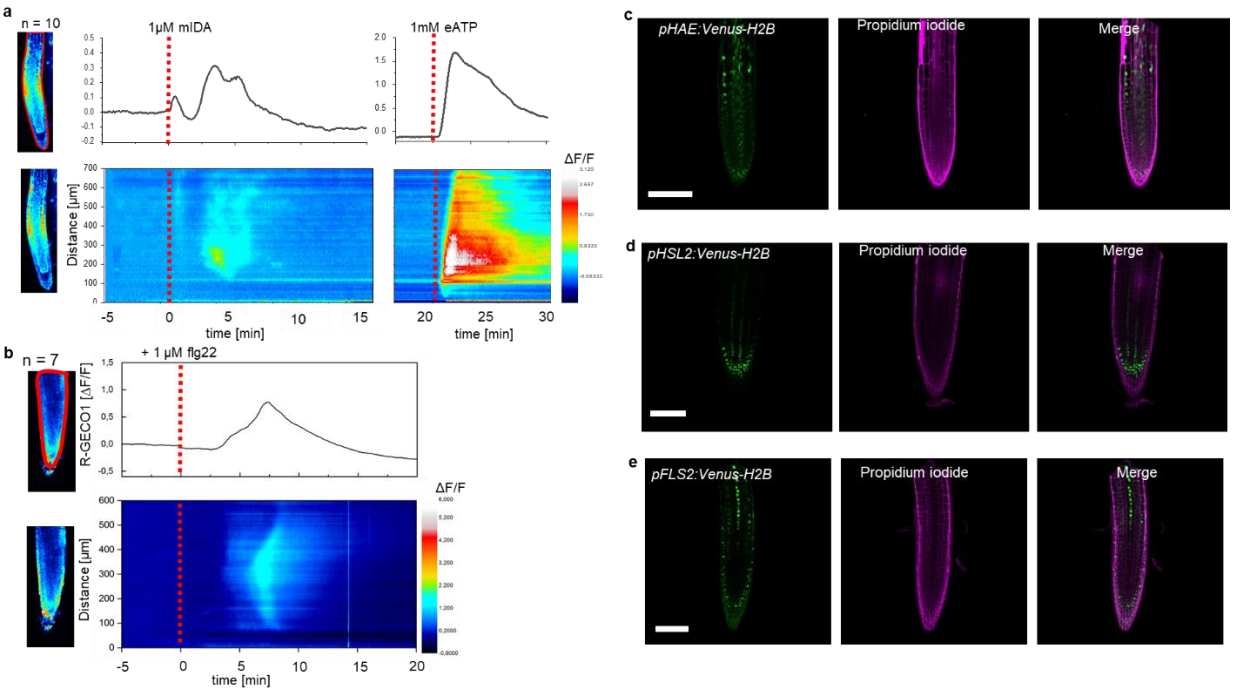


Fig. 1: mIDA-induced $[Ca^{2+}]_{cyt}$ release in Arabidopsis roots correlates with *pHAE* and *pHSL2* activity

a, Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from regions of interest (ROI) (upper panel, outlined in red) in the meristematic and elongation zone of the root. Fluorescence intensities (ΔF/F) over time of the whole root represented in a heat map (lower panel). Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the ROI in response to 1 µM mIDA over time. (see also Movie 1). Red lines at 5 minutes (min) indicates application of mIDA peptide or application of eATP at 22 min. Representative response from 10 roots (Sup Fig. 2). The increase in $[Ca^{2+}]_{cyt}$ response propagates through the roots as two waves. **b,** For comparison; Normalized R-GECO1 fluorescence intensities (ΔF/F) measured from regions of interest (ROI) (outlined in red, upper panel) in response to 1µM flg22 over time. Fluorescence intensities (ΔF/F) over time of the whole root represented in a heat map (lower panel). Red line at 0 min (min) indicates application of flg22 peptide. Representative response from 7 roots (Sup Fig. 3). The increase in $[Ca^{2+}]_{cyt}$ response propagates through the roots as a single wave seen as normalized R-GECO1 fluorescence intensities (ΔF/F) shown as a heat map (see also movie 2). **c,d,e,** Expression of the receptors **c**, *pHAE:Venus-H2B* **d**, *pHSL2:Venus-H2B* and **e**, *pFLS2:Venus-H2B* in 7 days-old roots. Representative pictures of n = 8. Scale bar = 50 µm, single plane image, magenta = propidium iodide stain.

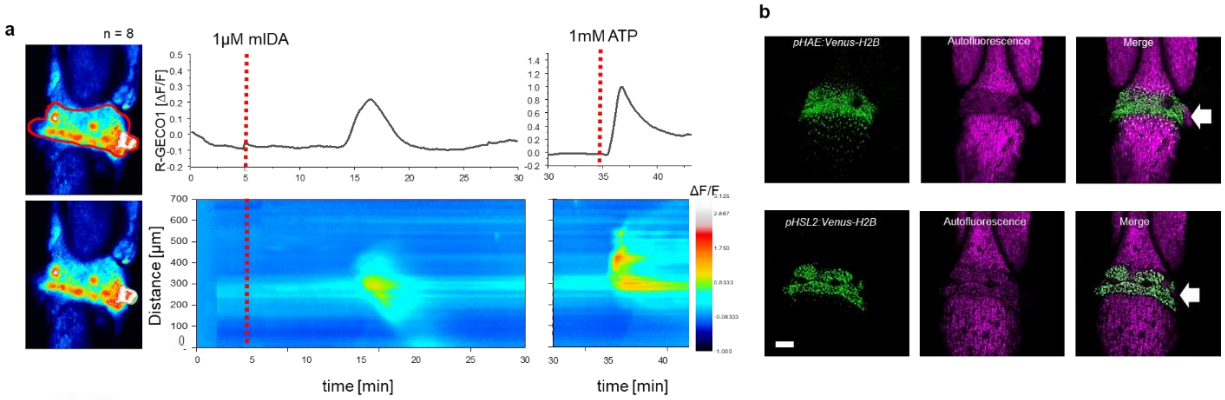
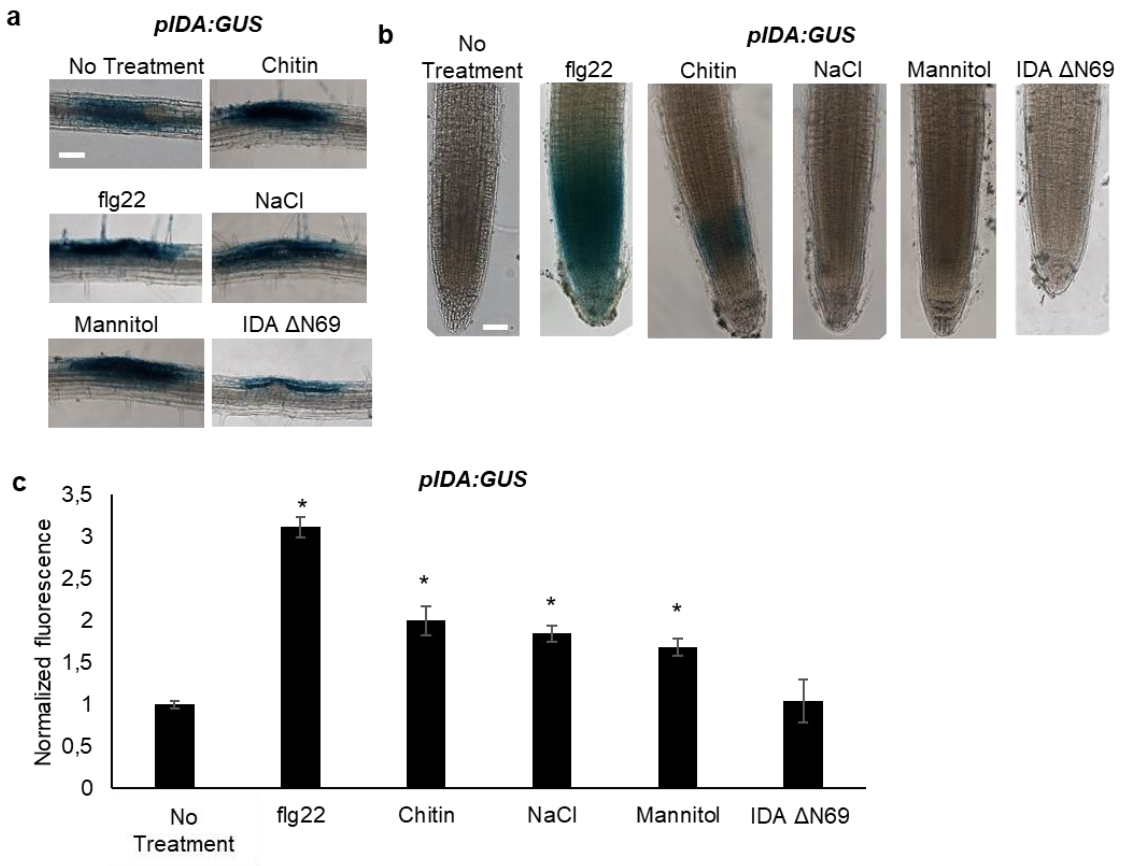


Fig. 2 : mIDA-induced $[Ca^{2+}]_{cyt}$ release in Arabidopsis abscission zones.

a, Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from regions of interest (ROI) (outlined in red) in floral abscission zone (AZ)s. Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the ROI in response to 1 µM mIDA over time (see also Movie 3). Representative response from 8 flowers (Sup. Fig 6). Red lines at 5 minutes (min) indicates application of mIDA peptide or application of eATP at 35 min (for AZs), respectively. The increase in $[Ca^{2+}]_{cyt}$ response propagates through the AZ as a single wave. **b**, Expression of *pHAE:Venus-H2B* and *pHSL2:Venus-H2B* in flowers at position 6 (See Sup Fig. 5a for positions) (arrowhead indicates AZ). Representative pictures of n = 8, scale bar = 100 µm, maximum intensity projections of z-stacks.



911

912 Fig. 3: *IDA* is induced by biotic and abiotic stress

913 Representative pictures of *pIDA:GUS* expression after 12 h treatment with 1 µM flg22, chitin, NaCl,
914 Mannitol and 1 µM IDAΔN69 in **a,** cells surrounding emerging lateral roots, and **b,** in the main root . **c**,
915 Normalized emitted fluorescence of fluorochrome 4-methyl umbelliferone (4-MU) in 7 days-old
916 seedlings after 12 h treatment with 1 µM flg22, chitin, NaCl, Mannitol and 1 µM of the inactive IDA
917 peptide, IDAΔN69. Normalized to 1 on No Treatment sample. Controls were not subjected to any stimuli
918 (No treatment). n = 6, experiment repeated 3 times. * = significantly different from the non-treated (No
919 treatment) sample ($p < 0.05$, student t-test, two tailed). Controls were not subjected to any stimuli (No
920 treatment). **a,b**, Representative picture of n = 10, experiment repeated 3 times, scale bar = 50 µm.

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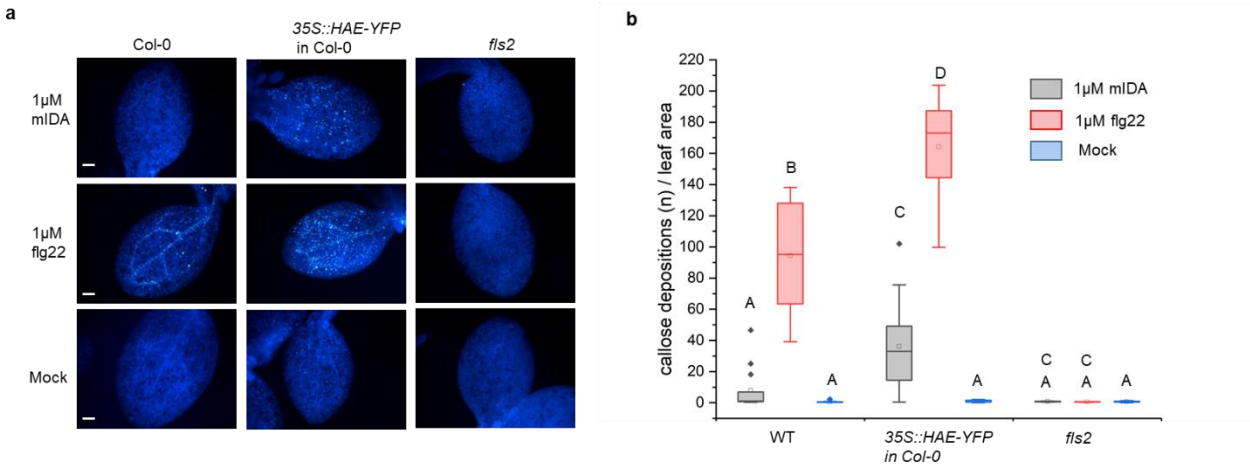
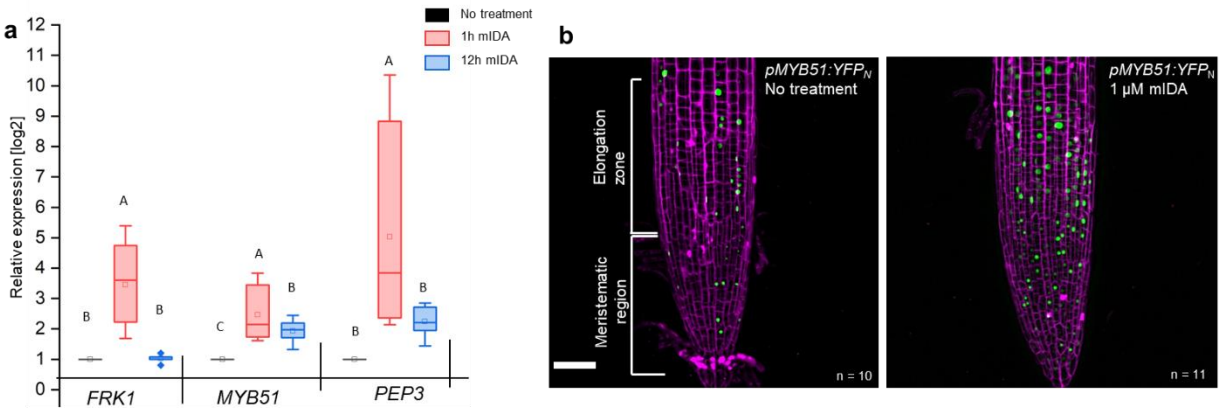


Fig. 4: mIDA induce callose deposition in Arabidopsis cotelydons expressing 35S::HAE-YFP

Callose deposition in Col-0 WT, 35S:HAE in Col-0 and *fls2* treated with water (mock treatment), 1 µM mIDA or 1uM flg22. **a**, Callose deposition could be observed in cotyledons of eight day old Col-0 WT and *35S:HAE in* seedlings treated with 1 µM flg22, and to a smaller extend in 35S:HAE treated with 1uM mIDA. No callose deposition could be detected in *fls2* mutant treated with…... Representative images of 9-12 seedlings per genotype. Scale bar 500 µm. **b**, Total callose depositions for the different genotypes treated with water (mock treatment), 1 µM mIDA or 1 µM flg22. Statistically significant difference at ($p < 0,05$). N = 9-12

933 

934 Fig. 5: mIDA induced transcription of defense-associated marker genes

935 **a**, Transcripts of *FRK1*, *MYB51*, and *PEP3* in WT Col-0 seedlings exposed to 1 µM mIDA for 1 h (red) and
936 to 1 µM mIDA for 12 h (blue) compared to untreated tissue (black). RNA levels were measured by RT-
937 qPCR analysis. *ACTIN* was used to normalize mRNA levels Figure represent three biological replicates
938 with four technical replicates. Statistical analyses was performed comparing induction times of
939 individual genes using one-way ANOVA and post-hoc Tukey's test ($p < 0.05$). **b**,*pMYB51:YFPN* expression
940 is enhanced in roots after 7 h exposure to 1 µM mIDA peptide compared to untreated roots (control),
941 scale bar = 50 µm, maximum intensity projections of z-stacks, magenta = propidium iodide stain.

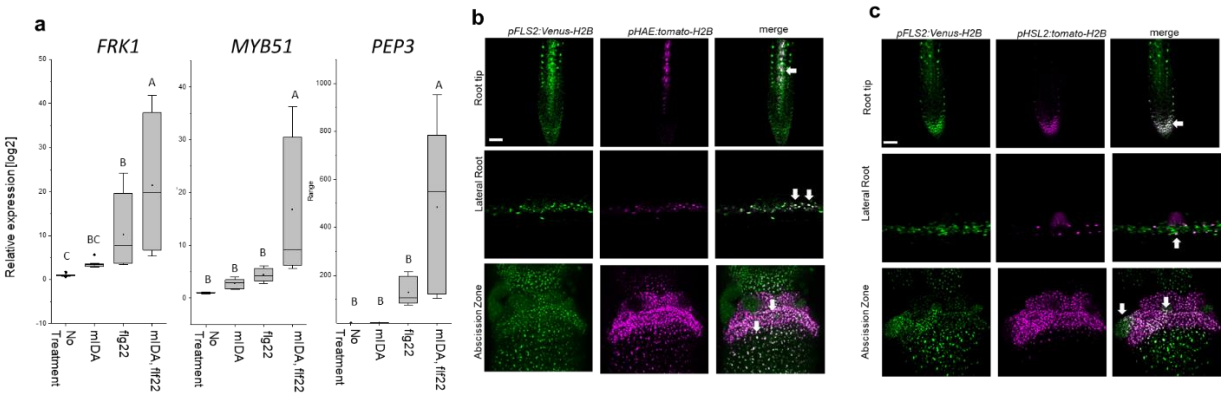
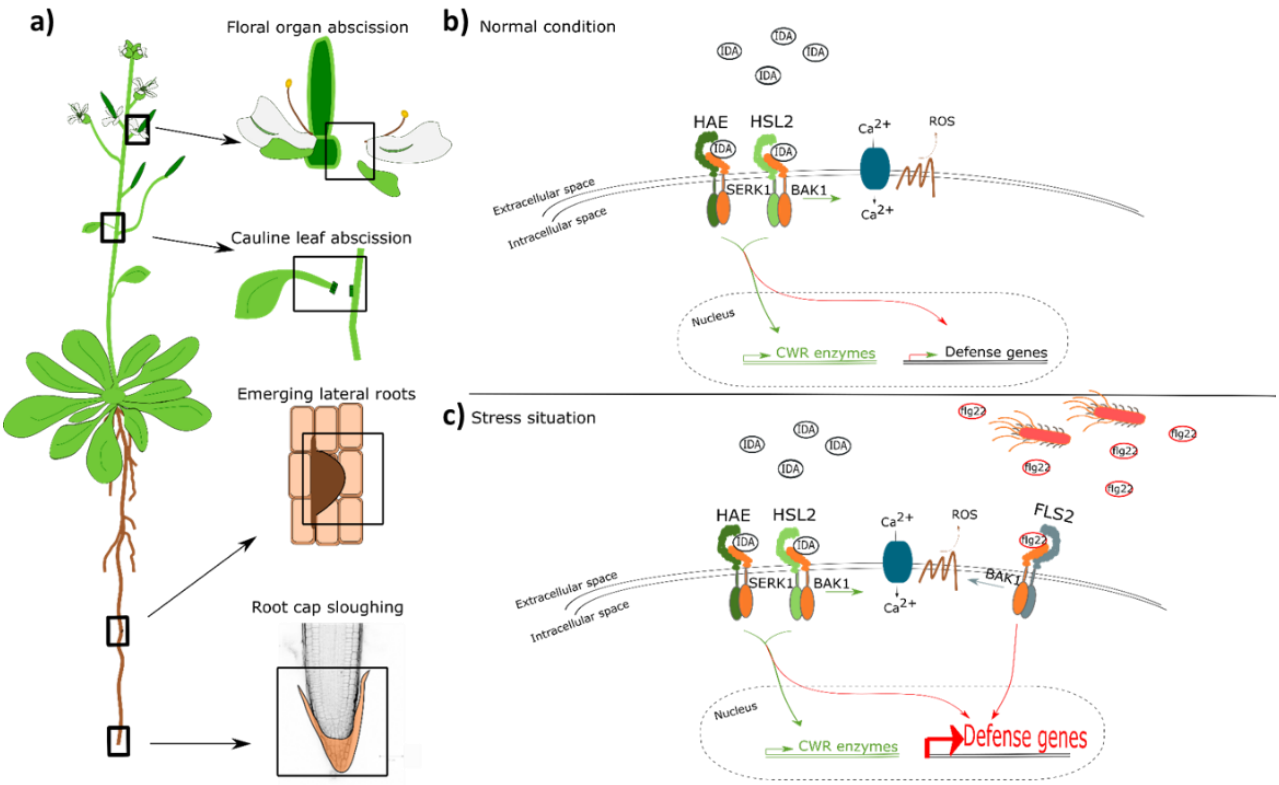


Fig. 6: mIDA and flg22 co-treatment extremely enhances the transcription of defense-associated marker genes

a, RT-qPCR data showing transcription of *FRK1*, *MYB51*, and *PEP3* in Col-0 WT seedlings exposed to 1 µM mIDA, 1 µM flg22 or a combination of 1 µM mIDA and 1 µM flg22 for 1 h compared to untreated seedlings (No treatment control). *ACTIN* was used to normalize mRNA levels. Figure represent three biological replicates with four technical replicates. Statistical analyses comparing No Treatment to peptide treated samples was performed using one-way ANOVA and post-hoc Tukey's test ($p < 0.05$). **b,** Microscopic analysis of 7-days-old *pFLS2:Venus-H2B pHAE:Tomato-H2B* expressing plants of root tip (upper panel) and lateral root (middle panel) of 7 days old plants, and flowers at position 6 (lower panel). Fluorescent nuclei representing co-expression of the Venus and Tomato marker could be observed in cells surrounding emerging LRs, in the stele of the root, as well as in the abscission zone. **c,** Microscopic analysis of 7-days-old *pFLS2:Venus-H2B pHSL2:Tomato-H2B* expressing plants of root tip (upper panel) and lateral root (middle panel) of 7 days old plants, and flowers at position 6 (lower panel). Fluorescent nuclei representing co-expression of the Venus and Tomato marker could be observed in the root tip, in cells surrounding emerging LRs, as well as in the abscission zone. Root pictures scale bar = 50 µm, single plane image. Abscission zone images= maximum intensity projections of z-stacks. Scale = 50µm.



962

963 **Fig. 7: IDA regulates cell separation processes, but is also involved in major transcription of defense**
964 **genes upon pathogen attack.**

965 **a,** IDA and the IDL peptides control cell separation processes during plant development and in response
966 to abiotic and biotic stress (Butenko et al. 2003; Patharkar and Walker 2016; Patharkar et al. 2017; Shi
967 et al. 2018). Tissue undergoing cell separation includes floral organ abscission, cauline leaf abscission,
968 emerging of lateral roots and root cap sloughing. **b**, During normal conditions, IDA control floral organ
969 abscission and emergence of lateral roots by relaying a signal through receptor complexes including
970 HAE, HSL2, SERK1 and BAK1 to modulate the expression of cell wall remodeling (CWR) genes as well as
971 moderately expression of defense genes. IDA activates a receptor dependent production of ROS and an
972 increase in $[Ca^{2+}]_{cyt}$. **b,** Upon stress, such as pathogen attack, the activation of HAE and HSL2 acts in
973 addition to activation of defense related receptors, such as FLS2, to enhance the expression of defense
974 related genes significantly. This ensure optimal protection of cells undergoing cell separation, which
975 may be major entry routes during a pathogen attack.

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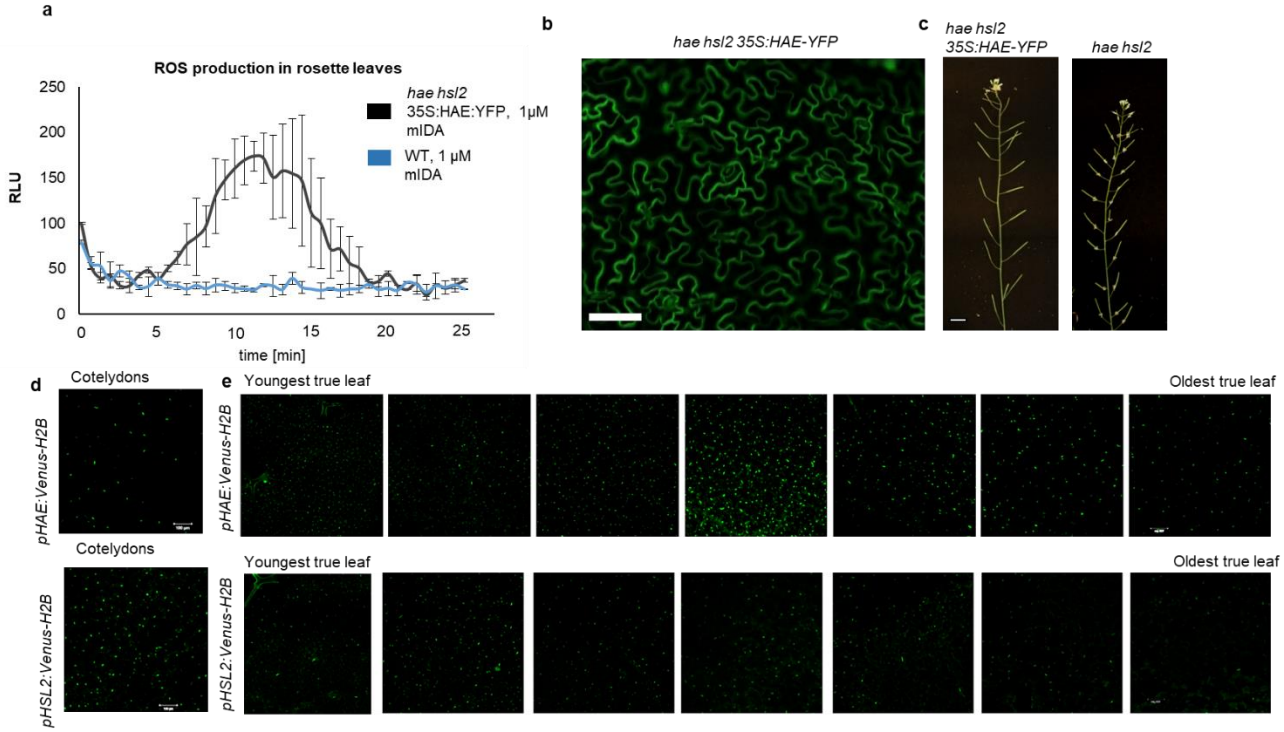
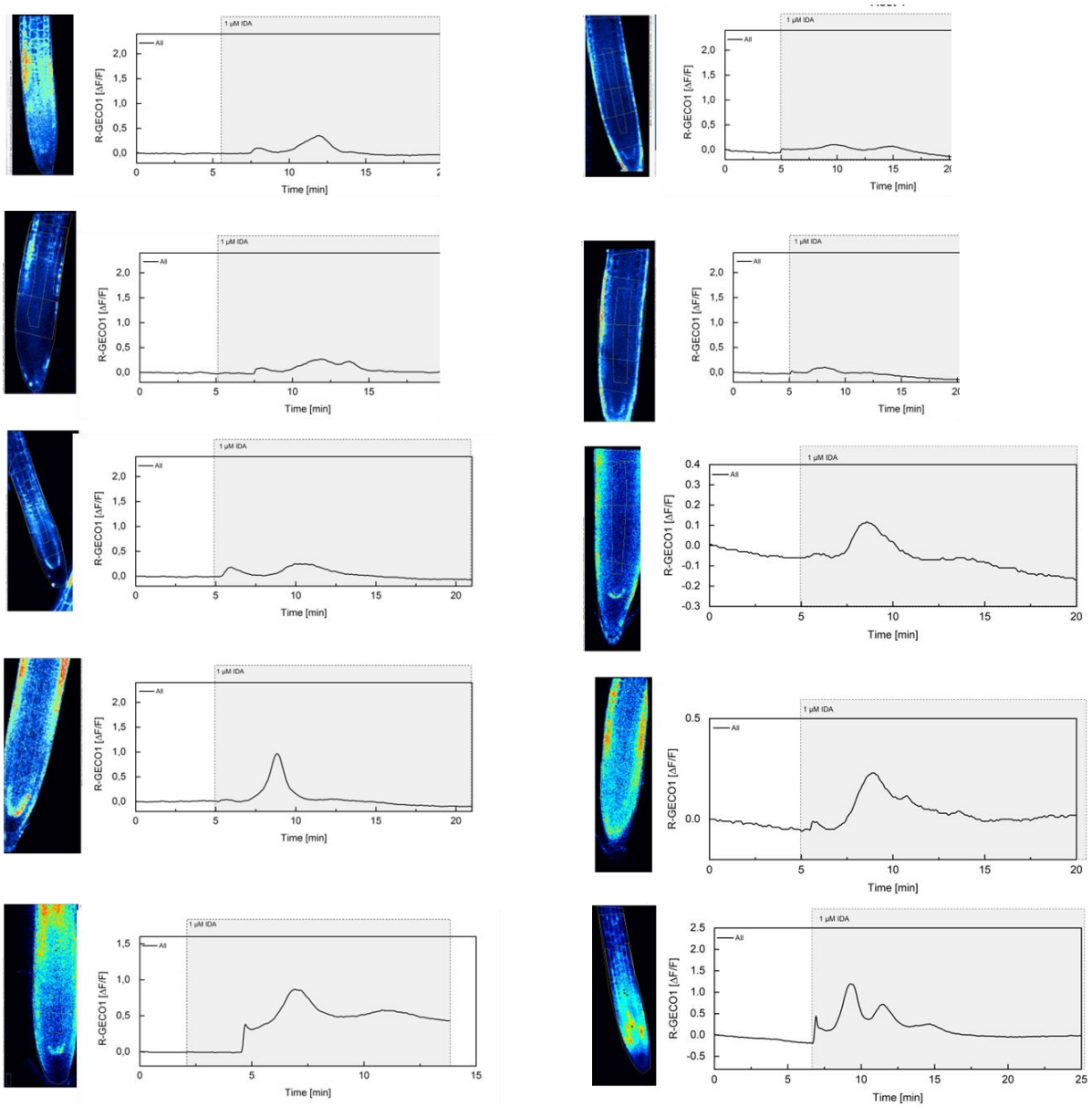
Supplementary figures - A dual function of the IDA peptide in regulating cell separation and modulating plant immunity at the molecular level



Sup Fig. 1: IDA induces ROS production in Arabidopsis

a, ROS production detected by the luminol-based assay was monitored over time as RLU. ROS production from *hae hsl2* leaf disks expressing *35S:HAE-YFP* in response to 1 µM mIDA (black) and in WT control (blue). **b**, Fluorescent image of *hae hsl2* rosette leaves expressing *35S:HAE:YFP*. Signal is detected in the plasma membrane of cells in the epidermal layer. Representative picture. Scale bar = 50 µm. **c,** *35S:HAE-YFP* complements the *hae hsl2* mutant abscission phenotype of observed. Scale = 1 cm. **d,e,** Microscopic analysis of *pHAE:Venus-H2B* (upper panel) and *pHSL2-Venus-H2B* (lower panel) in **d,** cotelydons of 7 days old seedlings and in **e,** rosette leaves of a 22 days old Arabidopsis plant containing 7 true leaves. Expression in each leaf is shown from youngest (left) to oldest (right).Scale = 100 µm



996

997 **Sup Fig. 2: mIDA-induced $[Ca^{2+}]_{cyt}$ release in Arabidopsis roots**

998 Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the ROI in response to 1 µM mIDA
999 over time. Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from a region of
1000 interest (ROI) containing the meristematic and elongation zone of the root.

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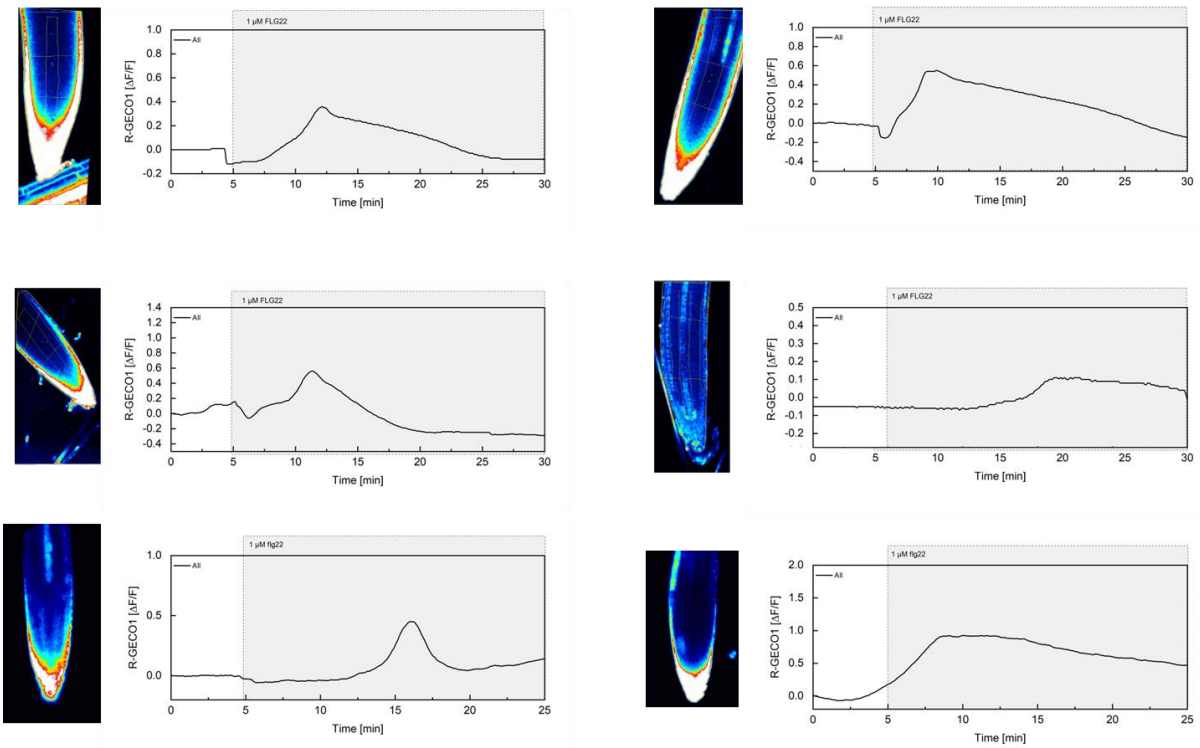

1002

1003 **Sup Fig. 3: flg22-induced $[Ca^{2+}]_{cyt}$ release in Arabidopsis roots**

1004 Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the ROI in response to 1 µM flg22
1005 over time. Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from a region of
1006 interest (ROI) containing the meristematic and elongation zone of the root.

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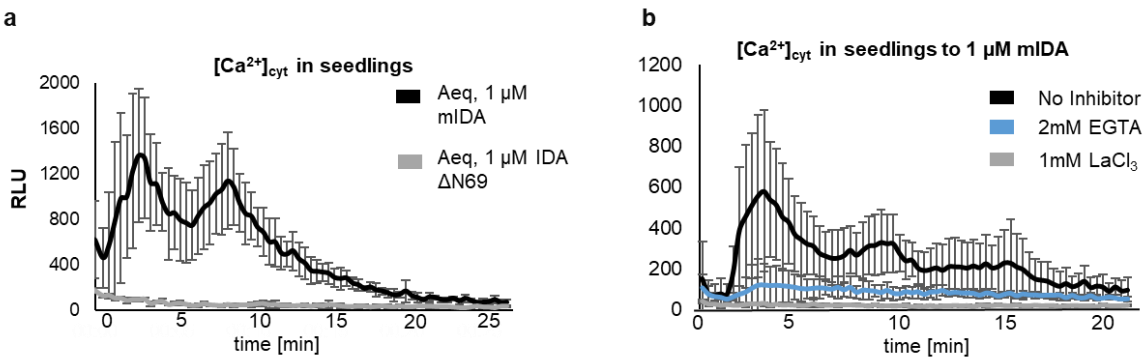
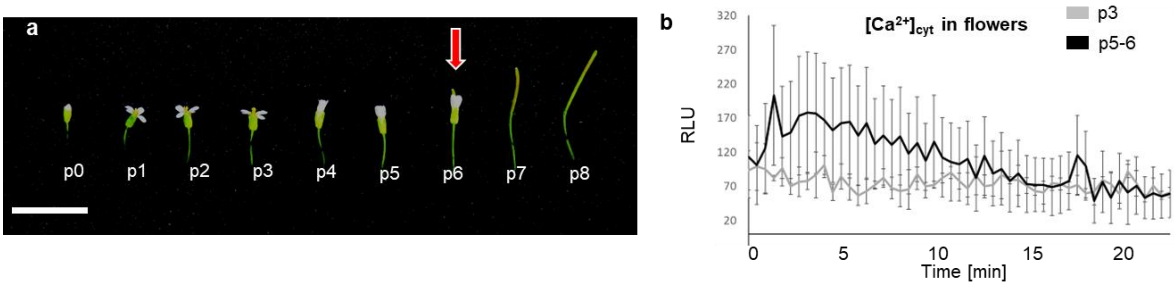


Sup Fig. 4 : The IDA triggered increase in $[Ca2+]_{cyt}$ **is abolished in the presence of** Ca^{2+} **inhibitors**

a, Increase in $[Ca^{2+}]_{cyt}$ in 7 days old seedlings expressing the cytosolic localized *Aequorin*-based luminescence Ca^{2+} sensor (*Aeq*) measured in relative light units (RLU) treated with 1 µM mIDA (black). No response is observed in *Aeq* seedlings treated with 1 µM IDAΔN69 (gray) or in *hae hsl2 Aeq* seedlings treated with 1 µM mIDA (blue). **b,** Increase in $[Ca^{2+}]_{cyt}$ in 7 days old seedlings expressing the cytosolic localized *Aequorin*-based luminescence Ca^{2+} sensor (*Aeq*) measured in relative light units (RLU) treated with 1 µM mIDA . No response is observed in *Aeq* seedlings treated with 1 mM EGTA (blue) or 1mM $LaCl_3$ (grey). Curves represent average of 3 independent experiments with 4-6 replicates in each experiment.



1024

1025 Sup Fig. 5: mIDA induces a Ca^{2+} response in flowers

1026 **a**, Arabidopsis flowers and siliques at different developmental stages. Flowers along the main
1027 inflorescence are counted from the first flower with visible white petals at the top of the inflorescence
1028 and is defined as p 1. Subsequently older flowers along the inflorescence (p0-p8). Cell wall remodeling
1029 in AZ cells, accompanied with a reduction in petal breakstrength (the force required to remove a petal
1030 from the receptacle) is initiated at p6 (red arrow). By p7 AZ cells have undergone cell separation and
1031 the floral organs have abscised. P=position **b**, Increase in $[Ca^{2+}]_{cyt}$ in flowers expressing the cytosolic
1032 localized *Aequorin*-based luminescence Ca^{2+} sensor (*Aeq*) measured in relative light units (RLU) treated
1033 with 1 µM mIDA. A $[Ca^{2+}]_{cyt}$ increase is observed in flowers where there is an initial weakening of the
1034 cell walls (p5-6) (black). No increase in luminescence was observed in *Aeq* expressing flowers of an
1035 earlier developmental stage (p3) (gray). Curves show representative measurements. Three
1036 independent experiments were performed with 4-6 replicates in each experiment.

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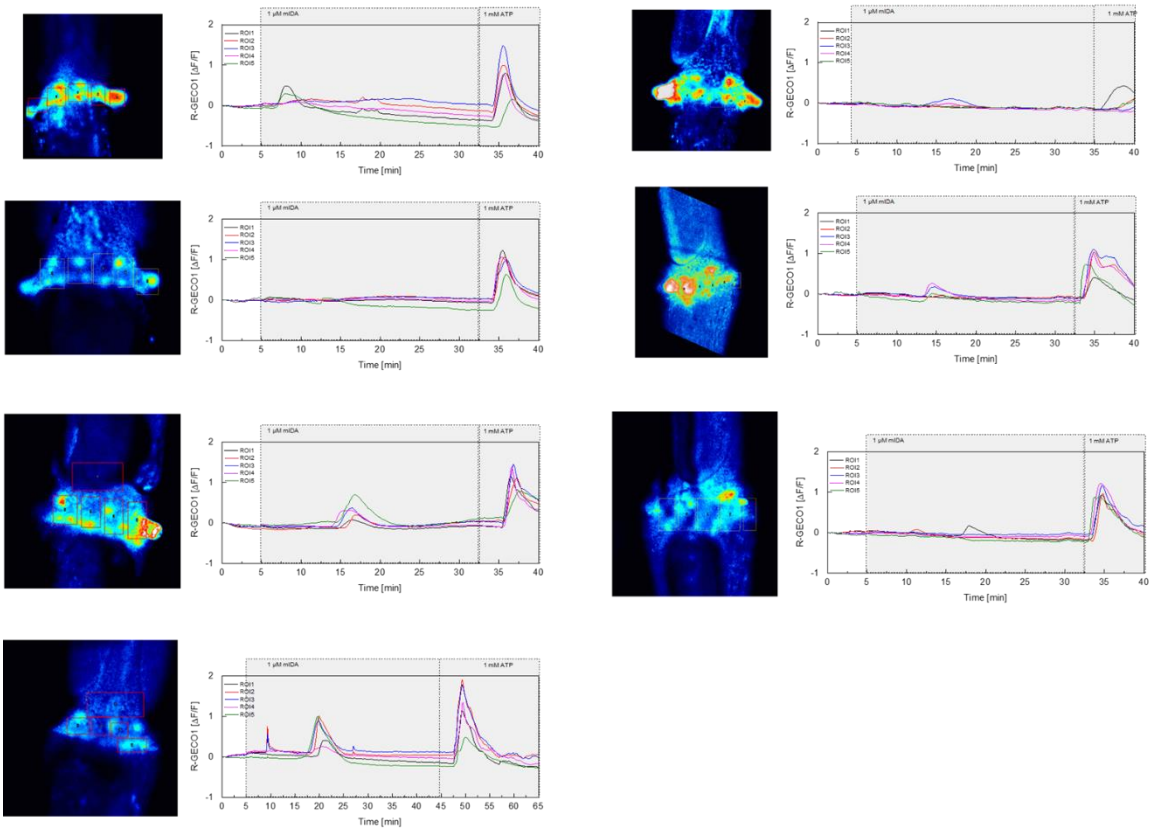

Sup Fig. 6: mIDA-induced $[Ca^{2+}]_{cyt}$ release in Arabidopsis abscission zones. Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from regions of interest (ROIs) in floral abscission zone (AZ)s. Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the ROI1-5 in response to 1 µM mIDA over time.

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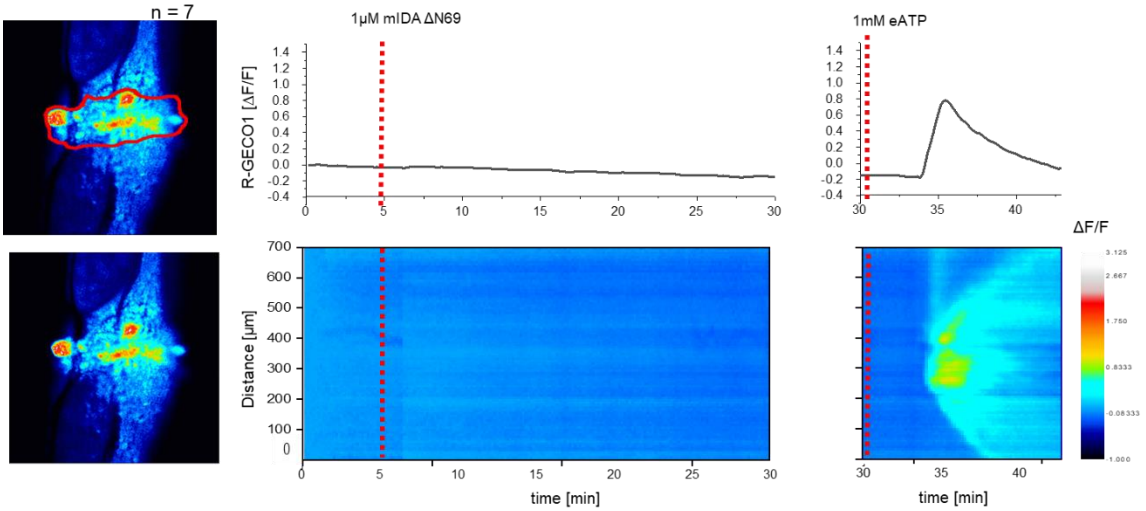

1045

1046 **Sup Fig. 7 : mIDAΔN69 does not induce a $[Ca^{2+}]_{cyt}$ release in Arabidopsis abscission zones (AZ)**

1047 Normalized R-GECO1 fluorescence intensities (ΔF/F) were measured from regions of interest (ROI)
1048 (outlined in red) in the floral AZs. Shown are cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) dynamics in the
1049 ROI in response to application of 1 µM mIDAΔN69 or eATP over time. Red lines at 5 minutes (min)
1050 indicates application of mIDAΔN69 peptide or application of eATP at 30 min (see also Movie 4).
1051 Representative response from 10 flowers. The increase in $[Ca^{2+}]_{cyt}$ response to eATP propagates
1052 through the AZ as a single wave seen as normalized R-GECO1 fluorescence intensities (ΔF/F) shown as
1053 a heat map.

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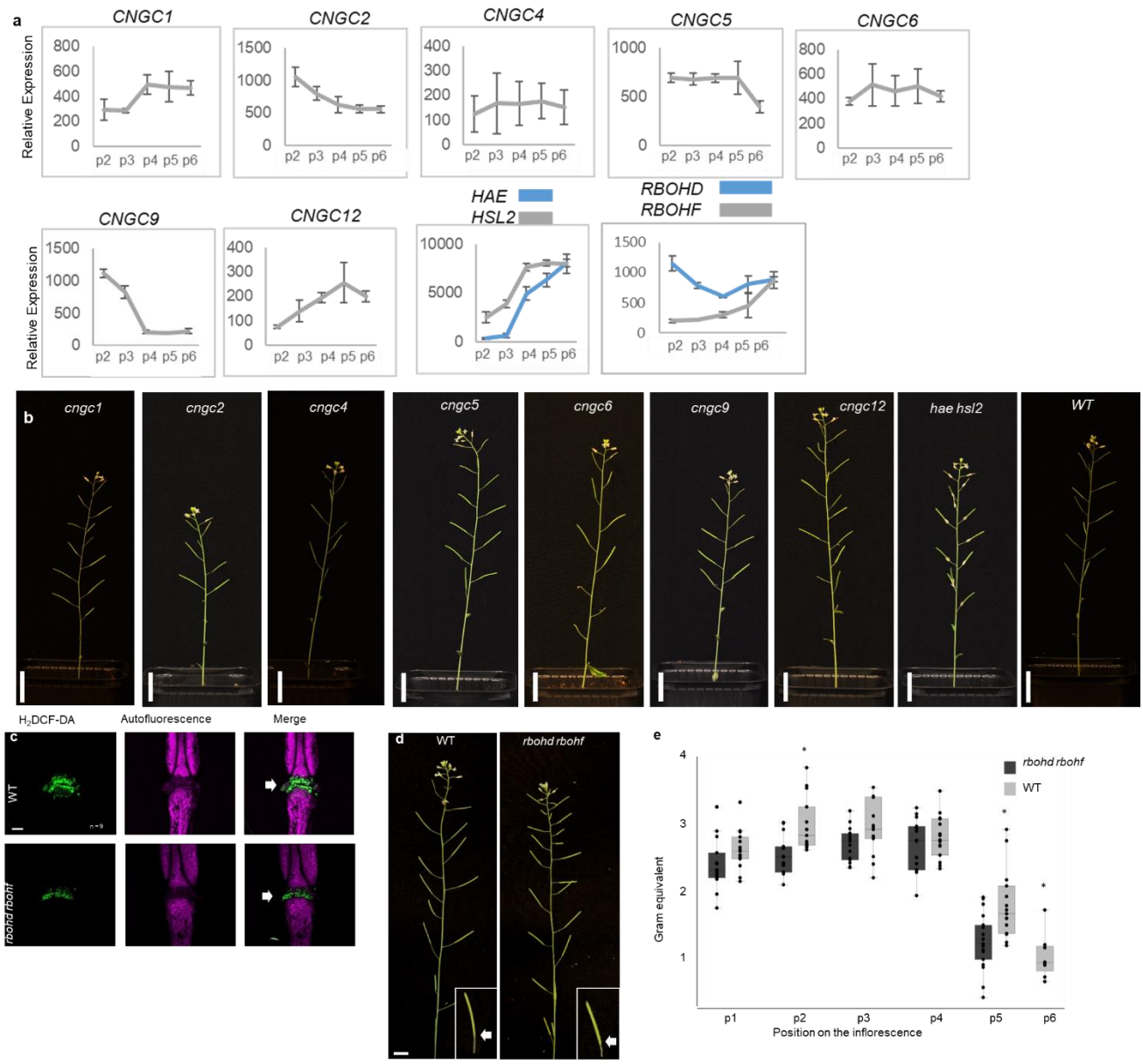
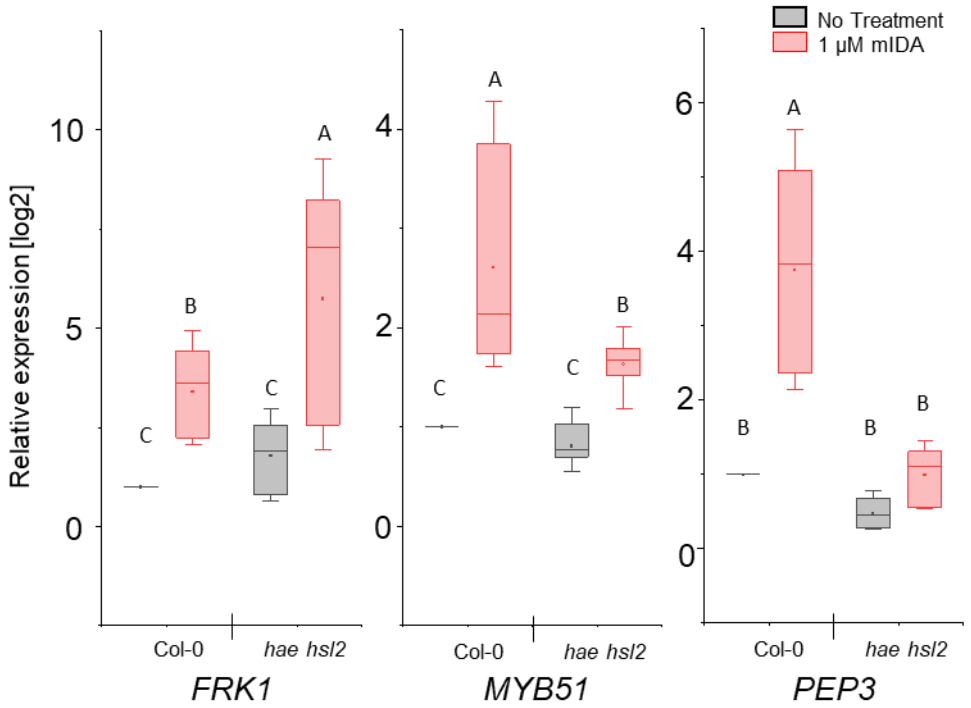


Sup Fig. 8: Investigation of CNGCs and RBOHs in the abscission process

a, Relative expression of *CNGC1*, *CNGC2*, *CNGC4*, *CNGC5*, *CNGC6*, *CNGC9*, *CNGC12, RBOHD* and *RBOHF* in the floral organs during the onset of abscission (Cai and lashbrook 2008). X-axis represents the developmental stages of abscission where position 12 represent flowers where the stamen and petals are of the same length, position 13 represent flowers at anthesis, position 15 represent flowers with stigma extended above the anthers. Floral organ abscission occurs at position 15. Relative expression of *HAE* and *HSL2* added for comparison. Nomenclature for the abscission process and relative expression values taken from (Cai and Lashbrook 2008). **b**, No floral organ abscission phenotype is observed in the single *cngc* mutant plants compared to the *hae hsl2* mutant which retains the floral organ indefinitely. Scale bar = 2cm, 5 weeks old plants. **c**, ROS production in the AZ (white arrow head) detected by using the fluorescent dye H_2DCF-DA in WT and *rbohd rbohf* flowers at position 6 (See

1069 Supplementary Fig. 5a for positions). Scale bar = 100 µm, maximum intensity projections of z-stacks.
1070 Representative pictures from 9 flowers. **d**, Representative pictures of WT and *rbohd rbohf*
1071 inflorescences, scale bar for inflorescences = 1 cm. **e**, Petal break strength (pBS) measurements of WT
1072 and the *rbohd rbohf* mutant at position 1-6 along the inflorescence. Force required to remove petals
1073 from the receptacle represented in gram equivalent. * = significantly different from WT at the given
1074 position ($p < 0.05$, student t-test, two tailed).



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1076 **Sup Fig. 9: mIDA induced enhanced transcription of defense-associated marker genes is only partially**
1077 **dependent on the HAE and HSL2 receptors**

1078 RT-qPCR data showing transcription of *FRK1*, *MYB51*, and *PEP3* in Col-0 WT and *hae hsl2* seedlings
1079 exposed to 1 µM mIDA for 1 h compared to untreated seedlings (No treatment). *ACTIN* was used to
1080 normalize mRNA levels. Figure represent 3 biological replicates with 4 technical replicates. Statistical
1081 analyses was performed comparing samples within same gene, using two-way ANOVA and post-hoc
1082 Tukey's test ($p < 0.05$).

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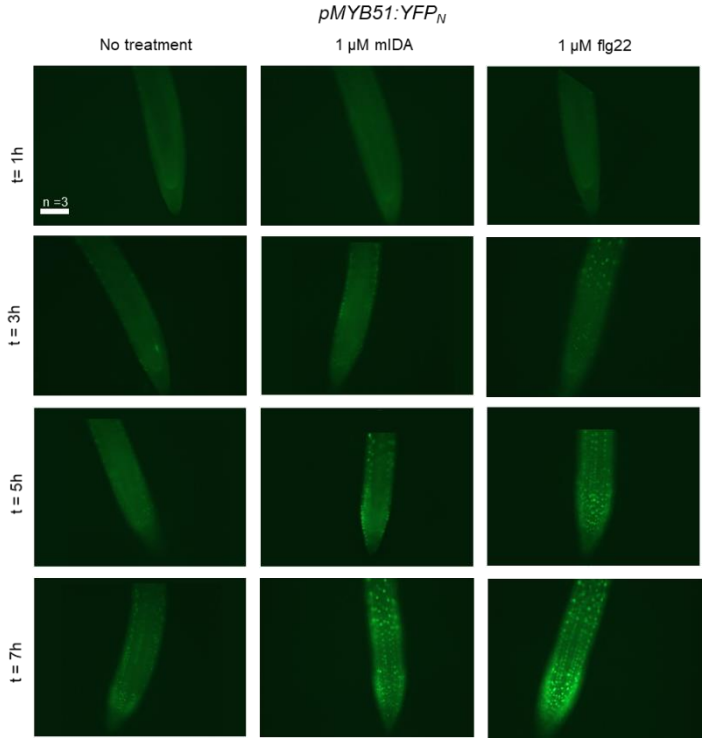

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1086 Sup Fig. 10: mIDA and flg22 induced expression of *pMYB51:YFP*

1087 Representative microscopic pictures of 7-days-old *pMYB51:YFPN* roots from seedlings transferred to
1088 liquid medium with 1 µM flg22 or 1 µM mIDA for 1, 3, 5 and 7 h using a fluorescent microscope.
1089 Control seedlings were transferred to medium with no peptide and imaged in the same time frame.
1090 Fluorescent nuclei could be observed in a similar temporal pattern in roots subjected to mIDA and
1091 flg22. Scale bar = 100 µm, single plane images using a Zeiss Axioplan2 microscope with an AxioCam
1092 HRc, 20x air objective and a YFP filter (Excitation: 500/20, Beamsplitter: FT515, Emission: 535/50), no
1093 imaging processing was performed, t = time in h. n = 3, experiment repeated 3 times.

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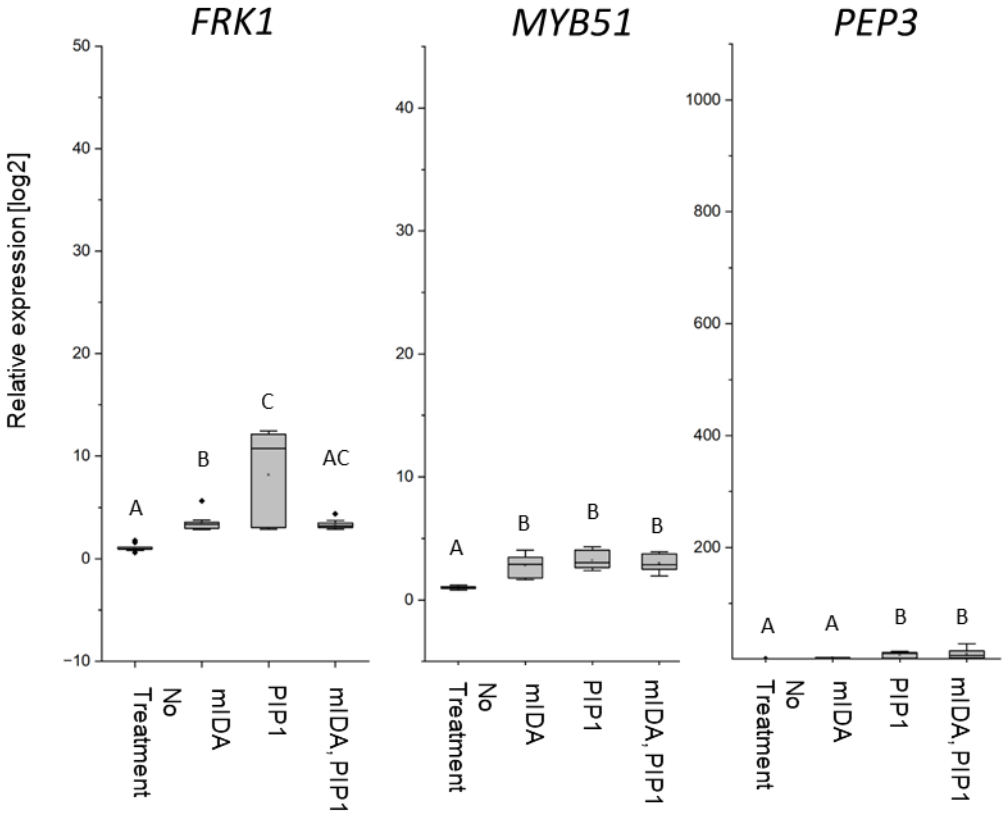

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1098 **Sup Fig. 11 : mIDA and PIP1 co-treatment do not enhances transcription of defense-associated**
1099 **marker genes**

1100 RT-qPCR data showing transcription of *FRK1*, *MYB51*, and *PEP3* in Col-0 WT seedlings exposed to 1 µM
1101 mIDA, 1 µM PIP1 or a combination of 1 µM mIDA and 1 µM PIP1 for 1 h compared to untreated
1102 seedlings (No treatment control). *ACTIN* was used to normalize mRNA levels. Figure showing extended
1103 results from experiment shown in Fig 6. For comparison, No treatment and mIDA treated samples
1104 were included in the figure (same results presented in Fig 6). Scale on y axis was kept as in Fig 6.
1105 Figure represents 2-3 biological replicates with 4 technical replicates. Statistical analyses comparing
1106 the samples was performed using one-way ANOVA and post-hoc Tukey's test ($p < 0.05$).

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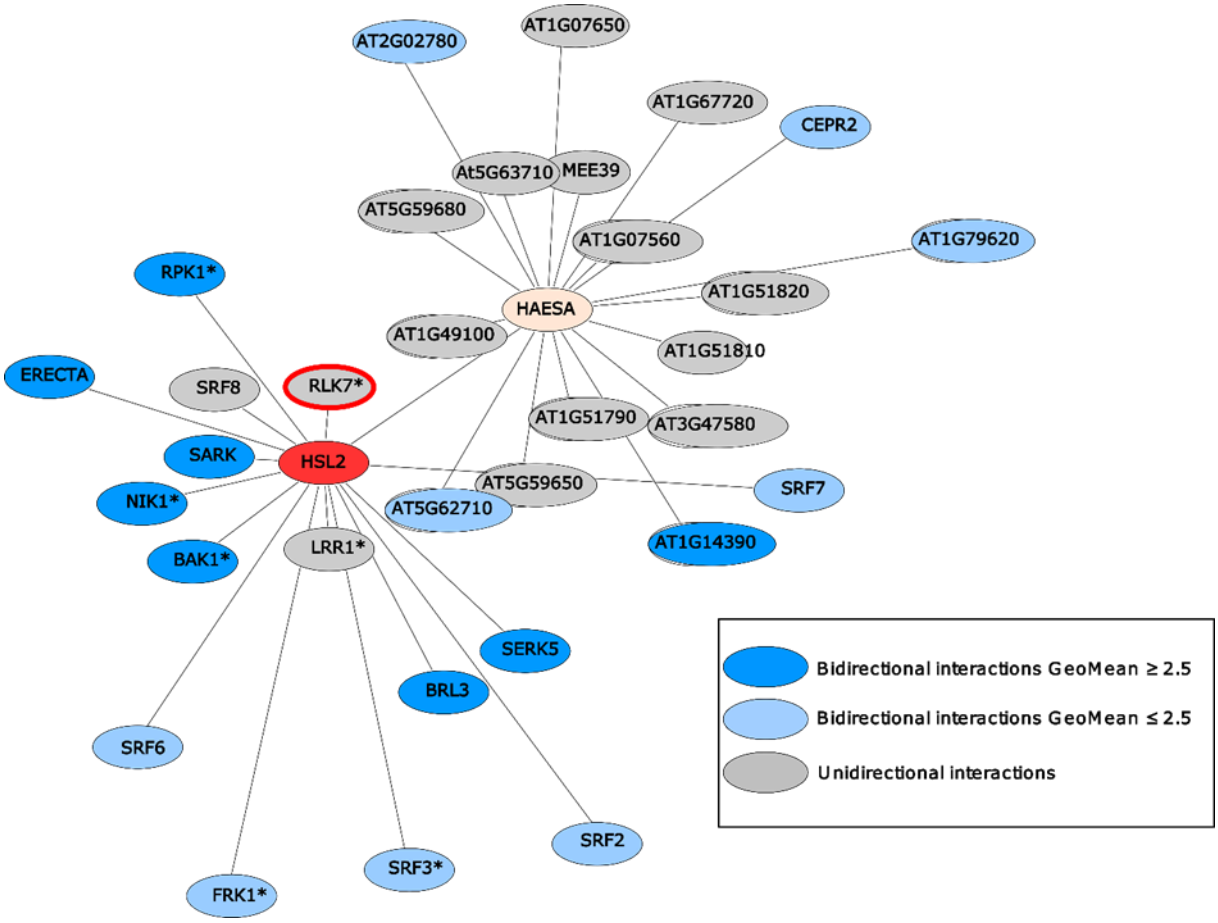

Sup Fig. 12: HAE (yellow) and HSL2 (red) display very distinct repertoire of immune and growth-related interacting LRR-RKs.

HEA and HSL2 specific interacting partners determined using a sensitized high-throughput extracellular domain interaction assay (Smakowska-Luzan et al,.2018). HSL2 and HAE show distinct interacting LRR-RKs where only HSL2 was found to interact with RLK7 (red halo). * indicates receptors known to play a function in biotic and/or abiotic responses in planta based on previous published work, dark blue nodes refer to bidirectional interactions with GeoMean ≥2.5, light blue nodes- refer to bidirectional interactions with GeoMean ≤2.5, and grey nodes refer to unidirectional interactions.