

# 1 pGG-PIP: A GreenGate (GG) entry vector collection with Plant Immune system Promoters 2 (PIP)

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## 16 Abstract

17 The regulatory sequences controlling the expression of a gene (i.e., the promoter) are essential to  
18 properly understand a gene's function. From their use in mutant complementation assays, to  
19 studying their responsiveness to different stimuli via transcriptional reporter lines or using them  
20 as proxy for the activation of certain pathways, assays using promoter sequences are valuable tools  
21 for insight into the genetic architecture underlying plant life. The GreenGate (GG) system is a  
22 plant-specific variant of the Golden Gate assembly method, a modular cloning system that allows  
23 the hierarchical assembly of individual donor DNA fragments into one expression clone via a  
24 single reaction step. Here, we present a collection of 75 GG entry vectors carrying putative  
25 regulatory sequences for *Arabidopsis thaliana* genes involved in many different pathways of the  
26 plant immune system, designated Plant Immune system Promoters (PIP). This pGG-PIP entry  
27 vector set enables the rapid assembly of expression vectors to be used for transcriptional reporters  
28 of plant immune system components, mutant complementation assays when coupled with coding  
29 sequences, mis-expression experiments for genes of interest, or the targeted use of CRISPR/Cas9  
30 genome editing. We used pGG-PIP vectors to create fluorescent transcriptional reporters in *A.*  
31 *thaliana* and demonstrated the potential of these reporters to image the responsiveness of specific

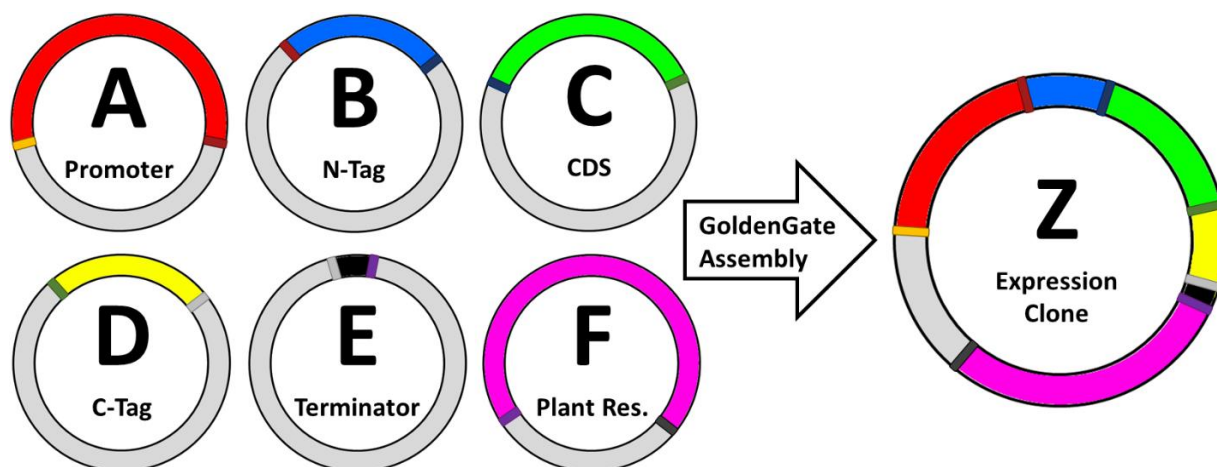
32 plant immunity genes to infection and colonization by the fungal pathogen *Fusarium oxysporum*.  
33 Using the PLANT ELICITOR PEPTIDE (PEP) pathway as an example, we show that several  
34 components of this pathway are locally activated in response to colonization by the fungus.

### 35 **Keywords**

36 Golden Gate; GreenGate; plant immunity; *Arabidopsis thaliana*; plant-microbe interactions;  
37 promoters; transcriptional reporters; PLANT ELICITOR PEPTIDE; synthetic biology; *Fusarium*  
38 *oxysporum*

### 39 **Introduction**

40 Since the development of molecular cloning in the early 1970s, the isolation of genes and  
41 promoters, and subsequent transgenesis of model organisms, has become standard practice in the  
42 life sciences (Somssich, 2022). The development of more advanced cloning methods, particularly  
43 the recombination-based Gateway technology in the year 2000, made the creation of expression  
44 clones ready for transformation ever easier (Hartley *et al.*, 2000). However, with the rise of the  
45 synthetic biology field, it is now no longer sufficient for these methods to facilitate the cloning of  
46 individual DNA fragments. To recreate entire pathways and gene circuits in plants and other model  
47 organisms, larger DNA constructs need to be readily assembled from individual components, and  
48 these distinct building blocks need to be compatible to allow the flexibility to mix and match  
49 different promoters, coding sequences, protein tags, terminators, and resistance genes for selection  
50 of transgenic lines (Meng and Ellis, 2020). This requirement was met with the new modular  
51 cloning techniques which use recombination-based hierarchical assembly of multiple donor-  
52 modules (each containing, for example, a promoter of choice, tag of choice, gene of interest, etc.)  
53 into one ordered expression clone to be used for transgenesis (Fig. 1) (Bird *et al.*, 2022). Among  
54 the developed modular cloning methods, the Golden Gate system has emerged as the most widely  
55 utilized version, and in 2013 Lampropoulos *et al.* developed the plant-specific GreenGate variant  
56 of the Golden Gate technique (Engler *et al.*, 2008, 2009; Weber *et al.*, 2011; Lampropoulos *et al.*,  
57 2013). The GreenGate toolkit provides users with a wide range of entry vectors that serve as donors  
58 for standard promoters (e.g. CaMV35S, UBQ10), protein tags (e.g. GFP, NLS, HDEL),  
59 terminators (e.g. CaMV35S, UBQ10) and plant resistance cassettes (e.g. BastaR, HygR, KanR),  
60 to build basic gene expression modules for plants.



**Figure 1: Overview of the modular Golden/GreenGate cloning principle**

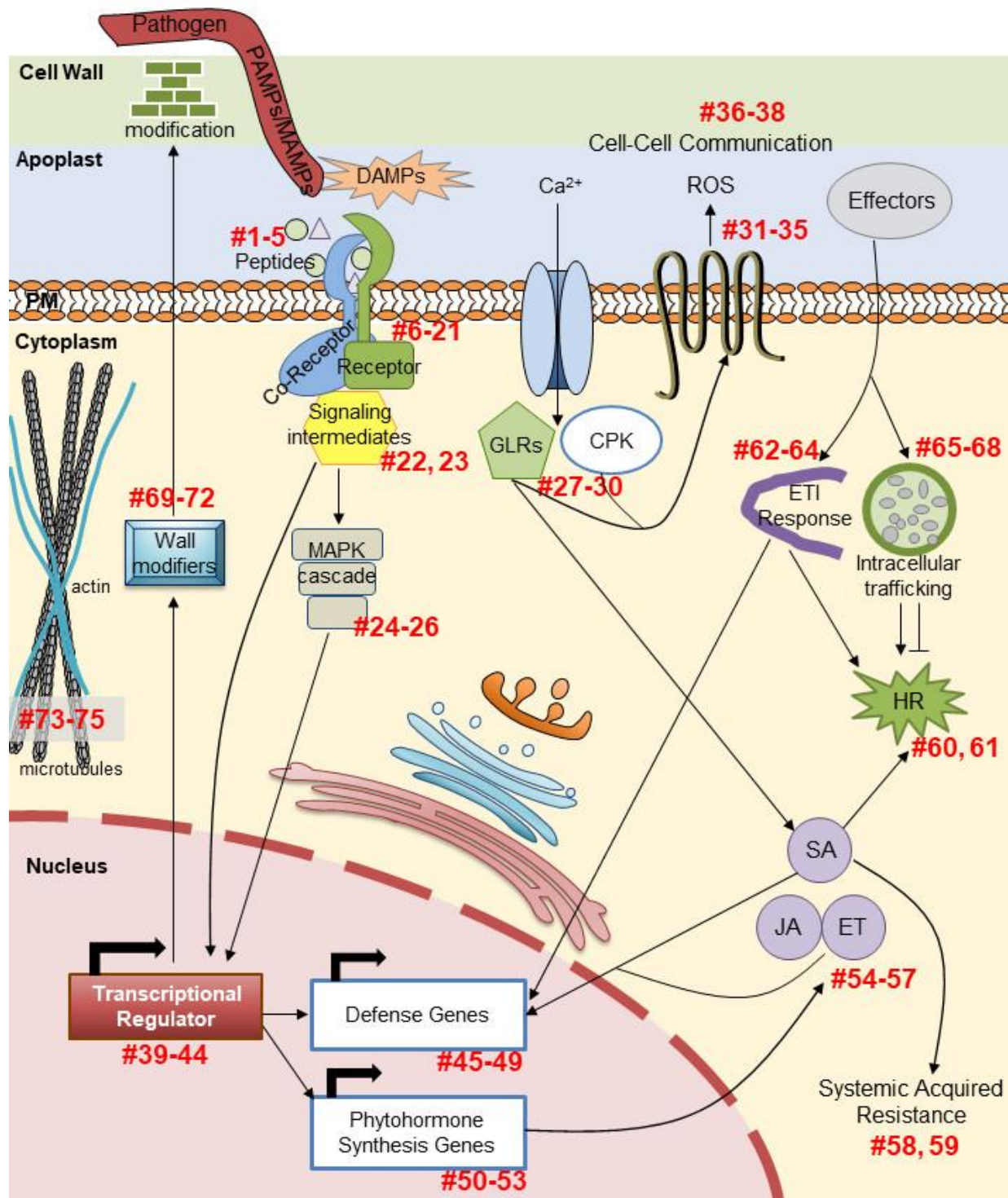
The promoter, N- and C-terminal tags, the gene-of-interest coding sequence (CDS), a terminator, and a plant resistance cassette are all cloned into individual entry clones (A-F), which are then combined with an 'empty' destination clone (Z) in the Golden Gate assembly mix. During the assembly reaction, the *Bsa*I restriction enzyme cuts the different fragments from their respective entry clones. These fragments subsequently self-align via complementary four base pair overhangs (indicated by the thin colored borders on each entry vector; complementary overhangs that will align have the same color), and the T4 DNA-ligase fuses the individual fragments in the final expression clone (Z) that can be used to transform plants.

61 Additional kits have been developed and added to the GreenGate toolbox since 2013, providing  
62 entry vector sets to build plasmids for CRISPR/Cas9-guided genome editing (Wu *et al.*, 2018),  
63 CRISPR/Cas9-guided tissue-specific gene knockout (Decaestecker *et al.*, 2019) and inducible and  
64 cell-type specific gene expression (Schürholz *et al.*, 2018). In addition, a toolbox of fluorescent  
65 proteins suitable for work in plants (Denay *et al.*, 2019), along with the necessary clones for *in*  
66 *planta* proximity ligation assays using the biotin ligase (Goslin *et al.*, 2021), and destination  
67 vectors with an already integrated plasma membrane-marker (Kümpers *et al.*, 2022) have been  
68 designed. The development and availability of these various toolboxes, all compatible with each  
69 other, demonstrate the usefulness of such modular systems to enable researchers to quickly adopt  
70 new technologies, providing the flexibility and versatility to combine and recombine their existing  
71 vectors (i.e., modules) with new vectors from all other kits.

72 In our work we use a microscopy-based live-imaging approach to monitor the *Arabidopsis*  
73 *thaliana*'s defense responses to infection and colonization by the pathogenic fungus *Fusarium*  
74 *oxysporum* strain Fo5176 (Fo5176) on an individual cell level (Calabria *et al.*, 2022; Wang *et al.*,  
75 2022a). Fluorescent transcriptional reporters are a good tool for such studies, as activation of  
76 certain pathways is typically associated with transcriptional upregulation (Ngou *et al.*, 2021).  
77 However, most studies investigating the transcriptional responsiveness of certain pathways to

78 pathogens tend to use transcriptomic analyses of whole tissues, organs, or seedlings, therefore  
79 losing all spatial resolution and differences between cell groups and even individual cells. The  
80 responses of only the few cells that could be the major contributors to a host-microbe outcome are  
81 also typically lost in such experiments, as they are below the threshold of background noise. A  
82 microscopy-based approach that allows for the spatial resolution and observation of responses in  
83 individual cells therefore closes this knowledge gap. One reason why such an approach is not more  
84 common is that a prerequisite for this work is the availability of fluorescent reporter lines for all  
85 the different plant immune pathways to be investigated. To this end, we have cloned the putative  
86 regulatory sequences (i.e., promoters) of 75 *A. thaliana* genes, representing many of the major  
87 branches of the plant immune system (Fig. 2 and Table 1). We have done so using the GreenGate  
88 cloning system, therefore creating a GreenGate plant promoter entry vector set that is compatible  
89 with all other GreenGate-based toolkits, and that we believe will be a valuable tool for the scientific  
90 community.

91 As a proof-of-principle, we selected a number of these promoters to create fluorescent  
92 transcriptional reporter lines for genes involved in the PLANT ELICITOR PEPTIDE (PEP)-  
93 pathway. The PEP pathway is involved in the immune response to several plant pathogens,  
94 including bacteria, fungi and oomycetes (Saijo *et al.*, 2018). The PEP1-6 peptides function as  
95 phytosulfokines. They are expressed as part of the plant's pattern-triggered immunity (PTI)  
96 response and trigger cell autonomous and non-autonomous defense responses when they are  
97 perceived by the plasma membrane localized receptors PEPR1 and PEPR2 (Yamaguchi *et al.*,  
98 2006, 2010; Saijo *et al.*, 2018). For their function, they require the intracellular kinase BIK1, which  
99 activates the NADPH oxidase RBOHD to trigger a ROS-burst, and also induces defense gene  
100 expression (Liu *et al.*, 2013; Kadota *et al.*, 2014; Saijo *et al.*, 2018; Jing *et al.*, 2020). A role for  
101 the PEP pathway in the defense against fungal disease has been hypothesized, based on large-scale  
102 transcriptomic data. Plants carrying mutations in *MEDIATOR18* and *20* show increased resistance  
103 to infection by *F. oxysporum* strain *Fo5176*, and mRNA sequencing has demonstrated that *PEP1*  
104 is among the genes no longer induced after infection of these mutant plants (Fallath *et al.*, 2017;  
105 Wang *et al.*, 2022a). Similarly, *PEPR1* and *2* were among the genes suppressed by the endophytic  
106 *F. oxysporum* strain *Fo47*, indicating that the PEP pathway normally acts to restrict fungal  
107 colonization (Guo *et al.*, 2021; Wang *et al.*, 2022a). However, no detailed data is available. Here,  
108 we show that colonization of the *A. thaliana* vasculature is accompanied by a highly localized



**Figure 2: Overview of the different immune pathways represented in the pGG-PIP collection**

The red numbers correspond to the pGG-PIP plasmid numbers in column one of [Table 1](#).

109 activation of this pathway via upregulation of genes encoding the peptide ligand PEP1, as well as  
 110 the cognate PEP RECEPTORS 1 and 2, the downstream signaling intermediate BIK1 and the  
 111 NADPH oxidase RBOHD.

## 112 **Results**

### 113 **Selection and cloning of the promoters**

114 By using these putative promoter sequences for the construction of transcriptional reporter lines,  
115 our main aim is to obtain information about the specific localized activation of different immune  
116 pathways in response to infection by a pathogen, in our case *Fo5176*. Once we have identified  
117 pathways involved in the plant's defense against this strain, we plan to further extend these  
118 experiments based on what is known about the function of these pathways. Thus, it is important to  
119 select promoters from many different pathways of the plant immune system, but also from  
120 pathways that are particularly well understood, since this existing knowledge will guide our future  
121 work. Some of the best studied immune pathways in the plant are the pattern-recognition flagellin  
122 (flg) and elongation factor thermos unstable (EF-Tu) pathways, the phytohormones jasmonic acid  
123 (JA), salicylic acid (SA) and ethylene (ET), and the transcriptional activity of several WRKY  
124 transcription factors. We therefore selected representative gene promoters from such well  
125 understood pathways, but also additional candidates such as MAP and calcium-dependent protein  
126 kinases (MPKs, CPKs), genes involved in production and signaling via reactive oxygen species  
127 (ROS), and genes involved in effector-triggered immunity (ETI) responses via the EDS1-PAD4  
128 module. **Figure 2** and **Table 1** provide a complete overview of the 75 promoters included in the  
129 pGG-PIP collection. The numbers in **Fig. 2** correspond to the pGG-PIP plasmid number in the first  
130 column of **Table 1**.

131 For the construction of the pGG-PIP plasmids, we considered the entire stretch of DNA from the  
132 3' upstream neighboring gene's coding sequence to the ATG of the gene of interest as the gene's  
133 putative regulatory sequences, and thus its 'promoter'. There are a few exceptions to this rule,  
134 where we used a defined stretch of DNA that has previously been described to complement a  
135 mutant (e.g., *FMO1*, *RPS4* (Wirthmueller *et al.*, 2007; Joglekar *et al.*, 2018)). Where present, this  
136 may include untranslated regions (UTRs), pseudogenes, or transposons, since these elements could  
137 indeed affect expression of a gene *in planta*. For the recombination-based cloning, the *BsaI*  
138 recognition site and standard four base pair GreenGate A overhangs (5'-ACCT and TTGT-3')  
139 were added via primers during amplification of the promoter sequences (see **Supplementary Table**  
140 **1** for a list of primer sequences). If the promoter sequence contained an internal *BsaI* restriction  
141 site (GGTCTC), we mutated a single base, usually a G to a C via scar-free *BsaI* cloning, i.e., we

142 inserted the base mutation by making it part of the four base pair overhang used for the scar-free  
 143 cloning (see **Table 1** for the exact sequence edits made, and **Supplementary Table 1** for primer  
 144 sequences). The promoters were then transferred into the GreenGate promoter entry vector  
 145 pGGA000 via the four base pair A overhangs in a Golden Gate assembly reaction.

GG-PIP plasmid	Gene	Bases	BsaI site edits	Protein function	Full gene name	Gene code
pGG-PIP01	<i>PEP1</i>	1907		Activate defense genes (Yamada <i>et al.</i> , 2016)	<i>PLANT ELICITOR PEPTIDE 1</i>	AT5G64900
pGG-PIP02	<i>PEP2</i>	769		Activate defense genes (Yamada <i>et al.</i> , 2016)	<i>PLANT ELICITOR PEPTIDE 2</i>	AT5G64890
pGG-PIP03	<i>PEP3</i>	1694		Activate defense genes (Yamada <i>et al.</i> , 2016)	<i>PLANT ELICITOR PEPTIDE 3</i>	AT5G64905
pGG-PIP04	<i>RALF23</i>	2027	1458 C → G	Phytosulfokine sensed by FER (Stegmann <i>et al.</i> , 2017)	<i>ARABIDOPSIS RAPID ALKALINIZATION FACTOR 23</i>	AT3G16570
pGG-PIP05	<i>SCOOP12</i>	2900		Phytosulfokine sensed by MIK2/BAK1 (Hou <i>et al.</i> , 2021; Rhodes <i>et al.</i> , 2021)	<i>PRECURSOR OF SERINE-RICH ENDOGENOUS PEPTIDE 12</i>	AT5G44585
pGG-PIP06	<i>BAK1</i>	1731		Co-receptor for several defense & development pathways (Greenwood and Williams, 2022)	<i>BRI1-ASSOCIATED RECEPTOR KINASE</i>	AT4G33430
pGG-PIP07	<i>CERK1</i>	493		Chitin-receptor (Cao <i>et al.</i> , 2014)	<i>CHITIN ELICITOR RECEPTOR KINASE 1</i>	AT3G21630
pGG-PIP08	<i>CIPP1</i>	3003		CERK1 co-receptor (Liu <i>et al.</i> , 2018)	<i>CERK-1 INTERACTING PROTEIN PHOSPHATASE 1</i>	AT1G34750
pGG-PIP09	<i>CORK1</i>	3376		DAMP-receptor (Tseng <i>et al.</i> , 2022)	<i>CELLOOLIGOMER RECEPTOR KINASE 1</i>	AT1G56145
pGG-PIP10	<i>EFR</i>	2376		Receptor for EF-Tu (Couto and Zipfel, 2016)	<i>EF-TU RECEPTOR</i>	AT5G20480
pGG-PIP11	<i>FER</i>	1243		Co-receptor for several defense & development pathways (Duan <i>et al.</i> , 2022)	<i>FERONIA</i>	AT3G51550
pGG-PIP12	<i>FLS2</i>	2913		Receptor for bacterial flagellin (Couto and Zipfel, 2016)	<i>FLAGELLIN-SENSITIVE 2</i>	AT5G46330
pGG-PIP13	<i>LYK5</i>	1560	1215 G → C	CERK1 co-receptor (Cao <i>et al.</i> , 2014)	<i>LYSM-CONTAINING RECEPTOR-LIKE KINASE 5</i>	AT2G33580
pGG-PIP14	<i>LYM1</i>	991		Fungal MAMP receptor (Zipfel and Oldroyd, 2017)	<i>LYSM DOMAIN GPI-ANCHORED PROTEIN 1 PRECURSOR</i>	AT1G21880
pGG-PIP15	<i>MIK2</i>	2513		SCOOP peptide receptor (Hou <i>et al.</i> , 2021; Rhodes <i>et al.</i> , 2021)	<i>MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2</i>	AT4G08850
pGG-PIP16	<i>PEPR1</i>	931	180 G → A	Receptor for PEP1-6 (Yamada <i>et al.</i> , 2016)	<i>PEP1 RECEPTOR 1</i>	AT1G73080
pGG-PIP17	<i>PEPR2</i>	1676		Receptor for PEP1 & 2 (Yamada <i>et al.</i> , 2016)	<i>PEP1 RECEPTOR 2</i>	AT1G17750
pGG-PIP18	<i>RFO1</i>	850		Cell wall-associated kinase (Huerta <i>et al.</i> , 2023)	<i>RESISTANCE TO FUSARIUM OXYSPORUM 1</i>	AT1G79670
pGG-PIP19	<i>RLP26</i>	822		Co-receptor for PRR receptors (Wu <i>et al.</i> , 2016)	<i>RECEPTOR LIKE PROTEIN 26</i>	AT2G33050
pGG-PIP20	<i>RLP29</i>	2790		Co-receptor for PRR receptors (Wu <i>et al.</i> , 2016)	<i>RECEPTOR LIKE PROTEIN 29</i>	AT2G42800
pGG-PIP21	<i>SOBIR1</i>	1163		Co-receptor for several defense pathways (Cho <i>et al.</i> , 2022)	<i>SUPPRESSOR OF BIR 1 / EVERSHERD</i>	AT2G31880
pGG-PIP22	<i>BIK1</i>	2668		Defense signaling (Gonçalves Dias <i>et al.</i> , 2022)	<i>BOTRYTIS-INDUCED KINASE1</i>	AT2G39660
pGG-PIP23	<i>XLG2</i>	1256		G protein involved in immunity (Petutschnig <i>et al.</i> , 2022)	<i>EXTRA-LARGE GTP-BINDING PROTEIN 2</i>	AT4G34390
pGG-PIP24	<i>MPK3</i>	654		Activates immune response (Tsuda and Somssich, 2015)	<i>MITOGEN-ACTIVATED PROTEIN KINASE 3</i>	AT3G45640
pGG-PIP25	<i>MPK4</i>	1025		Activates immune response (Tsuda and Somssich, 2015)	<i>MITOGEN-ACTIVATED PROTEIN KINASE 4</i>	AT4G01370

pGG-PIP26	<i>MPK6</i>	811		Activates immune response (Tsuda and Somssich, 2015)	<i>MITOGEN-ACTIVATED PROTEIN KINASE 6</i>	<i>AT2G43790</i>
pGG-PIP27	<i>CPK5</i>	1983		Involved in calcium-dependent stress responses (Gao and He, 2013)	<i>CALMODULIN-DOMAIN PROTEIN KINASE 5</i>	<i>AT4G35310</i>
pGG-PIP28	<i>CPK29</i>	826	325 G->C	Involved in calcium-dependent stress responses (Patil and Senthil-Kumar, 2020)	<i>CALCIUM-DEPENDENT PROTEIN KINASE 29</i>	<i>AT1G76040</i>
pGG-PIP29	<i>GLR2.5</i>	2488	1488, 1489 G->C	Involved in defense-development balancing (Birkenbihl <i>et al.</i> , 2017a)	<i>GLUTAMATE RECEPTOR 2.5</i>	<i>AT5G11210</i>
pGG-PIP30	<i>GLR2.7</i>	4000		Involved in defense-development balancing (Birkenbihl <i>et al.</i> , 2017a)	<i>GLUTAMATE RECEPTOR 2.7</i>	<i>AT2G29120</i>
pGG-PIP31	<i>RBOHD</i>	2309		Produces ROS-burst (Kadota <i>et al.</i> , 2014)	<i>RESPIRATORY BURST OXIDASE HOMOLOGUE D</i>	<i>AT5G47910</i>
pGG-PIP32	<i>RBOHF</i>	4119		Produces ROS-burst (Morales <i>et al.</i> , 2016)	<i>RESPIRATORY BURST OXIDASE HOMOLOG F</i>	<i>AT1G64060</i>
pGG-PIP33	<i>EX1</i>	989		Produces ROS from chloroplasts (Dogra <i>et al.</i> , 2022)	<i>EXECUTER 1</i>	<i>AT4G33630</i>
pGG-PIP34	<i>HPCA1</i>	2944		ROS-sensor (Sun and Zhang, 2021)	<i>HP-INDUCED Ca2+ INCREASES 1</i>	<i>AT5G49760</i>
pGG-PIP35	<i>RCD1</i>	3137	2888 G->C	Modulator of root-to-shoot ROS-signaling (Jin <i>et al.</i> , 2022)	<i>RADICAL-INDUCED CELL DEATH1</i>	<i>AT1G32230</i>
pGG-PIP36	<i>DORN1</i>	2325		eATP DAMP-receptor (Sun and Zhang, 2021)	<i>DOES NOT RESPOND TO NUCLEOTIDES 1</i>	<i>AT5G60300</i>
pGG-PIP37	<i>LECRK-VI.2</i>	1183		NAD(P)-receptor (Sun and Zhang, 2021)	<i>L-TYPE LECTIN RECEPTOR KINASE-VI.2</i>	<i>AT5G01540</i>
pGG-PIP38	<i>BPS1</i>	2142		Regulator of root-to-shoot communication (Lee <i>et al.</i> , 2016)	<i>BYPASS 1</i>	<i>AT1G01550</i>
pGG-PIP39	<i>WRKY11</i>	2026	240 C->G	Regulates defense gene expression (Journot-Catalino <i>et al.</i> , 2006)	<i>WRKY DNA-BINDING PROTEIN 11</i>	<i>AT4G31550</i>
pGG-PIP40	<i>WRKY17</i>	4565	4325 G->C	Regulates defense gene expression (Birkenbihl <i>et al.</i> , 2018)	<i>WRKY DNA-BINDING PROTEIN 17</i>	<i>AT2G24570</i>
pGG-PIP41	<i>WRKY33</i>	1665		Regulates defense gene expression (Birkenbihl <i>et al.</i> , 2018)	<i>WRKY DNA-BINDING PROTEIN 33</i>	<i>AT4G23810</i>
pGG-PIP42	<i>WRKY40</i>	4158		Regulates defense gene expression (Birkenbihl <i>et al.</i> , 2018)	<i>WRKY DNA-BINDING PROTEIN 40</i>	<i>AT1G80840</i>
pGG-PIP43	<i>WRKY53</i>	2515		Regulates defense gene expression (Birkenbihl <i>et al.</i> , 2017b)	<i>WRKY DNA-BINDING PROTEIN 53</i>	<i>AT2G38470</i>
pGG-PIP44	<i>WRKY70</i>	4099		Regulates defense gene expression (Journot-Catalino <i>et al.</i> , 2006)	<i>WRKY DNA-BINDING PROTEIN 70</i>	<i>AT3G56400</i>
pGG-PIP45	<i>ELI-3</i>	524		Elicitor-response gene (Tanaka <i>et al.</i> , 2018)	<i>CINNAMYL-ALCOHOL DEHYDROGENASE 7 / ELICITOR-ACTIVATED GENE3</i>	<i>AT4G37980</i>
pGG-PIP46	<i>FRK1</i>	1271		Defense gene (Birkenbihl <i>et al.</i> , 2017b)	<i>FLG22-INDUCED RECEPTOR-LIKE KINASE 1</i>	<i>AT2G19190</i>
pGG-PIP47	<i>MLO6</i>	3042		Defense gene (Acevedo-Garcia <i>et al.</i> , 2017)	<i>MILDEW RESISTANCE LOCUS O 6</i>	<i>AT1G61560</i>
pGG-PIP48	<i>PER5</i>	2007	558 C->G	Marker for activated immune signalling (Chuberre <i>et al.</i> , 2018)	<i>PEROXIDASE 5</i>	<i>AT1G14550</i>
pGG-PIP49	<i>PLP1</i>	2490		Pathogen-induced (Yang <i>et al.</i> , 2007)	<i>PATATIN-LIKE PROTEIN 1</i>	<i>AT4G37070</i>
pGG-PIP50	<i>ACS2</i>	2914	150 C->G	ET biosynthesis (Wang <i>et al.</i> , 2022c)	<i>1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2</i>	<i>AT1G01480</i>
pGG-PIP51	<i>AOS</i>	2093	2088 G->C ;	JA biosynthesis (Yang <i>et al.</i> , 2019)	<i>ALLENE OXIDE SYNTHASE</i>	<i>AT5G42650</i>



			1434 G->C			
pGG- PIP52	<i>ARR5</i>	2229	639 C - > G	Cytokinin signaling (Lee <i>et al.</i> , 2016)	<i>ARABIDOPSIS RESPONSE REGULATOR 5</i>	<i>AT3G48100</i>
pGG- PIP53	<i>EDS16</i>	2975	1579 G->C ; 1049 C->G	SA biosynthesis (Calabria <i>et al.</i> , 2022)	<i>ENHANCED DISEASE SUSCEPTIBILITY TO ERYSPHE ORONTII 16</i>	<i>AT1G74710</i>
pGG- PIP54	<i>ERF1</i>	2682		ET & JA response regulator (Wang <i>et al.</i> , 2022c)	<i>ETHYLENE RESPONSE FACTOR 1</i>	<i>AT3G23240</i>
pGG- PIP55	<i>PDF1.2</i>	1540		ET & JA-induced defense gene (Yang <i>et al.</i> , 2019)	<i>PLANT DEFENSIN 1.2</i>	<i>AT5G44420</i>
pGG- PIP56	<i>PR1</i>	2343		SA-induced defense gene (Yang <i>et al.</i> , 2019)	<i>PATHOGENESIS-RELATED GENE 1</i>	<i>AT2G14610</i>
pGG- PIP57	<i>VSP2</i>	1471		JA-induced defense gene (Yang <i>et al.</i> , 2019)	<i>VEGETATIVE STORAGE PROTEIN 2</i>	<i>AT5G24770</i>
pGG- PIP58	<i>FMO1</i>	1726		Systemic acquired resistance marker (Joglekar <i>et al.</i> , 2018)	<i>FLAVIN-DEPENDENT MONOOXYGENASE 1</i>	<i>AT1G19250</i>
pGG- PIP59	<i>MYB72</i>	4619		Involved in induced systemic resistance	<i>ARABIDOPSIS THALIANA MYB DOMAIN PROTEIN 72</i>	<i>AT1G56160</i>
pGG- PIP60	<i>CSLD2</i>	1963		Marker for cell death, possibly via ET-signaling (Salguero-Linares <i>et al.</i> , 2022)	<i>CELLULOSE-SYNTHASE LIKE D2</i>	<i>AT5G16910</i>
pGG- PIP61	<i>HRM1</i>	2238		Marker for cell death (Salguero-Linares <i>et al.</i> , 2022)	<i>HYPERSENSITIVE RESPONSE MARKER 1</i>	<i>AT5G17760</i>
pGG- PIP62	<i>EDS1</i>	1419	848 A- >T ; 1269 G->C	Involved in ETI-signaling (Jia <i>et al.</i> , 2022)	<i>ENHANCED DISEASE SUSCEPTIBILITY 1</i>	<i>AT3G48090</i>
pGG- PIP63	<i>PAD4</i>	1596		Involved in ETI-signaling (Jia <i>et al.</i> , 2022)	<i>PHYTOALEXIN DEFICIENT 4</i>	<i>AT3G52430</i>
pGG- PIP64	<i>RPS4</i>	511		NLR effector receptor (Jia <i>et al.</i> , 2022)	<i>RESISTANT TO P. SYRINGAE 4</i>	<i>AT5G45250</i>
pGG- PIP65	<i>ATG8A</i>	1408		Autophagy marker (Yang <i>et al.</i> , 2018)	<i>AUTOPHAGY-RELATED 8A</i>	<i>AT4G21980</i>
pGG- PIP66	<i>TET8</i>	2068		Targets vesicular transport to infection sites (Cai <i>et al.</i> , 2018)	<i>TETRASPANIN8</i>	<i>AT2G23810</i>
pGG- PIP67	<i>SULTR4;1</i>	2087		Sulfate transporter (Wang <i>et al.</i> , 2022b)	<i>SULFATE TRANSPORTER 4.1</i>	<i>AT5G13550</i>
pGG- PIP68	<i>SULTR4;2</i>	712		Sulfate transporter (Wang <i>et al.</i> , 2022b)	<i>SULFATE TRANSPORTER 4.2</i>	<i>AT3G12520</i>
pGG- PIP69	<i>CAD5</i>	1134		Lignin biosynthesis (Kim <i>et al.</i> , 2020)	<i>CINNAMYL ALCOHOL DEHYDROGENASE 5</i>	<i>AT4G34230</i>
pGG- PIP70	<i>GPAT5</i>	1587		Suberin biosynthesis (Andersen <i>et al.</i> , 2015)	<i>GLYCEROL-3-PHOSPHATE SN-2- ACYLTRANSFERASE 5</i>	<i>AT3G11430</i>
pGG- PIP71	<i>MYB15</i>	2050		Lignin biosynthesis in response to pathogens (Kim <i>et al.</i> , 2020)	<i>ARABIDOPSIS THALIANA MYB DOMAIN PROTEIN 15</i>	<i>AT3G23250</i>
pGG- PIP72	<i>PMR4</i>	1954		PAMP-induced defense, callose-deposition (Blümke <i>et al.</i> , 2013)	<i>POWDERY MILDEW RESISTANT 4</i>	<i>AT4G03550</i>
pGG- PIP73	<i>FH16</i>	1158		Involved in microtubule/actin-network reorganisation (Wang <i>et al.</i> , 2013)	<i>FORMIN HOMOLOG 16</i>	<i>AT5G07770</i>
pGG- PIP74	<i>NET4A</i>	1718		Modulates the vacuole (Kaiser <i>et al.</i> , 2019)	<i>NETWORKED 4A</i>	<i>AT5G58320</i>
pGG- PIP75	<i>TUB6</i>	3349	2358 A->G	Component of microtubules (Liu <i>et al.</i> , 2019)	<i>BETA-6 TUBULIN</i>	<i>AT5G12250</i>

146 **Table 1: List of the 75 pGG-PIP promoter entry vectors in the set described in this paper.**

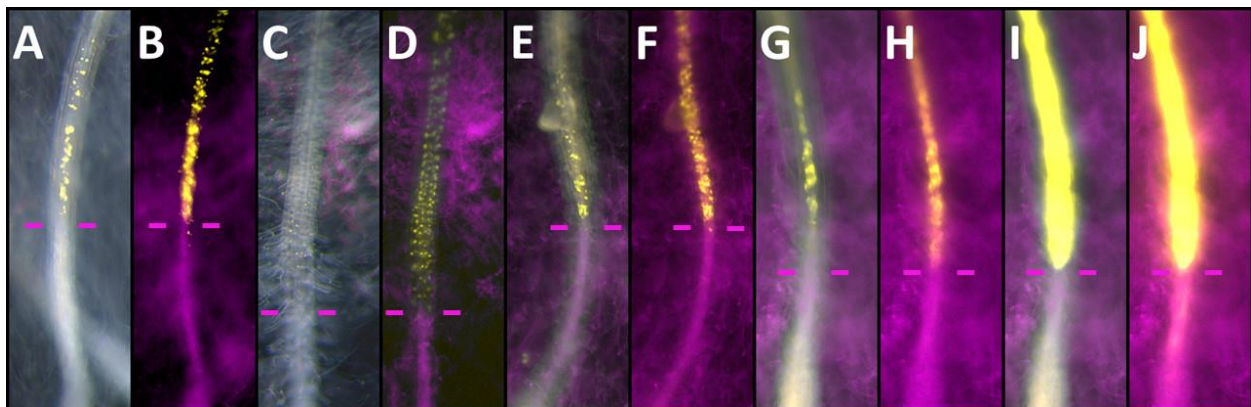
147 The pGG-PIP numbers correspond to the red numbers in Fig. 2.

## 148 Using pGG-PIP vectors to create transcriptional reporters for the PEP pathway

149 To test the usefulness of these promoters to map local individual cell responses upon attack by a  
150 microbe, we created transcriptional reporter lines for genes involved in the PEP pathway. We used

151 a nuclear localized tandem of mTurquoise2 (mT2) fluorophores as a reporter, since mT2 has been  
152 shown to be very bright and photostable in plant cells (Goedhart *et al.*, 2012; Long *et al.*, 2018;  
153 Denay *et al.*, 2019). The nuclear localization enhances the brightness even further due to molecular  
154 crowding, and furthermore helps to identify individual cells.

155 We first probed our reporters for the PEP1 and PEP2 peptides (pGG-PIP01 and 02), as well as the  
156 PEPR1 and PEPR2 receptors (pGG-PIP16 and 17), assessing their local responsiveness to  
157 colonization by *Fo5176*. Under control uninfected conditions, *PEP1* was weakly expressed in  
158 inner tissue cells of the root differentiation zone (DZ) (Fig. S1A-D). No expression was detectable  
159 in the root tip, the meristematic or elongation zones (MZ and EZ). *PEP2* was robustly expressed  
160 in the DZ, but in contrast to *PEP1*, its expression was stronger in the outer tissues (Fig. S1G-H).  
161 In addition, it was also expressed in the root tip, around the MZ and early EZ (Fig. S1E-F). *PEPR1*  
162 was generally expressed at a very weak level. In the root tip, starting with the EZ, expression was  
163 limited to the vasculature, while from the young DZ (or root hair zone) onward, expression was  
164 restricted to the outer tissues (Fig. S2A-D). *PEPR2* was expressed only in the DZ, and only in the  
165 vasculature, but expression was much stronger compared to *PEPR1* (Fig. S2E-H). Thus, in  
166 vascular cells, *PEPR1* and *PEPR2* complemented each other under uninfected conditions.  
167 Following colonization of the vasculature by *Fo5176*, *PEP1*, as well as both *PEPRs*, showed a  
168 strong upregulation in the cells adjacent to the colonization site (Fig. 3). Interestingly, this



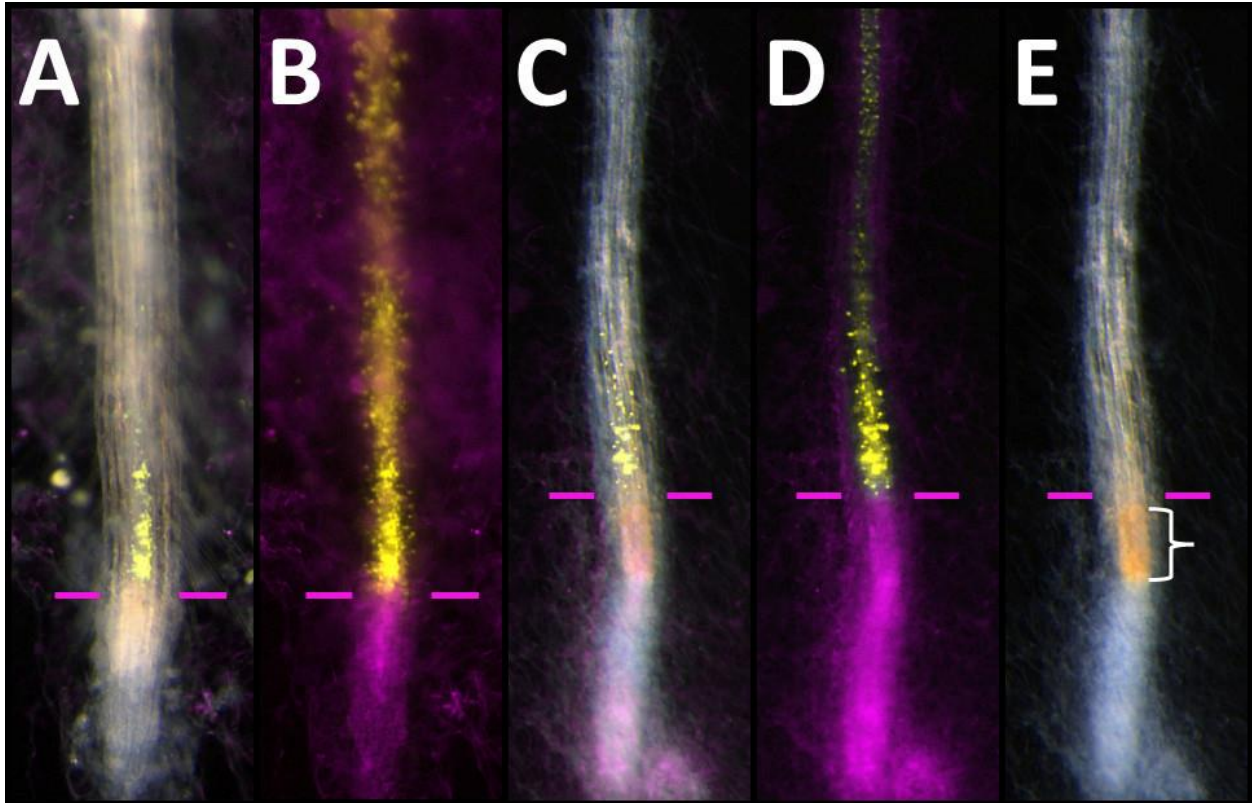
**Figure 3: Local transcriptional responses in *PEP1*, *PEP2*, *PEPR1* and *PEPR2* expression to colonization by *Fo5176***

Bright field and fluorescence (A, C, E, G, I) and fluorescence only (B, D, F, H, J) images of colonized roots expressing the *PEP1* (A, B), *PEP2* (C, D), *PEPR1* (E, F) or *PEPR2* (G-J) reporters. *Fo5176* is shown in magenta, the reporters in yellow. The purple bars indicate the fungal colonization front. All images were recorded with comparable imaging settings, except for G and H, which are the same as I and J, just with reduced exposure time. Expression of the *PEPR2* reporter was so strong (I, J), that we reduced the exposure time to visualize individual cells in G and H.

169 upregulation was limited to the cells of the vasculature, the exact tissue that is targeted and  
170 colonized by *Fo5176*, showing how precisely the plant targets its response. Importantly, the  
171 induction of *PEPR2* was much stronger than the induction of *PEP1* and *PEPR1* (Fig. 3G-J).  
172 Interestingly, *PEP2* also showed an induction next to the colonized tissue, but its expression  
173 appeared to be limited to the outer tissues, the epidermis and cortex, rather than the colonized  
174 vasculature (Fig. 3C, D). The strong induction of the *PEPs* and *PEPRs* remained visible for  
175 roughly 10 to 12 cells, and then tapered out, eventually returning to the expression pattern of the  
176 uninfected control.

177 Since it was shown that the ability of the *PEPs* and *PEPRs* to induce any signaling downstream of  
178 the receptors is strictly dependent on the activity of the cytoplasmic kinase *BIK1*, we also  
179 investigated the responsiveness of a *BIK1* (pGG-PIP22) transcriptional reporter (Liu *et al.*, 2013;  
180 Yamada *et al.*, 2016). Under uninfected control conditions, *BIK1* is expressed in the MZ and DZ,  
181 with stronger expression in the outer tissues, and only weak expression in the vascular tissue of  
182 the MZ, somewhat resembling the expression pattern of *PEP2* (compare Fig. S3A-D and S1E-H).  
183 Following colonization of the root by *Fo5176*, this pattern changed significantly, with *BIK1* now  
184 expressed specifically in the cells immediately bordering the colonized tissue, with a clearly visible  
185 expression maximum in the vasculature (Fig. 4A, B). Hence, the expression maxima of *BIK1*,  
186 *PEP1*, *PEPR1* and *PEPR2* all overlapped in a small group of vascular cells closest to the colonized  
187 tissue. *BIK1* however, also showed expression in the endodermis and cortex, albeit at a much lower  
188 level than in the vasculature, while *PEP1* and *PEPRs* expression were restricted to the vasculature  
189 (Fig. 3 and Fig. 4A, B).

190 One of the downstream outputs of *PEP*-activated *BIK1* is the activation of the NADPH oxidase  
191 *RBOHD* to trigger an apoplastic ROS-burst (Holmes *et al.*, 2018; Jing *et al.*, 2020). We therefore  
192 investigated if *RBOHD* (pGG-PIP31) is also activated in the cells expressing *PEP1*, the *PEPRs*  
193 and *BIK1*. Under uninfected control conditions, *RBOHD* is expressed in all cells and tissues of the  
194 DZ, but not in the root tip (MZ, EZ) (Fig. S3E-H). Upon infection by *Fo5176*, expression is  
195 activated in the cells next to the colonization site, even if they are still part of the EZ, which  
196 normally does not express *RBOHD* (Fig. 4C, D). The pattern we observed very much resembled



**Figure 4: Transcriptional responses of *BIK1* and *RBOHD* expression to colonization by *Fo5176***

Bright field and fluorescence (A, C), fluorescence only (B, D), or bright field only (E) images of a colonized root expressing the *BIK1* (A, B), or *RBOHD* (C, D, E) reporters. The *Fo5176* is shown in magenta, the reporters in yellow. The purple bars indicate the fungal colonization front. The white bracket in E indicates the area with 'root browning'.

197 the pattern of *BIK1* expression in response to colonization, with a maximum in a small group of  
198 vascular cells next to the colonization site (Fig. 4). However, in contrast to the expression of *BIK1*  
199 and the *PEPs* and *PEPRs*, upregulation of *RBOHD* appeared to be more spatially restricted to the  
200 cells closest to the colonization site. *RBOHD* expression quickly returned to control levels behind  
201 this small group of cells, while the activation of the other markers appeared to taper out more  
202 gradually, across a longer stretch of cells.

203 While imaging the fungal infected reporter lines, we also regularly observed a discoloration of the  
204 root in the colonized tissue (white bracket in Fig. 4E). This has previously been described as 'root  
205 browning', and has been observed in response to infection by *F. oxysporum* or treatment of the  
206 plant with SERINE-RICH ENDOGENOUS PEPTIDES (Tintor *et al.*, 2020; Hou *et al.*, 2021).  
207 While it remains unclear what the exact cause for this discoloration is, redox/oxidative stress  
208 induced by ROS is one possibility. In our assay, the browning does not completely overlap with

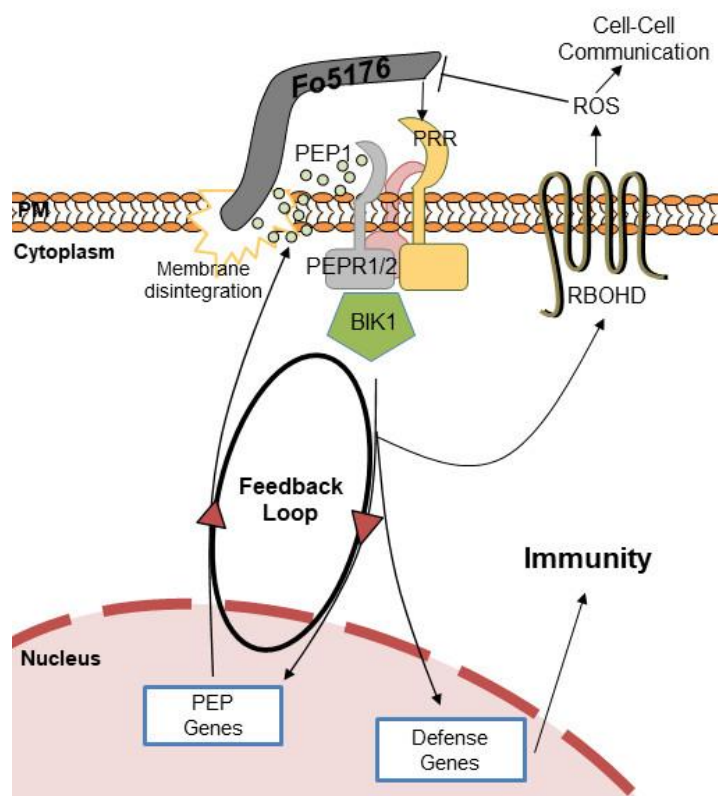
209 the expression maximum of *RBOHD*, but as the colonization of the fungus progresses upward  
210 through the vasculature, the *RBOHD* expression also extends along adjacent to the colonization  
211 front. Therefore, the area showing root browning would be the region that last expressed *RBOHD*  
212 at high levels, and thus could have been under redox/oxidative stress, but this requires further  
213 examination.

## 214 **Discussion**

215 In this paper we present a GreenGate-based entry vector set with 75 plant immune system  
216 promoters. In combination with the basic GreenGate kit from Lampropoulos et al. (2013) this is  
217 already sufficient to build simple tools, such as transcriptional reporter lines, that can then be  
218 probed for their responsiveness to a pathogen, elicitor, or other stimulant. Furthermore, owing to  
219 the universal compatibility of these entry vectors with all other GreenGate-based systems, this  
220 vector set can be used to screen for interactors of proteins in their native expression domain using  
221 the proximity ligation kit from Goslin *et al.* (2021), or for tissue-specific gene knockouts by  
222 combining them with the CRISPR-TSKO kit from Decaestecker *et al.* (2019), to name just two  
223 possibilities.

224 As a straightforward proof-of-principle, we used some of the pGG-PIPs to create fluorescent  
225 reporter lines for the PEP pathway and demonstrate how these can be used to gain insights into  
226 *spatial immunity* – the highly localized activation of an immune pathway. Using these lines, we  
227 showed that *PEP1*, and to some degree *PEP2*, were responsive to infection and colonization by  
228 the vascular pathogen *Fo5176*. The danger signal PEP1 was upregulated in a specific group of  
229 vascular cells immediately adjacent to the fungal colonization site, as were the two receptors  
230 *PEPR1* and *2*. We thus hypothesize that activation of the PEP pathway is part of the plant's  
231 immune response to infection and colonization by *Fo5176*. Importantly, we could show the highly  
232 targeted activation of this pathway in just a couple of vascular cells immediately adjacent to a  
233 colonization site. Such a local response has rarely been shown *in vivo* and *in planta* before, and is  
234 typically overlooked in large-scale transcriptomic analyses.

235 Since the PEPRs are unlikely to serve as pattern-recognition receptors (PRRs) to sense the  
236 pathogen, it is more likely that the PEPRs are part of a larger complex containing the PRR co-  
237 receptor BAK1, as well as other PRRs, such as MIK2 or FER, since the interaction between BAK1



**Figure 5: Model of the PEP pathway in response to Fo5176**

Alkalinization of the apoplast via *Fo5176*-derived alkalinization factors induces interaction of the PEPRs with their PRR co-receptors. Intracellularly the receptors phosphorylate and activate BIK1. BIK1 activates expression of PEP1, thereby amplifying the PEP signal and creating a positive feedback loop. BIK1 also activates other defense genes, possibly via a MAPK cascade, and activates RBOHD to produce an apoplastic ROS-burst, which is damaging to the pathogen, and functions as cell-cell communication signal.

and the PEPRs has been previously shown, and all of these PRRs have been implicated to function in the defense against *Fo5176* (Yamada *et al.*, 2016; Wang *et al.*, 2022a) (Fig. 5). Possibly,

243 these co-receptors are all co-localized in plasma membrane nanodomains to facilitate the rapid  
244 assembly of larger receptor platforms (Somssich *et al.*, 2015; McKenna *et al.*, 2019; Somssich,  
245 2020; Gronnier *et al.*, 2022). Indeed, rather than functioning as a PRR sensing the pathogen  
246 directly, work from a recent publication suggests that the PEPRs act as extracellular pH sensors  
247 (Liu *et al.*, 2022). Alkalinization of the apoplast is a hallmark of activated immunity, and Liu *et*  
248 *al.*, (2022) show that the PEPRs specifically interact with their co-receptor BAK1 under alkaline  
249 conditions, while they only show a weak affinity for interaction under acidic conditions (Felix *et*  
250 *al.*, 1993; Liu *et al.*, 2022). Interestingly, *F. oxysporum* uses functional homologs of plant RAPID  
251 ALKALINIZATION FACTORS to induce alkalinization of the apoplast, as this also stimulates  
252 infectious growth of the fungus (Masachis *et al.*, 2016; Wang *et al.*, 2022a). Thus, the PEPRs  
253 pathway may act as pH sensors to counteract this action by the fungus, improving the plant's  
254 defense under these alkaline conditions. Extracellular binding of the PEPs to their receptors  
255 generally leads to the activation of the PEPRs intracellular kinase domain and trans-  
256 phosphorylation of the interacting cytoplasmic kinase BIK1 (Liu *et al.*, 2013). Indeed, we could  
257 show that our *BIK1* reporter is also activated in the same pattern as the *PEP1* and the *PEPR*  
258 reporters. BIK1 activation then facilitates downstream intracellular signaling (Yamada *et al.*,

259 2016). These downstream signaling events eventually result in the transcriptional activation of  
260 defense genes, but also auto-activation of the *PEP* genes (Fig. 5). Since the PEPs do not have an  
261 identifiable secretion signal that would direct them into the secretory pathway, it is assumed that  
262 they are released into the apoplast indirectly when a pathogen breaches the plasma membrane and  
263 causes local membrane disintegration (Fig. 5) (Yamaguchi and Huffaker, 2011; Yamada *et al.*,  
264 2016). Once in the apoplast, they are then perceived by the PEPRs, creating a positive feedback-  
265 loop of PEP-signaling and an auto-amplification of the plant's defense response (Fig. 5). A second  
266 intracellular pathway activated via BIK1 leads to activation of the NADPH oxidase RBOHD (Fig.  
267 5) (Kadota *et al.*, 2014; Holmes *et al.*, 2018; Jing *et al.*, 2020). This pathway was proposed to be  
268 primarily driven by PEP1 and the PEPR2 (Jing *et al.*, 2020) and indeed, *PEP1* and *PEPR2* are also  
269 the two reporters showing the strongest response to colonization by *Fo5176*, while *PEP2* was  
270 primarily upregulated in the cells around the vasculature. *PEPR1* was also activated in the same  
271 pattern as *PEP1*, *PEPR2*, and *BIK1*, but at a much lower level compared to *PEPR2*. Further, we  
272 could also confirm that *RBOHD* expression is activated in these same cells, indicating that this  
273 *BIK1-RBOHD*-dependent pathway for ROS-release is also potentially involved in the defense  
274 against *Fo5176* (Fig. 5). The activation of RBOHD, and the resulting ROS-burst, would aid in the  
275 plant's defense against *Fo5176*, with ROS being directly harmful to the pathogen. Additionally,  
276 ROS is known to also function as a signal in cell-cell communication to prime the surrounding  
277 tissue for impending attack (Waszczak *et al.*, 2018). We believe that the local 'root browning' that  
278 we observed in the infected roots is likely the result of this apoplastic ROS-burst.

279 A role for the PEP-pathway in the defense against *F. oxysporum* has previously been hypothesized  
280 on the basis of transcriptomic data showing up- or downregulation of some of the components in  
281 response to infection (Fallath *et al.*, 2017; Guo *et al.*, 2021; Wang *et al.*, 2022a). By investigating  
282 the responsiveness of our transcriptional reporters for PEP1 and 2, as well as PEPR1 and 2, we  
283 added spatial resolution to the available transcriptomic data. We could show that PEP1 and the  
284 PEPRs act in a clearly defined group of vascular cells immediately next to the fungal colonization  
285 site, and this localized response overlaps with the activation of downstream signaling factors and  
286 a local ROS-burst. *PEP2* was also induced in response to colonization, but it appeared to be  
287 upregulated in the outer tissues, not the infected vasculature. While this may indicate a role for  
288 *PEP2* in priming these neighboring tissues, the fact that neither of the two *PEPRs* were expressed  
289 there, makes it unclear how *PEP2* would be perceived in these cells. Importantly, when Fallath *et*

290 *al.* (2017) investigated the transcriptional response of *med* mutants to infection by *Fo5176*, they  
291 also only noted a deregulation of *PEP1* in these mutants, but not *PEP2*, indicating that *PEP1* is  
292 indeed the major PEP signal acting in response to colonization by *Fo5176*.

293 Finally, we believe that the pGG-PIP plant immune promoter resource introduced in this article  
294 will be valuable and helpful to the community for various research approaches. We will make the  
295 75-plasmid collection available via AddGene for quick and simple distribution to the community.  
296 We aim to provide further detailed ‘spatial immunity’ information by using these pGG-PIPs in our  
297 future work.

## 298 **Material & Methods**

### 299 **Cloning of the pGG-PIPs and transcriptional reporter constructs**

300 The pGG-PIP entry vectors are based on the pGGA000 vector from the original GreenGate kit  
301 (Lampropoulos *et al.*, 2013). The different promoters were amplified from total cellular *A. thaliana*  
302 (natural accession Columbia) DNA, extracted from rosette leaves with the Qiagen DNeasy Plant  
303 Kit, and using the primers in supplementary table 1. For error-free amplification, the Phusion high-  
304 fidelity DNA polymerase with proofreading (New England Biolabs) was used. The fragments were  
305 then transferred into pGGA000 in a Golden Gate assembly reaction using the NEBridge Golden  
306 Gate Assembly Kit (BsaI-HF v2) (New England Biolabs). To clone the transcriptional reporter  
307 lines, we used the destination vector pGGZ003, as well as the donor vectors pGGD007 (*linker-*  
308 *NLS* (Nuclear Localization Signal)), pGGE009 (*UBQ10* terminator), and pGGF005  
309 (*pUBQ10::HygR:tOCS*) from the original GreenGate kit from Lampropoulos *et al.* (2013). pGGB-  
310 mT2 (*mTurquoise2*) and pGGC-mT2 (*mTurquoise2*) were created by cloning the *mT2* coding  
311 sequence from an AddGene-derived template into pGGB000 and pGGC000 from the original  
312 GreenGate kit (Goedhart *et al.*, 2012; Lampropoulos *et al.*, 2013). In combination with our pGG-  
313 PIPs, this yielded the *pPIP::mT2-mT2-NLS:tUBQ10:pUBQ10::HygR:tOCS* reporters.

### 314 **Plant growth and transformation**

315 *A. thaliana* Columbia plants (Somssich, 2018) were grown at 16-hour light conditions, with 21 °C  
316 during the light hours, and 18 °C during the dark hours. Light intensity was 120 mmol m<sup>-2</sup>s<sup>-1</sup>, and  
317 humidity was 70%. Plant transformation was done via the *Agrobacterium tumefaciens* host strain  
318 GV3101 *pMP90 pSoup* carrying one of the plasmids in table 1 using the floral dip method (Holsters  
319 *et al.*, 1980; Koncz and Schell, 1986; Clough and Bent, 1998; Hellens *et al.*, 2000; Somssich,



2019). Individual resistant bacteria colonies were selected after two days of growth at 28° C on  
YT plates (Miller, 1972) supplemented with 50 µg/ml rifampicin, 25 µg/ml gentamycin, 5 µg/ml  
tetracyclin, and 100 µg/ml spectinomycin, then grown overnight in 250 ml of liquid 2×YT medium  
with 50 µg/ml rifampicin, 25 µg/ml gentamycin, and 100 µg/ml spectinomycin, shaking at 200  
rpm. Cells were harvested by centrifugation at 3200 g for 20 min, and resuspended in 300 ml of a  
5% sucrose solution with 0.08% Silwet L-77. Plants were then dipped into the solution for  
approximately 30 seconds with gentle agitation and laid out into a tray covered with cling wrap  
overnight to maintain humidity. The next day they were returned to upright, and the procedure was  
repeated after seven days. Once the siliques had ripened, the seeds were harvested and dried for at  
least two weeks. For the selection of positive transformants, seeds were surface-sterilized using  
75% ethanol with 0.1% Triton X-100 on a rotating incubator for at least two hours, after which the  
seeds were decanted onto filter paper and the ethanol left to evaporate. Once the seeds were dry,  
they were sprinkled onto a plate of half-strength basal Murashige & Skoog (MS) medium with  
vitamins and 30 µg/ml hygromycin B, wrapped in aluminum foil, and stratified at 4 °C for three  
days (Murashige and Skoog, 1962). They were then placed into a growth cabinet for 10 to 14 days,  
after which healthy looking seedlings were transferred to half-strength MS plates without the  
hygromycin, and grown for another two weeks, at which stage they were transferred to soil.

### 337 **Fungal growth and transformation**

338 The *F. oxysporum* f. sp. *conglutinans* strain 5176 was collected in 1971 from white cabbage  
339 (*Brassica oleracea* var. *capitata* (L.)) in Australia (Wang *et al.*, 2022a). It has since been  
340 maintained by the Brisbane Pathology (BRIP) Plant Pathology Herbarium in Queensland,  
341 Australia under the accession number BRIP 5176 a. It was first used as a model pathogen for *A.*  
342 *thaliana* by (Campbell *et al.*, 2003). To generate a *Fo5176* line expressing cytoplasmic tdTomato  
343 (tdT), an expression clone was generated by cloning the *tdT* coding sequence (amplified from an  
344 AddGene-derived plasmid (Shaner *et al.*, 2004)) into the *Bg*III cloning site of plasmid pLAU2  
345 using Gibson assembly (Idnurm *et al.*, 2017). This places the gene under the control of a  
346 constitutive promoter from the *actin* gene of ascomycete *Leptosphaeria maculans*. After  
347 replication in *E. coli*, this plasmid pMAI32 was electroporated into *A. tumefaciens* strain EHA105  
348 with selection on LB medium with kanamycin (50 µg/ml). *Fo5176* was routinely cultured on  
349 potato dextrose agar (PDA) plates. To generate spores for transformation, five plugs of about 5  
350 mm diameter were inoculated into 50 ml half strength potato dextrose broth and cultured at 150

351 rpm for five days at room temperature, then filtered through miracloth, centrifuged at 3000 g for  
352 5 min, and resuspended in sterile H<sub>2</sub>O. The spores were then transformed with the *A. tumefaciens*  
353 strain that had been cultured overnight in LB broth with kanamycin, using standard methods  
354 (Idnurm *et al.*, 2017). That is, fungal spores and bacteria were mixed on induction medium plates  
355 and cocultured for three days. Selection for transformants used an overlay of PDA supplemented  
356 with hygromycin and cefotaxime (50 µg/ml and 100 µg/ml, respectively). Transformants that grew  
357 through the overlaid medium, were subcultured onto PDA containing hygromycin and cefotaxime  
358 and allowed to produce conidia, which were separated with a metal loop to generate strains derived  
359 from a single conidium.

### 360 **Plant-fungus co-cultivation and infection**

361 To obtain fungal spores, *Fo5176* was grown for at least seven days on PDA plates at room  
362 temperature, at which time five pieces of roughly 1 mm<sup>2</sup> size were cut from the plate and dropped  
363 into a flask containing 50 ml yeast nitrogen base (YNB) medium with 1% sucrose. The liquid  
364 cultures were incubated for four days at room temperature with shaking at 120 rpm. The solution  
365 was then filtered through miracloth, the spores harvested by centrifugation at 3000 rcf for 10 min  
366 and resuspended in 25 ml sterile MilliQ water. For the co-cultivation of plant and fungus, *A.*  
367 *thaliana* seedlings were grown on a vertical petri dish containing half-strength basal MS medium  
368 with vitamins for 11 days and then transferred to a horizontal petri dish with a 2-3 cm strip of half-  
369 strength basal MS medium with vitamins at the top end, while the rest of the plate was filled  
370 roughly 2-3 mm high with liquid quarter-strength basal MS medium with vitamins (this is a setup  
371 with slight modifications as described in (Tintor *et al.*, 2020)). The seedlings were placed onto the  
372 thin MS medium strip at the top end, with the root in the liquid medium. Fungal spores were then  
373 added to the liquid medium. The plates were covered with aluminum foil up until the leaves of the  
374 plant, and then placed into a growth chamber.

### 375 **Microscopy**

376 We imaged the infection and the progression of colonization on a Leica M205 FA  
377 stereomicroscope. Infection could usually be observed on day three after spored addition, and at  
378 day 5 there was robust colonization. We usually imaged daily from day 3 to 11 dpi. For the  
379 fluorescence coming from the plant 2xmT2 reporters, we used the Leica ET CFP (ET436/20x  
380 ET480/40m) filter, and for the fungal tdT reporter the Leica ET mCHER (ET560/40x ET630/75m)

381 filter. We use 80× magnification for the images used in this paper. The settings for the imaging  
382 (illumination strength, exposure time, gain, etc.) were kept constant for all imaging sessions, to  
383 allow for at least semi-quantitative imaging, and to make the images at least relatively comparable.  
384 The images were recorded using the Leica Application Suite software, and processed using Fiji Is  
385 Just ImageJ (FIJI) and the GNU Image Manipulation Program (GIMP) (Schindelin *et al.*, 2012).

### 386 **Data availability**

387 We have donated the pGG-PIP vector collection to AddGene (Deposit-ID: 82532, Catalog-#: 196739-196813).

### 389 **Acknowledgements**

390 This work was funded by the Australian Research Council (grant no. DE200101560) and was  
391 further supported by a seed grant from the Melbourne University Botany Foundation. LW was  
392 supported by the China Scholarship Council. H-WC was supported by a Graduate Research  
393 Scholarship from the University of Melbourne. Imaging was done on instruments maintained by  
394 the Biological Optical Microscopy Platform (BOMP) and the BioSciences Microscopy Unit at the  
395 University of Melbourne. The authors would like to thank Dr. Imre E. Somssich for critical reading  
396 of the manuscript.

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652 336.

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655 **Supplementary information:**

656 **Supplementary table 1: List of primers used to clone the promoters**

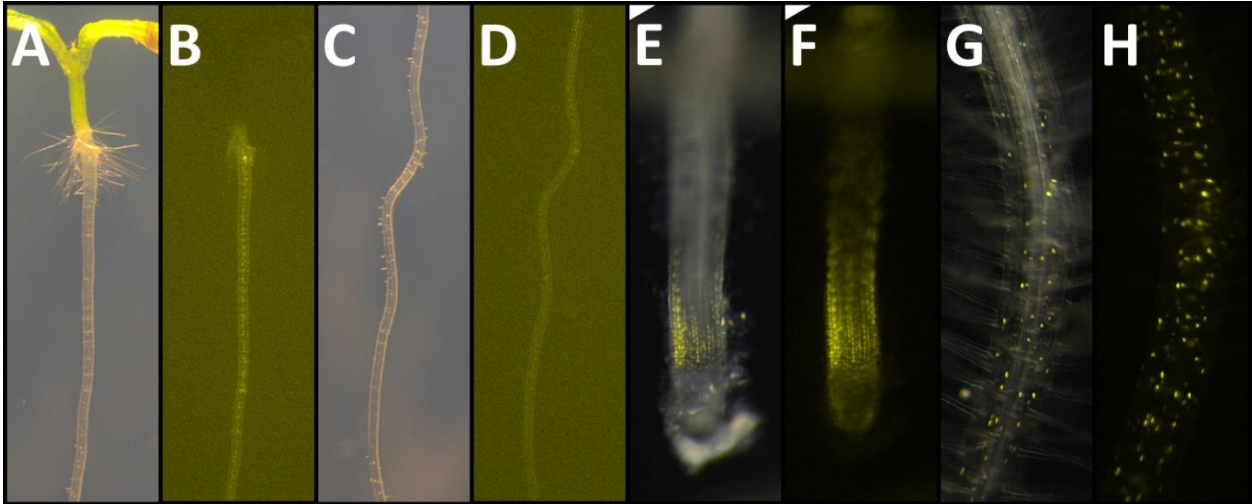
657 MAS-GG-ACS2p-F AACAGGTCTCAACCTCGGTCGATGTAAATGGATTAAATTTTATA  
658 MAS-GG-ACS2p-R AACAGGTCTCATGTTGCTGTGTCAATTCTCACTTCTTTG  
659 MAS-GG-ACS2pm-F AACAGGTCTCAGGTGTCCTCAAGGTTTCTGTTTCAAC  
660 MAS-GG-ACS2pm-R AACAGGTCTCACACCAAATAAAGTTGATGTGGGGTC  
661 MAS-GG-AOSp-F AACAGGTCTCAACCTACACTTAGACACCCCAATATTTTAGATTT  
662 MAS-GG-AOSp-R AACAGGTCTCATGTTCTATTCGAAACAGTGGCGAGT  
663 MAS-GG-AOSpm1-F AACAGGTCTCAGCCAATATTTTAGATTTTACTTTAAAGAAAT  
664 MAS-GG-AOSpm1-R AACAGGTCTCATGGCGTCTCTAAGTGTTTTTTTTT  
665 MAS-GG-AOSpm2-F AACAGGTCTCAGACACCTAAGTATTTTCTTTCCAACAA  
666 MAS-GG-AOSpm2-R AACAGGTCTCATGTCCTTCTCTTTGAATAAACCTCA  
667 MAS-GG-ARR5p-F AACAGGTCTCAACCTATATGATTTTTTCAAAGAAAACACCATTTAGT  
668 MAS-GG-ARR5p-R AACAGGTCTCATGTTATCAAGAAGAGTAGGATCGTGACTCGT  
669 MAS-GG-ARR5pm-F AACAGGTCTCAGATTAGGATTATTTCTTTATAGAATGTTTTGGTGC  
670 MAS-GG-ARR5pm-R AACAGGTCTCAAATCGTCTCGGTTTTTACCTCTCAAATAGTAT  
671 MAS-GG-ATG8Ap-F AACAGGTCTCAACCTTTAGCAGTGCTTAGTGAGCTTAAATTATAGTT  
672 MAS-GG-ATG8Ap-R AACAGGTCTCATGTTAATTAATAAACTCGATCGTCTGCTAGATCG  
673 MAS-GG-BAK1p-F AACAGGTCTCAACCTGTTTTTTGGAAACAGAGAGAAAACCTCA  
674 MAS-GG-BAK1p-R AACAGGTCTCATGTTTATCCTCAAGAGATTA AAAACAAACCCTA  
675 MAS-GG-BIK1p-F AACAGGTCTCAACCTCGTTCCCAAATCTCGGTCAATTG  
676 MAS-GG-BIK1p-R AACAGGTCTCATGTTCAAAGCTAAGAACAGATTCGTTTTCTTCTT  
677 MAS-GG-BIK1pm-F AACAGGTCTCAGTACATTCATTTTGATTGGGATTTATCTTTTTA  
678 MAS-GG-BIK1pm-R AACAGGTCTCAGTACAGACCAAAAACAGTGGACTTGGG  
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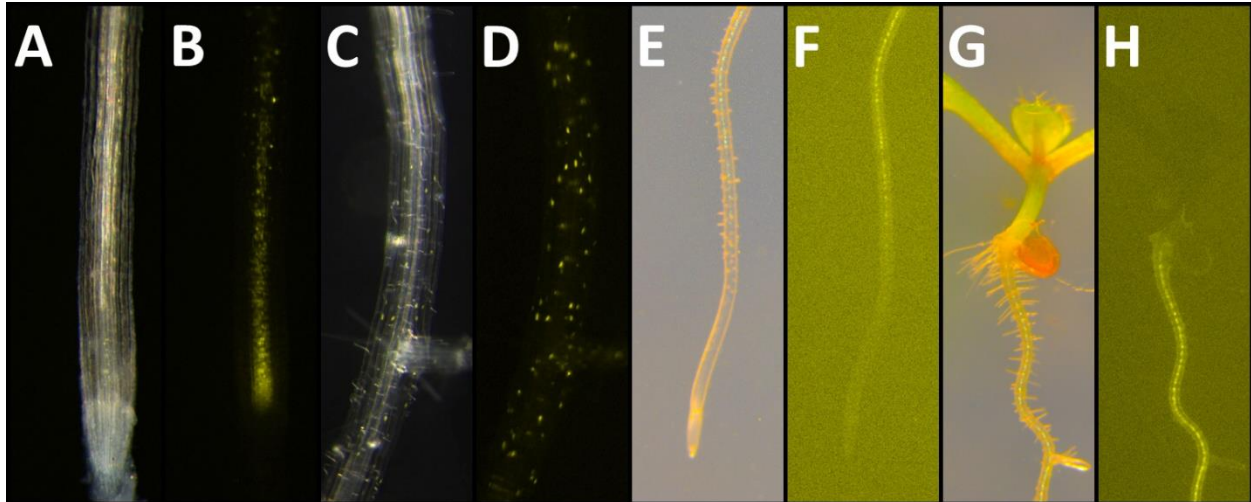
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**Figure S1: Expression pattern of *PEP1* and *PEP2***

**A-D** shows the expression of *PEP1* (yellow). Robust expression is found in the inner root tissues of the mature DZ (A, B). From the young to mature DZ, expression gradually becomes stronger (C, D). No expression in the root tip, MZ or EZ. **E-H** shows the expression of *PEP2* (yellow). Expression is found in the EZ of the tip, then disappears in the young DZ (E, F), and returns to all tissues in the mature DZ (G, H). A, C, E, G are bright field images plus fluorescence, B, D, F, H are fluorescence only.

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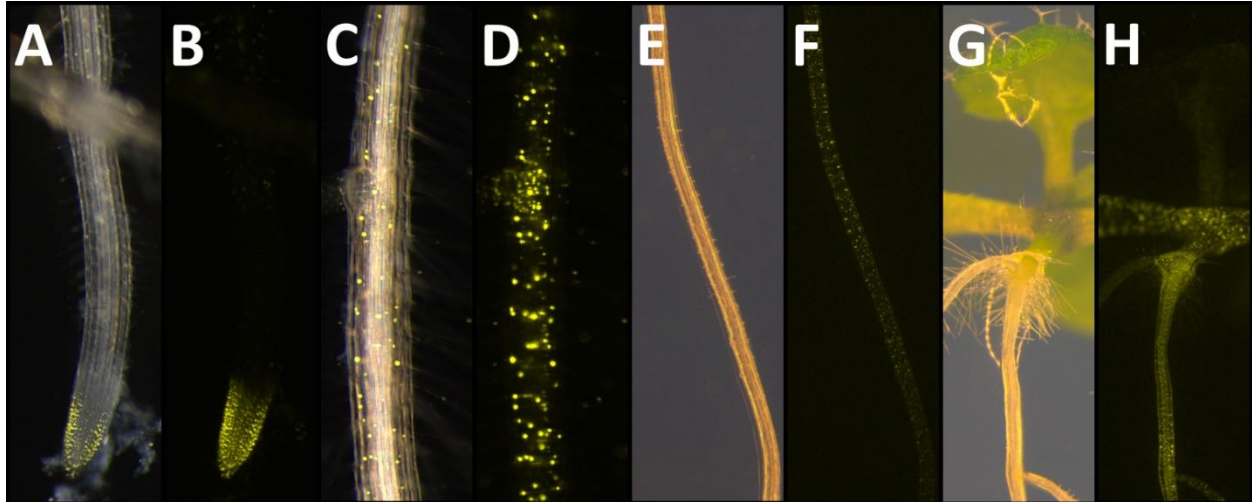


**Figure S2: Expression pattern of *PEPR1* and *PEPR2***

**A-D** shows the expression of *PEPR1* (yellow). Weak expression is found in vasculature of the MZ, EZ and young DZ (A, B). In the mature DZ, weak expression is found in all tissues (C, D). **E-H** shows the expression of *PEPR2* (yellow). Strong expression is found in the vasculature of the DZ, starting in the root hair zone (E, F) and becoming stronger in the mature DZ (G, H). A, C, E, G are bright field plus fluorescence, B, D, F, H are fluorescence only.

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**Figure S3: Expression pattern of *BIK1* and *RBOHD***

**A-D** shows the expression of *BIK1* (yellow). Expression is found in the root tip around the meristem, including the root cap and part of the EZ (A, B). Expression appears stronger in the outer tissues compared to the vasculature. Further up the root, expression is robust in the mature DZ, but still stronger in the outer tissues (C, D). **E-H** shows the expression of *RBOHD* (yellow). *RBOHD* is expressed in all cells and tissues from the young DZ onwards (E, F). Expression is strongest in differentiated tissue (G, H), A, C, E, G are bright field plus fluorescence, B, D, F, H are fluorescence only.

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