1 **Plant SYP12 syntaxins mediate an evolutionarily conserved general** 2 **immunity to filamentous pathogens**

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15 **Abstract**

16 Filamentous fungal and oomycete plant pathogens that invade by direct penetration through the leaf
17 epidermal cell wall cause devastating plant diseases. Plant pre-invasive immunity towards non-
18 adapted filamentous pathogens is highly effective and durable. Pre- and post-invasive immunity
19 correlates with the formation of evolutionarily conserved and cell-autonomous cell wall structures,
20 named papillae and encasements, respectively. Yet, it is still unresolved how papillae/encasements
21 are formed and whether these defense structures prevent pathogen ingress. Here we show that in
22 *Arabidopsis*, the two closely related members of the SYP12 clade of syntaxins (PEN1 and SYP122)
23 are indispensable for the formation of papillae and encasements. Moreover, loss-of-function
24 mutants were hampered in pre-invasive immunity towards a range of phylogenetically distant non-
25 adapted filamentous pathogens, underlining the versatility and efficacy of this defense.
26 Complementation studies using SYP12s from the early diverging land plant, *Marchantia*
27 *polymorpha*, showed that the SYP12 clade immunity function has survived 470 My of independent
28 evolution. These results suggest that ancestral land plants evolved the SYP12 clade to provide a

29 broad and durable pre-invasive immunity to facilitate their life on land, and pave the way to a better
30 understanding of how adapted pathogens overcome this ubiquitous plant defense strategy.

31

32 **Introduction**

33 In response to attack by filamentous pathogens, plants assemble localized pre-invasive papillae and
34 post-invasive encasements at sites of attempted cell entry (Figure 1-figure supplement 1A-B)
35 (Hückelhoven and Panstruga, 2011). These conserved defense structures are thought to contain
36 antimicrobial cargo that provide effective and durable immunity against diverse filamentous
37 pathogens, and fossil evidence suggests that these structures appeared very early in the evolution of
38 land plants (O'Connell and Panstruga, 2006; Krings et al., 2007; Overdijk et al., 2016; Hansen and
39 Nielsen, 2018). However, although their frequent association with resistance to non-adapted
40 pathogens is well-known, direct evidence that papillae/encasements contribute to immunity against
41 these pathogens is still lacking. The discovery that the secretory syntaxin PEN1 is required for the
42 timely formation of papillae to hamper penetration by non-adapted powdery mildew fungi
43 highlighted the key role played by membrane trafficking in plant immunity (Collins et al., 2003;
44 Nielsen et al., 2012; Nielsen and Thordal-Christensen, 2013). PEN1 (also referred to as syntaxin of
45 plants 121 or SYP121) is a Qa-SNARE primarily located at the plasma membrane. On the other
46 hand, the ARF-GEF, GNOM, regulates recycling of PEN1 between the plasma membrane and the
47 *trans*-Golgi network, where it is needed for a fast papilla response (Nielsen et al., 2012).
48 Accordingly, PEN1 is thought to mediate membrane fusion both at the plasma membrane and at the
49 *trans*-Golgi network. The ROR2 syntaxin in barley is orthologous to PEN1 (Collins et al., 2003),
50 suggesting that the pre-invasive immunity dependent on PEN1 or ROR2 was present in early
51 angiosperms before the divergence of monocots and dicots, spanning some 140 million years (My)
52 of evolution. Focal accumulation of GFP-PEN1 at attack sites in response to *Blumeria graminis*
53 f.sp. *hordei* (*Bgh*) is also seen for ROR2 in barley and highlights a likely conserved functionality in
54 plant defense against powdery mildews (Bhat et. al, 2005). The strong extracellular accumulation of
55 GFP-PEN1 signal localized to fungal attack sites can be explained by the secretion of extracellular
56 vesicles, also known as exosomes, which accumulate to a high degree in both papillae and
57 encasements (An et al., 2006; Meyer et al., 2009; Nielsen et al., 2012). While exosomes are
58 suggested to contain miRNAs directed against the invading pathogen, their topology makes it
59 difficult to associate syntaxin functionality (Meyer et al., 2009; Hansen and Nielsen, 2018). GFP-

PEN1 is generally considered a valid exosomal marker yet the biogenesis pathway of PEN1-labeled exosomes remains unclear (Rutter and Innes, 2017). Loss of PEN1 or ROR2 delays, but does not prevent, the papilla response upon attack by the barley powdery mildew fungus *Bgh* in *Arabidopsis* or barley, respectively (Assaad et al., 2004; Böhlenius et al., 2010). In contrast, the deposition of encasements around developing intracellular pathogenic structures (IPS) is unaffected by the loss of PEN1 (Wen et al., 2011; Nielsen et al., 2017). Furthermore, although many other filamentous pathogens also penetrate directly through the plant epidermal cell wall, the PEN1- and ROR2-dependent immunity appears dedicated towards the haustorium-forming powdery mildew and rust fungi (Collins et al., 2003; Jarosch et al. 2005; Lipka et al., 2005; Loehrer et al., 2008; Hoefle et al., 2009). Thus, the molecular mechanisms underlying papilla/encasement formation, as well as their role in general plant pre- and post-invasive immunity towards other filamentous pathogens, remain obscure.

72

PEN1 and ROR2 belong to the SYP12 clade of secretory syntaxins, which first appears in embryophytes and is conserved in all land plants (Slane et al., 2017). Consequently, the appearance of the SYP12 clade was proposed to play a key role in the terrestrialization of plants by enabling specialization of the secretory pathway (Sanderfoot, 2007). This is supported by the fact that four of the five SYP12 clade members in *Arabidopsis* function in specialized forms of polarized secretion, these being pre-invasive immunity towards powdery mildew fungi (PEN1), root hair tip growth (SYP123), and pollen tube tip growth (SYP124 and SYP125) (Collins et al., 2003; Slane et al., 2017; Ichikawa et al., 2014). Recently, PEN1 and SYP122, which is the closest homologue of PEN1, were suggested to mediate distinct but complementary secretory pathways during vegetative plant growth (Waghamare et al., 2018). Indeed, *syp122* mutants show an altered cell wall composition but in contrast to *pen1* mutants there is no effect on the pre-invasive immunity towards *Bgh* (Assaad et al., 2004). Interestingly, the *pen1 syp122* double mutant is autoimmune, developing a severe necrotic phenotype and accumulating the defense hormone salicylic acid (Assaad et al., 2004; Zhang et al., 2007, 2008). However, prior to the onset of necrosis, *pen1 syp122* double mutant plants are indistinguishable from wild-type plants. This underlines that PEN1 and SYP122 likely mediate a highly specialized form of secretion whereas general secretion relies on the evolutionarily ancient Qa-SNARE SYP132 (Park et al., 2018; Karnahl et al., 2018). The development of necrotic lesions in *pen1 syp122* double mutant plants is markedly delayed when the accumulation of salicylic acid is hampered (Zhang et al., 2007, 2008). Accordingly, the *pen1*

92 *syp122* double mutant autoimmune phenotype was suggested to result from activation of
93 nucleotide-binding leucine-rich repeat receptors (NLRs), immune sensors that recognize pathogen
94 effectors, directly or indirectly, which leads to a programmed cell death response (Wang et al.,
95 2019a; Wang et al., 2019b; Horsefield et al., 2019; Wan et al., 2019). In support of this, mutations
96 in the deubiquitinase AMSH3 that impairs the cell death reaction caused by activation of coiled-coil
97 (CC)-NLRs, also suppresses the autoimmune phenotype of the *pen1 syp122* double mutant
98 (Schultz-Larsen et al., 2018). The autoimmune phenotype shows that while PEN1 and SYP122 are
99 likely to have separate functions in the pre-invasive immunity towards *Bgh*, the two syntaxins also
100 share an as-yet unresolved function which is likely related to defense and not general development.

101

102 Here we show that the formation of papillae and encasements in *Arabidopsis* requires either of the
103 two SYP12 clade members, PEN1 and SYP122, because plants lacking both syntaxins are
104 completely devoid of both types of defense structure. We show that PEN1 and SYP122 provide pre-
105 and post-invasive immunity not only against the powdery mildew *Bgh* but also against distantly-
106 related filamentous pathogens that invade host epidermal cells, namely the fungus *Colletotrichum*
107 *destructivum* and the oomycete *Phytophthora infestans*. Complementation studies show that
108 SYP12A from *Marchantia polymorpha* functions and behaves like SYP122 in *Arabidopsis*, thereby
109 revealing a conserved ancestral mechanism. We conclude that papilla and encasement formation is
110 mediated by a specialized function of the SYP12 clade of syntaxins and that these defense
111 structures correlate with a broad and effective resistance towards filamentous pathogens that
112 penetrate the epidermal cell layer. Moreover, we suggest that early land plants evolved the SYP12
113 clade of secretory syntaxins to provide a durable immunity that helped plants move on to land and
114 possibly shape mutualistic plant-fungus interactions.

115

116 **Results**

117 The role of PEN1 in timely papilla deposition in response to *Bgh* attack prompted us to investigate
118 the involvement of other syntaxins in pre-invasive immunity, notably SYP122 that shares a
119 currently unknown function with PEN1. This shared function is apparent from the autoimmune
120 lesion-mimic phenotype of the *pen1 syp122* double mutant (Assaad et al., 2004; Zhang et al., 2007,
121 2008). Yet, during the first ~two weeks of growth, the *pen1 syp122* mutant develops no visible

122 symptoms, leaving a short window in which to study the interaction with *Bgh* before the onset of
123 necrosis. From analysis of unattacked plants, we found that the *pen1 syp122* mutant developed
124 spontaneous callose deposits despite having a wild-type like appearance (Figure 1A-B). Callose is a
125 polysaccharide (beta-1,3-glucan) secreted in response to various biotic and abiotic stresses and is a
126 major constituent of papillae and encasements. Although the deposition of callose is not required to
127 block the penetration event by *Bgh per se*, it serves as a reliable visual marker for easy detection of
128 papillae and encasements (Ellinger et al., 2013). We found that in response to attack by *Bgh*, plants
129 lacking PEN1 and SYP122 were unable to form papillae at sites of unsuccessful attack by fungal
130 appressoria, as visualized by detection of callose (Figure 1C-D). Surprisingly, while the
131 appressorial germtube did not induce a papilla response in *pen1 syp122* mutants, we observed large
132 callosic deposits induced by the non-invasive primary germ tube, which were not observed in any
133 other genetic background (Figure 1D, F-G, Figure 1-figure supplement S1A). Thus, SYP122 seems
134 to play a complementary role to PEN1 in papilla formation upon attack by *Bgh*. However, despite
135 being unable to form a papilla at the attempted entry site, the *pen1 syp122* mutant remains capable
136 of generating focused depositions of callose elsewhere and seems to be highly sensitive to signals
137 released by *Bgh*.

138 In response to a successful penetration attempt by *Bgh*, the host cell initiates post-invasive
139 immunity, where an encasement forms as an extension of the penetrated papilla (Figure 1-figure
140 supplement 1B). The encasement expands and, eventually, completely encloses the IPS and likely
141 prevents the pathogen from proliferating. Previous observations have shown that although *pen1*
142 mutants display a delayed papilla formation response to attack by *Bgh*, these plants seem unaffected
143 in their ability to form encasements when successfully penetrated (Assaad et al., 2004; Wen et al.,
144 2011; Nielsen et al., 2017). In contrast to our observations of unsuccessful penetration sites, the
145 successful penetration of *pen1 syp122* cells often induced a strong but diffuse, unfocused
146 accumulation of callose at the fungal entry site (Figure 1E-G, Figure 1 - video 1-2). Interestingly,
147 we did not observe any encasement-like structures. Instead, the callosic deposits in the *pen1 syp122*
148 mutant were superficial and did not extend inwards from the outer surface of the host cell
149 enveloping the growing IPS. This would suggest that the penetration event by *Bgh* triggers a strong
150 callose response but formation of the encasement is dependent on a functional overlap of the PEN1
151 and SYP122 syntaxins. Furthermore we tested the adapted powdery mildew *Golovinomyces orontii*
152 (*G.orontii*) which also induces encasement formation, although at a later stage and less frequently
153 than *Bgh*. For *G. orontii*, IPS formation seemed unaffected in *pen1 syp122* and no encasements

154 were found. Interestingly, successful penetration by *G. orontii* did not induce the strong, diffuse
 155 callose response described for *Bgh* (Figure 1-figure supplement 1C-D, Figure 1 - video 3-4).

156 The lack of papillae and encasements in the *pen1 syp122* mutant, prompted us to investigate the
 157 secretion of exosomes in response to *Bgh* attack. We speculated that the plant exosomal marker
 158 TET8-GFP (TETRASPANIN 8), which accumulates at sites of attack by *Botrytis*, would also
 159 accumulate in response to attack by *Bgh* (Boavida et al., 2013; Cai et al., 2018). In unattacked
 160 epidermal cells of wild-type plants, we found TET8-GFP localized mainly on the plasma membrane
 161 (Figure 2A). TET8-GFP signal focally accumulated in response to successful penetration by *Bgh*
 162 and seems a reliable marker for the encasement (Figure 2B). In unattacked epidermal cells of the
 163 *pen1 syp122* mutant, the TET8-GFP localization was similar to that seen in wild-type plants
 164 whereas successful penetration by *Bgh* did not induce focal accumulation of TET8-GFP (Figure 2C-
 165 D, Figure 2 - video 1-2). Thus, the general localization of TET8-GFP was not affected in the *pen1*
 166 *syp122* mutant. Only in the event of fungal attack where TET8-GFP is transported to the infection
 167 site in wild-type plants, this did not occur in the double mutant, supporting the idea that PEN1 and
 168 SYP122 play a specific role in pre- and post-invasive immunity.

169 We noticed that the spontaneous callose deposits formed in the lesion-mimic *pen1 syp122* mutant,
 170 made it difficult to compare pre- and post-invasive immunity responses during attack by *Bgh*.
 171 Furthermore, successful penetration of epidermal cells in *pen1 syp122* by *Bgh*, and even *G. orontii*,
 172 resulted in a fast-developing cell death response which would likely compromise encasement
 173 formation. To overcome the difficulties of working with the *pen1 syp122* mutant, we made use of
 174 the partially rescued triple mutant, *fmo1 pen1 syp122* (Zhang et al., 2008). Flavin-dependent-
 175 monooxygenase1 (FMO1) enables *Arabidopsis* plants to generate N-hydroxypipecolic acid, which
 176 plays a central role in pathogen-inducible plant immunity (Hartman et al., 2018). Similar to
 177 introducing a number of SA-signalling mutations, the loss of FMO1 clearly relieves the
 178 downstream effects of the *pen1 syp122* lesion-mimic mutant (Figure 3-figure supplement 1A-B).
 179 However, importantly, these mutations do not affect pre-invasive immunity towards *Bgh*, thereby
 180 clearly separating the two phenotypes mechanistically (Zhang et al., 2007 and this work).
 181 Moreover, *fmo1 pen1 syp122* plants did not display the large callosic deposits induced by the non-
 182 invasive primary germ tube. To ensure that the autoimmune phenotype of *fmo1 pen1 syp122* would
 183 not compromise fungal development, we first tested *G. orontii*. During the initial 48 hours after
 184 inoculation, we found that formation of the IPS and secondary hyphae in *fmo1 pen1 syp122* was
 185 indistinguishable from the *fmo1* control plants (Figure 3-figure supplement 1C-D). In response to

186 attack by *Bgh*, we found that *fmo1* plants formed papillae at the site of attack which were identical
 187 to wild-type as visualized by detection of callose (Figure 3A-C, Figure 3-figure supplement 2A-D).
 188 Instead, plants lacking PEN1 and SYP122 were unable to form discrete papillae at sites of
 189 unsuccessful fungal attack. To support these findings, we also tested the ability of these plants to
 190 accumulate papillary H₂O₂ in response to attack by *Bgh* using DAB (Thordal-Christensen *et al.*,
 191 1997). Similar to the staining of callose, the accumulation of H₂O₂ is used as a convenient marker
 192 of the defense structure. In accordance with our observations on callose deposition, we found that
 193 *fmo1 pen1 syp122* plants failed to accumulate papillary H₂O₂ (Figure 3D-F). Thus, these results
 194 confirmed that plants lacking PEN1 and SYP122 failed to form papillae at sites of attack by *Bgh*.

195 Similar to the papilla response, we found that *fmo1* mutants reacted like wild-type plants in
 196 response to successful penetration by *Bgh*, as visualized by the detection of callose encasements
 197 around the IPS (Figure 4A-C, Figure 4-figure supplement 1A-D). In stark contrast to this, *fmo1*
 198 *pen1 syp122* plants were unable to create an encasement around the developing IPS when
 199 penetrated by *Bgh*. To rule out the possibility that the lack of these defense structures is due to a
 200 host cell death response, dead cells stained by Trypan blue were excluded from our observations.
 201 Similar to our observations on papilla formation, we also assessed the ability of these plants to
 202 accumulate H₂O₂ in response to successful penetration by *Bgh* using DAB. We found that in *fmo1*
 203 *pen1 syp122* plants, successful penetration by *Bgh* induced a diffuse, superficial accumulation of
 204 H₂O₂ but we did not observe encasement-like structures (Figure 4D-E). This supported our finding
 205 that plants lacking PEN1 and SYP122 failed to form encasements at sites of attack. To verify the
 206 requirement for PEN1 and SYP122 in forming papillae and encasements, we included observations
 207 of *pad4 sid2 pen1 syp122* and *amsh3 pen1 syp122* mutants, where, similar to *fmo1*, the autoimmune
 208 phenotype of *pen1 syp122* is attenuated (Zhang *et al.*, 2008; Schultz-Larsen *et al.*, 2018). As
 209 expected, fungal development and host cell responses in these lines were similar to that observed in
 210 *fmo1 pen1 syp122* plants (Figure 4-figure supplement 2, Figure 4 - video 1-8). As SYP122 appears
 211 to have a significant, albeit less important, role in papilla and encasement formation, we wondered
 212 if the complete absence of these defense structures would lead to an increase in penetration
 213 frequency as compared to the delayed papilla response described for the *pen1* mutant. We found a
 214 significant increase in fungal penetration frequency on *fmo1 pen1 syp122* plants when compared to
 215 *pen1* single and *fmo1 pen1* double mutant plants (Figure 4F). This supports our previous finding of
 216 a minor, but significant PEN1-independent contribution to pre-invasive immunity that relates to
 217 proper formation of the encasement (Nielsen *et al.*, 2017). Taken together, these observations show

218 that the *Bgh*-induced formation of papillae and encasements at attack sites is dependent on a
219 functional overlap between the PEN1 and SYP122 syntaxins.

220 To further dissect the overlapping functions of PEN1 and SYP122 in immunity, we tested host cell
221 responses to another, non-adapted fungal pathogen, namely *C. destructivum* (an ascomycete fungal
222 pathogen causing alfalfa anthracnose disease). The initial invasion strategy of *C. destructivum* is
223 very similar to that of *Bgh* (Latunde-Dada et al., 1997). Nevertheless, while host cell responses
224 include papilla formation, the highly effective pre-invasive immunity of *Arabidopsis* towards non-
225 adapted *Colletotrichum* species such as *C. destructivum* does not require PEN1 (Shimada et al.,
226 2006; Yang et al., 2014). Moreover, attack by *C. destructivum* did not induce accumulation of GFP-
227 PEN1, GFP-SYP122 or TET8-GFP at sites of attempted penetration (Figure 5-figure supplement
228 1A-C). Nonetheless, we found that plants lacking both PEN1 and SYP122 showed a dramatic
229 decrease in the ability to form papillae at sites of unsuccessful penetration (Figure 5A-C). In
230 contrast to *Bgh*, the host cell response to penetration by *C. destructivum* only rarely resulted in the
231 formation of a detectable encasement. Nevertheless, penetrated host cells did respond by
232 accumulating callose in and around the site of penetration. In *fmo1 pen1 syp122*, the responses
233 varied from no detectable callose to a faint diffuse accumulation of callose at the penetration site
234 (Figure 5D-E, Figure 5-figure supplement 1D-E). Moreover, the lack of host cell responses
235 correlated with a remarkable increase in penetration frequency by *C. destructivum* on *fmo1 pen1*
236 *syp122* plants (Figure 5F, Figure 5-figure supplement 1F-G). Thus, pre-invasive immunity towards
237 *C. destructivum* requires a syntaxin function shared by PEN1 and SYP122 and this correlates with
238 the lack of defense structure formation in response to attack.

239 Finding that PEN1 and SYP122 facilitate a pre-invasive immunity that extends to a fungal pathogen
240 other than powdery mildews, we speculated that this immunity could be a general defense
241 mechanism effective against an even wider range of direct penetrating filamentous pathogens. One
242 such pathogen is *P. infestans* (the potato blight pathogen), which, as an oomycete, is thought to
243 have evolved completely independently of true fungi (Lévesque, 2011). Similar to the above
244 observations for *C. destructivum*, pre-invasive immunity towards *P. infestans* in *Arabidopsis* does
245 not rely on a functional PEN1 (Lipka et al., 2005). Instead, Rodriguez-Furlán et al. (2016) reported
246 that loss of the root-hair specific SYP123 affects the penetration rates by *P. infestans*. However,
247 SYP123 was suggested to regulate rhizobacterial priming of induced systemic resistance and is
248 unlikely to be directly involved in the pre-invasive response in leaves. We observed that attempted
249 penetration by *P. infestans* did not lead to accumulation of either GFP-PEN1, GFP-SYP122 or

250 TET8-GFP at the site of attack (Figure 6-figure supplement 1A-C). As expected, penetration
251 attempts by *P. infestans* incited papilla formation in all genotypes, except for *fmo1 pen1 syp122*
252 (Figure 6A-C). Successful penetration by *P. infestans* was often followed by host cell death.
253 Nevertheless, we found that host cells expressing PEN1 and/or SYP122 could initiate a strong
254 accumulation of callose and form an encasement around the IPS. In the *fmo1 pen1 syp122* triple
255 mutant, the penetrated cell, as well as neighboring cells, responded with a diffuse callose
256 accumulation but encasement of the IPS was not seen (Figure 6D-E). Similar to our observations
257 with *C. destructivum*, *fmo1 pen1 syp122* plants displayed elevated penetration frequencies when
258 attacked by *P. infestans* (Figure 6F, Figure 6-figure supplement 1D). Surprisingly, we could not
259 detect an increase in penetration events by *P. infestans* in the *syp123* mutant, even when in
260 combination with *pen1*. Taken together, these findings show that PEN1 or SYP122 are required for
261 papilla and encasement responses, which mediate immunity towards these phylogenetically distant,
262 non-adapted filamentous pathogens. Moreover, it is striking that the functionality of PEN1 and
263 SYP122 in immunity does not correlate with the ability of these syntaxins to visibly accumulate at
264 attack sites.

265 PEN1 and SYP122 belong to the SYP12 clade of secretory syntaxins, which evolved during plant
266 terrestrialization and is conserved in all land plants (Sanderfoot, 2007; Slane et al., 2017). To
267 evaluate whether the immunity function of the SYP12 clade is also evolutionarily conserved among
268 land plants, we investigated the response of the early diverging land plant *Marchantia* to attack by
269 non-adapted filamentous pathogens. Consistent with previous findings, the germinating spores of
270 *Bgh* were not able to properly differentiate a penetration hypha or appressorium on *Marchantia*
271 thalli (Figure 7-figure supplement 1A; Takikawa et al., 2013). Similarly, most spores of *P. infestans*
272 germinating on *Marchantia* seemed unable to identify a host cell and direct an attack. Nonetheless,
273 occasional sites of attempted and successful penetrations were detected, to which the host cell
274 responded by forming papillae and encasement-like defense structures, respectively (Figure 7A,
275 Figure 7-figure supplement 1B). Thus, neither *Bgh* nor *P. infestans* were suitable for studying non-
276 host interactions in *Marchantia*. In contrast, *C. destructivum* spores germinated and developed
277 characteristic darkly melanized appressoria on the thallus surface. In response to attack, *Marchantia*
278 epidermal cells formed callose-containing papillae similar to those found in *Arabidopsis* (Figure
279 7B). However, we never detected successful penetration of *Marchantia* cells by *C. destructivum*. A
280 previous report that the *Marchantia* syntaxin MpSYP13B accumulates in response to colonization
281 by *P. palmivora* prompted us to test whether candidate MpSYPs also accumulate in response to *C.*

282 *destructivum* (Carella et al., 2018). However, despite inducing papillae, attack by *C. destructivum*
283 did not induce focal accumulation of any of the four MpSYPs tested (Figure 7C-F; Kanazawa et al.,
284 2016). Thus, the cellular responses of *Marchantia* to *C. destructivum* were similar to those that we
285 observed in *Arabidopsis*.

286

287 In contrast to PEN1 and SYP122 in *Arabidopsis*, MpSYP12A seems to be essential in *Marchantia*,
288 thereby precluding the possibility for studying pre-invasive immunity in knock-out lines (Kanazawa
289 et al., 2020). Instead, to evaluate whether the immunity function of the SYP12 clade is
290 evolutionarily conserved among land plants, we introduced GFP-fused versions of the two
291 *Marchantia* SYP12s (*i.e.* MpSYP12A and MpSYP12B) into the *Arabidopsis pen1 syp122* mutant.
292 GFP-MpSYP12A fully suppressed the necrotic and dwarfed phenotypes of *pen1 syp122* plants,
293 while GFP-MpSYP12B only partially suppressed these phenotypes (Figure 8-figure supplement
294 1A-B). In contrast, expression of either of the two SYP13 clade members, MpSYP13A and
295 MpSYP13B, failed to rescue the *pen1 syp122* mutant. This suggests that MpSYP12A shares the
296 PEN1/SYP122 overlapping function and that MpSYP12B does so incompletely. Because
297 MpSYP12A complements the growth phenotype of *pen1 syp122* at the macroscopic level, we
298 decided to test how these plants react to attack by *Bgh*. We found that MpSYP12A completely, and
299 MpSYP12B partially, restored both papilla and encasement responses (Figure 8A-B, Figure 8-
300 figure supplement 2, Figure 8-figure supplement 3). As explained above, the papillary accumulation
301 of GFP-PEN1 in response to attack by *Bgh* is also seen for ROR2 in barley and highlights a likely
302 conserved functionality in plant defense against powdery mildews (Bhat et. al, 2005). In contrast,
303 neither GFP-SYP122 nor the two GFP-MpSYP12s accumulated in papillae at sites of attempted
304 penetration (Figure 8C, Figure 8-figure supplement 4). However, all the tested SYPs accumulated in
305 the encasement matrix at sites of successful penetration (Figure 8D, Figure 8-figure supplement 5).
306 Interestingly, pre-invasive immunity towards *Bgh* was not restored by GFP-MpSYP12A. Instead,
307 the penetration frequency of *Bgh* was similar to that of plants expressing GFP-SYP122 (Figure 8E).
308 Thus, MpSYP12A resembles SYP122, and not PEN1, both in its localization and functionality. In
309 support of this, GFP-MpSYP12A fully restored immunity towards both *P. infestans* and *C.*
310 *destructivum* (Figure 8F, Figure 8-figure supplement 6A-B). Also, when tested with the adapted
311 filamentous pathogens *G. orontii* and *C. higginsianum*, penetration frequencies were similar to
312 plants expressing PEN1 and SYP122 (Figure 8-figure supplement 6C-D). As described for PEN1
313 and SYP122, focal accumulation of GFP-MpSYP12A or GFP-MpSYP12B was not observed at

314 unsuccessful attack sites by either *C. destructivum* or *P. infestans* (Figure 8-figure supplement 7).
315 Taken together, these results reveal a conserved SYP12 clade functionality that has survived 470
316 My of independent evolution. Moreover, our findings suggest that the PEN1-dependent immunity
317 towards powdery mildew fungi evolved at a later stage of land plant evolution.

318

319 Discussion

320 We have revealed a highly conserved role for the SYP12 clade of plant syntaxins in mediating the
321 formation of pre- and post-invasive defense structures and provide immunity to highly diverged
322 filamentous pathogens. *Arabidopsis* plants lacking both PEN1 and SYP122 were incapable of
323 forming papillae and encasements in response to attack by the non-adapted pathogens *Bgh*, *C.*
324 *destructivum* and *P. infestans*, as based on staining for callose and H₂O₂, resulting in highly
325 elevated penetration frequencies. Instead, these plants responded to attack with diffuse and
326 unfocused accumulations of callose and H₂O₂. It is noteworthy that although the *pen1 syp122*
327 double mutant lacks these defense structures, epidermal cells are still able to respond to pathogen-
328 derived signals in a polarized fashion, as seen in responses to the non-penetrative primary germ tube
329 of *Bgh*. This underlines that neither the general ability to secrete callose, generate H₂O₂, nor the
330 underlying mechanism needed for polarized secretion are dependent on PEN1 and SYP122. Instead,
331 we suggest that the PEN1/SYP122-dependent papillae and encasements are defense structures that
332 enable a focused secretion, specifically aimed at blocking the ingress of filamentous pathogens. In
333 support of this, knock-down of StSYR1 in potato, a homolog of PEN1, results in reduced papilla
334 formation in response to *P. infestans* (Eschen-Lippold et al., 2012). A more indirect role of
335 syntaxins in pre-invasive immunity is the regulation of rhizobacterial priming of induced systemic
336 resistance by the root-hair specific SYP123 (Rodriguez-Furlán et al. 2016). The induction of
337 systemic resistance could affect the speed by which PEN1/SYP122 dependent papillae/encasements
338 form in response to attack. However, as we failed to see an effect of SYP123, we could not pursue
339 this question further. We are unsure of the reason for this discrepancy between our results and those
340 reported by Rodriguez-Furlán et al. (2016), but we speculate that differences in the soil composition
341 and microbiome could play a role in this. Instead, we have shown here that loss of
342 papillae/encasements correlates with the loss of general pre-invasive immunity. Yet, it is difficult to
343 explain how these cell wall depositions would prevent invasion by pathogens secreting cell wall
344 degrading enzymes. Also, we find it unlikely that accumulation of callose, H₂O₂, syntaxins or even

345 TET8 would have a direct effect on the pathogen. This is supported by our observations that neither
346 PEN1, SYP122 nor TET8 focally accumulate in response to attack by *C. destructivum* or *P.*
347 *infestans*, despite the formation of papillae. Instead our findings reinforce the idea that papillae and
348 encasements serve as defense structures that contain a complex mixture of exosomes, toxic
349 metabolites and/or antimicrobial enzymes and peptides that mediate immunity (Kwon et al., 2008).
350 It is likely that these cargo molecules are delivered in a PEN1- and SYP122-dependent manner to
351 the site of attack to form papillae/encasements and thus prevent pathogen ingress.

352

353 Previous studies on the interaction between *Arabidopsis* and *Bgh* have revealed that pre-invasive
354 immunity correlates with the timely formation of papillae. This fast response likely requires the
355 recycling of papilla material in a process involving PEN1 and GNOM (Nielsen et al., 2012). In
356 contrast, although SYP122 also locates to the plasma membrane and interacts with the same
357 SNARE partners as PEN1, *syp122* mutants show a wild-type response to *Bgh* (Assaad et al., 2004;
358 Pajonk et al., 2008). Previous studies have shown that PEN1 and SYP122 mediate the secretion of
359 distinct cargo subsets, which may explain their differing contributions to pre-invasive immunity
360 (Waghmare et al., 2018). Based on the work presented here, it is now evident that PEN1 and
361 SYP122 functionally overlap in the formation of papillae and encasements. This could be explained
362 by PEN1 serving two functions in pre-invasive immunity. The first mediates a fast mobilization of
363 papilla material, while the second function is shared with SYP122 and contributes to a more general
364 papilla and encasement response to filamentous pathogens (Figure 9). An alternative explanation
365 would be that although redundant with SYP122, PEN1 somehow plays a more significant role in
366 pre-invasive immunity against *Bgh*. The fast mobilization of papilla material is likely related to the
367 ability of PEN1 to shuttle between the plasma membrane and *trans*-Golgi network, which is less
368 prominent for SYP122 (Hansen and Nielsen, 2018; Nielsen and Thordal-Christensen, 2012; Nielsen
369 and Thordal-Christensen, 2013). Consequently, PEN1, and less so SYP122, could function at the
370 *trans*-Golgi network, receiving vesicles from the plasma membrane that mediate the fast GNOM-
371 dependent recycling of papilla material, warding off the powdery mildew fungus. We envisage that
372 the redundant function of PEN1 and SYP122 is at the plasma membrane, where either of the two
373 syntaxins receive the membrane material and vesicular cargo required for papilla/encasement
374 formation. In contrast to the fast-acting, PEN1-dependent papilla response explained above, the
375 slow-forming papilla uses either PEN1 or SYP122 already present at the site of attack in the plasma
376 membrane and does not necessitate a fast recycling of the syntaxin itself. This could help to explain

377 why PEN1 and SYP122 do not accumulate at sites of attack by *C. destructivum* and *P. infestans*
378 although both syntaxins clearly mediate pre-invasive immunity. The pathway that delivers cargo for
379 the slow-forming papilla is also likely to be involved in delivering cargo for the encasement,
380 because the formation of neither is affected by the lack of PEN1. Therefore, we speculate that the
381 differences in papilla content based on observations of GFP-PEN1, GFP-SYP122 and TET8-GFP,
382 result from the involvement of distinct pathways for delivering cargo vesicles to the papilla.
383 Alternatively, as the accumulation of some papilla cargos are triggered by only a subset of
384 pathogens, the host cell might be able to differentiate the response according to the attacker.
385 Nonetheless, these pathways share a common requirement for PEN1 or SYP122 to mediate vesicle
386 fusion with the plasma membrane at pathogen attack sites. In addition, because some spores fail to
387 penetrate, even in the complete absence of papillae and encasements, pre-invasive immunity
388 towards filamentous pathogens could also involve other molecular pathways that act independently
389 of PEN1 and SYP122. In this regard, pre-invasive immunity mediated by PEN2 and PEN3, as well
390 as the resistance gained by loss of MLO, are of particular interest, since both pathways seem to
391 function independently of PEN1 (Lipka et al., 2005; Stein et al., 2006; Kuhn et al., 2017). It remains
392 to be elucidated if these pre-invasive immunity pathways also function independently of SYP122.

393

394 The SYP12 clade of plant secretory syntaxins is thought to have evolved during colonization of the
395 land and is conserved in all land plants so far examined (Slane et al., 2017). Because
396 terrestrialization presented enormous challenges to plants, the SYP12 clade was suggested to have
397 acquired specialized roles in secretion needed to facilitate this transition (Sanderfoot, 2007). This is
398 consistent with the previously identified functions of PEN1 (powdery mildew resistance), SYP123
399 (root hair tip growth) and, SYP124 and SYP125 (pollen tube tip growth) all being associated with
400 specialized forms of polarized secretion (Collins et al., 2003; Slane et al., 2017; Ichikawa et al.,
401 2014). In addition, PEN1 promotes K⁺ uptake by direct interaction with the RYxxWE motif found
402 in a subset of Kv channels (Karnik et al., 2017). However, powdery mildews, roots hairs and pollen
403 tubes did not exist until long after the onset of plant terrestrialization. Also, the interaction motifs
404 needed for Kv channel interaction, is restricted to vascular plants (Karnik et al., 2017). Therefore,
405 this knowledge gives little insight into the original function of the SYP12 clade. Our discovery that
406 SYP12A from *Marchantia* can complement SYP122, but not PEN1, in *Arabidopsis* reveals a
407 conserved syntaxin functionality spanning 470 My of independent evolution. In accordance with
408 this, MpSYP12A showed a localization behavior identical to that of SYP122. Therefore, the

409 functionality of the original SYP12 clade syntaxins is likely to have been similar to that of
410 MpSYP12A and SYP122. At a later stage of plant evolution, PEN1 has acquired an additional
411 specialization needed for powdery mildew resistance, which could explain its distinctive subcellular
412 localization (Nielsen and Thordal-Christensen, 2012; 2013). Interestingly, in *Marchantia*,
413 MpSYP12A locates to the plasma membrane and seems vital for cell plate formation whereas
414 MpSYP12B locates to a type of oil body unique to liverworts (Kanazawa et al., 2016; 2020). This
415 indicates yet another functional specialization of the SYP12 clade similar to that which occurred in
416 *Arabidopsis*.

417 We suggest that MpSYP12A and SYP122 represent the original SYP12 clade functionality in land
418 plants, which provides a highly conserved and effective broad-spectrum immunity towards
419 filamentous pathogens. Before plant terrestrialization, the land was already colonized by
420 filamentous fungi that were able to degrade cell walls and to utilize living or dead plant tissue as a
421 nutrient source (Chang et al., 2015). Recent findings suggest that even before terrestrialization,
422 plants used nucleotide-binding leucine-rich repeat receptors (NLRs) to activate their defense
423 responses (Gao et al., 2018). Activation of plant NLRs leads to a fast, programmed cell death
424 response, which does not provide immunity against fungi growing on dead or dying plant tissue
425 (Wang et al., 2019a; Wang et al., 2019b; Horsefield et al., 2019; Wan et al., 2019). Instead, the
426 papilla/encasement defense structures would have conferred an effective defensive barrier against
427 attack by the early saprophytic filamentous fungi already present on the land. Moreover, it is
428 generally accepted that plant colonization of the land was aided by, or dependent on, mycorrhiza-
429 like fungi (Pirozynskij et al., 1975; Redecker et al., 2000; Heckman et al., 2001). These mutualistic
430 interactions are thought to have resulted from ancestral land plants being able to ‘tame’ early
431 saprophytic filamentous fungi (Chang et al., 2015; Berbee et al., 2017). In this context, hyphal
432 encasement may have provided a tool for plants to restrict and manipulate the intracellular growth
433 of these fungi. We envisage that the SYP12 syntaxins enabled early land plants to regulate fungal
434 entry and colonization, and that over time this led to the mutually beneficial interactions that
435 facilitated terrestrialization.

436 Adapted filamentous pathogens are able to overcome pre-invasive immunity in their respective
437 host-plants, but not in other plant species. Our finding that plants could rely on a common
438 membrane trafficking pathway to block pathogen entry should lead to new insights into how
439 successful pathogens defeat this immunity by means of effector proteins (Jones et al., 2016). A
440 future goal will be to identify the membrane trafficking targets of such effectors and to exchange

441 those between hosts and non-hosts to generate a pre-invasive immunity that pathogens cannot
442 overcome.

443

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649 **availability:** All materials are available on request.

650

651 Materials and Methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
gene (<i>Arabidopsis thaliana</i>)	<i>pen1-1 syp122-1</i>	Assaad et al., 2004		See Materials and methods
gene (<i>Arabidopsis thaliana</i>)	<i>fmo1-1 pen1-1 syp122-1</i>	Zhang et al., 2008		See Materials and methods
gene (<i>Arabidopsis thaliana</i>)	<i>amsh3-4 pen1-1 syp122-1</i>	Schultz-Larsen et al., 2018		See Materials and methods
gene (<i>Arabidopsis thaliana</i>)	<i>sid2 pad4 pen1 syp122</i>	Zhang et al., 2004		See Materials and methods
gene (<i>Arabidopsis thaliana</i>)	<i>TET8-GFP</i>	Boavida et al., 2013		See Materials and methods

652

653 Plant, fungal and oomycete growth

Plants of *Arabidopsis thaliana* and *Marchantia polymorpha* (Tak-1) were grown at 21°C, with 8 h of light at 125 micro Einstein s⁻¹m⁻² per day. All mutant plant lines were reported previously (Collins et al., 2003; Assaad et al., 2004; Zhang et al., 2008; Rodriguez-Furlan et al., 2016; Schultz-Larsen et al., 2018). The barley powdery mildew fungus, *Bgh* (*B. graminis* f.sp. *hordei*, isolate C15) and *Arabidopsis* powdery mildew, *G. orontii*, were propagated on barley and *Arabidopsis*, respectively. *C. destructivum* isolate LARS 709 and *C. higginsianum* isolate IMI 349063A was cultured on Mathur's medium containing glucose (2.8 g), MgSO₄·7H₂O (1.22 g), KH₂PO₄ (2.72 g), Oxoid Mycological peptone (2.18 g) and agar (30 g) in 1 liter of deionised water at 25 °C in the dark. The *P. infestans* (field isolate 2017-DK-31-03 and SW1_A1) was cultured on rye sucrose (2%) agar supplemented with β-sitosterol (50mg/L), Pimaricin (10mg/L) and Ampicillin (100mg/L) at 18 °C in the dark (Tzelepis et al., 2020).

665

666 Infection studies

Spores from *Bgh* and *G. orontii* were blown evenly onto the leaves of 4-week-old *Arabidopsis* plants and kept at 21°C. For *Colletotrichum*, spores were harvested in water from a 7-10 day old culture. Spores were adjusted to a final concentration of 5 x 10⁵ or 1 x 10⁶ spores ml⁻¹ and sprayed evenly onto the leaves of 5-week-old *Arabidopsis* plants. Following inoculation, the plants were sealed inside plastic bags and kept at 21°C. Zoospores of *P. infestans* were released from sporangia by addition of cold water and incubation at 4°C for 90 min. The zoospore suspension was diluted to 5 x 10⁵ or 1 x 10⁶ spores/ml and added to the abaxial side of attached leaves from 4-week-old *Arabidopsis* plants. The leaves were kept in a closed Petri dish on water-soaked filter paper at 21°C.

For scoring penetration success, leaf material was Trypan blue–stained at 24 h post inoculation for *Bgh* or *P. infestans*, 48 h post inoculation for *G. orontii* and 72 h post inoculation for *C. destructivum* (Keogh et al., 1980). Penetration was determined by the presence of a developing IPS inside epidermal cells using light microscopy. To avoid the possibility that cells undergoing programmed cell death would be restricted in their encasement response to IPS, these cells were excluded from our observations based on Trypan blue staining. Once the penetration status for a particular event was determined, only then was the callose response investigated. For staining of live fungal structures, infected leaves were vacuum infiltrated with propidium iodide (10μM) for 2 hours. Callose staining was performed using 0.01% Aniline blue in 1 M glycine [adjusted to pH 9.5 using NaOH] and visualized by UV epifluorescence. All experiments were repeated at least three

685 times with similar results. Data from the individual studies described above represent discrete
686 variables since it was recorded whether or not a certain event had taken place (e.g., whether or not a
687 spore caused penetration). Consequently, these data were analysed by logistic regression, assuming
688 a binomial distribution (corrected for over-dispersion when present) (Collet, 1991). Hypotheses
689 were rejected at $P < 0.001$. All data were analysed by PC-SAS (release 9.4; SAS Institute, Cary, NC).

690

691 RNA isolation and quantitative PCR

692 Total RNA was isolated from *A. thaliana* tissue using Monarch® Total RNA Miniprep Kit (NEB).
693 cDNA was synthesized using ProtoScript® II First Strand cDNA Synthesis Kit (NEB) and an
694 oligo(dT) primer. Transcript quantification was performed using the 5x HOT FIREPol EvaGreen
695 qPCR Mix Plus (SOLIS BIODYNE) on a Stratagene MX3000P real-time PCR detection system.
696 Gene specific primers are described below. We used the At4g26410 transcript as internal control.

697 REF1_F2 TGAAGCTGCTGATTTGATGGA

698 REF1_R2 CTCTAAGCTTGATAGCATCCCTTC

699 PR1-F2 GAACACGTGCAATGGAGTTTG

700 PR1-R2 CACTTTGGCACATCCGAGTCT

701

702 Constructs

703 Open reading frames (ORFs) of PEN1 and SYP122 were amplified by PCR from cDNA and the
704 amplified products were subcloned into pDONR221 (Invitrogen). Along with pENTR clones
705 containing MpSYP12A and MpSYP12B (Kanazawa et al., 2016), PEN1 and SYP122 were cloned
706 into pUBIN-GFP, and PEN1 also in pB7WGR2, (Karimi et al., 2002; Grefen et al., 2010)
707 destination vectors according to the manufacturer's instructions. For cloning, the following primers
708 were used:

709 attB1-for GGGGACAAGTTTGTACAAAAAAGCAGGCTAC

710 attB2-rev GGGGACCACTTTGTACAAGAAAGCTGGGTC

711 B1-PEN1-for AAAAAGCAGGCTACATGAACGATTTGTTTCC

712 B2-PEN1-rev AGAAAGCTGGGTCTCAACGCAATAGACGCCTTGC

713 B1-SYP122-for AAAAAGCAGGCTACATGAACGATCTTCTCTCC

714 B2-SYP122-rev AGAAAGCTGGGTCTTAGCGTAGTAGCCGCCG

715

716 Fluorescent protein, propidium iodide and callose detection using confocal microscopy

717 Samples were examined using a 63× water immersion lens mounted on a Leica CLSM TCS SP5
718 confocal microscope. For detection and localization of the fluorophores and stains, GFP and
719 propidium iodide were excited at 488 nm and detected between 500- and 520 nm, and 555- and 615
720 nm, respectively. Aniline blue-stained callose was excited at 405 nm and detected between 435- and
721 485 nm. Imaging data were collected at the Center for Advanced Bioimaging (CAB) Denmark,
722 University of Copenhagen. Projections of serial confocal sections, image overlays, and contrast
723 enhancement were performed using image processing software (GIMP 2).

724

725 **Figure legends:**

726

727 **Figure 1. PEN1/SYP122 are required for papilla and encasement responses to *Bgh*.** (A, B)
728 Spontaneous callose depositions in leaves of 3 week old plants. (C-G) Accumulation of callose in
729 response to *Bgh* attack at appressoria (arrows) and primary germ tube (arrowheads) in (C-D) non-
730 penetrated cells and (E-G) and cells following a successful penetration. Open arrows point to the
731 developing IPS. (G) Same as F, but optical section taken below the point of entry. * marks cells
732 with cell death response. Bars = 20 μm.

733 **Figure 1-figure supplement 1. Pre- and post-invasive immunity.** (A) Attack by non-adapted
734 filamentous pathogens such as *Bgh* on *Arabidopsis*, is met by a localized apposition (papilla)
735 formed between the plant cell wall (CW) and the plasma membrane (PM), containing callose,
736 phenolic compounds, reactive oxygen species, and exosomes, that likely prevents penetration
737 (Zeyen et al., 2002; Assaad et al., 2004; An et al., 2006). (B) Upon successful penetration, the host
738 cell forms an encasement, similar in composition to the papilla, that eventually encloses the
739 developing haustorium (H) and prevents nutrient uptake. Similar to *Bgh*, spores of *C. destructivum*

740 and *P. infestans* attempt to penetrate and form an intracellular pathogenic structure (IPS, haustoria,
741 biotrophic hyphae and infection vesicle, respectively). In the case of an ineffective encasement
742 response, the host cell initiates a cell death response that prevents pathogen growth. (C-D)
743 Accumulation of callose in response to successful penetration by *G. orontii* (24 h). Open arrows
744 point to the developing IPS. Bars = 20 μ m.

745 **Figure 1 – Video 1.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
746 Col-0.

747 **Figure 1 – Video 2.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
748 *pen1 syp122*.

749 **Figure 1 – Video 3.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
750 *orontii* in Col-0.

751 **Figure 1 – Video 4.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
752 *orontii* in *pen1 syp122*.

753 **Figure 2. PEN1/SYP122 are required for accumulation of TET8-GFP in response to**
754 **penetration by *Bgh*.** (A-D) Localization of TET8-GFP in (A, C) resting epidermal cells and in (B,
755 D) response to successful penetration by *Bgh* (stained with propidium iodide, PI) and initiation of
756 the IPS (arrows). Bars = 10 μ m.

757 **Figure 2 – Video 1.** Z-stack (GFP and propidium iodide overlay) of successful penetration by *Bgh*
758 in Col-0.

759 **Figure 2 – Video 2.** Z-stack (GFP and propidium iodide overlay) of successful penetration by *Bgh*
760 in *pen1 syp122*.

761

762 **Figure 3: PEN1/SYP122 are required for papilla responses towards *Bgh*.** (A-B, D-E)
763 Accumulation of callose (A-B) or H₂O₂ (D-E) in response to *Bgh* attack (arrows) at non-penetrated
764 attack sites. Bars = 10 μ m. (C, F) Frequency of papillae in response to *Bgh* in non-penetrated cells
765 as detected by staining for callose (C) or H₂O₂ (F). (C, F) All values are mean $\pm$ SD (n = 5 leaves per
766 genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using
767 logistic regression.

768 **Figure 3-figure supplement 1. Loss of FMO1 attenuates *pen1 syp122* autoimmunity.** (A) Plants
769 at 4 weeks, showing the growth phenotypes of the mutant lines used in these studies. (B) Relative
770 transcript levels of PR1 in four week old plants. (F) Values are mean $\pm$ SD (n = 3). ***, $P < 0.001$,
771 Student's t test. (C-D) Accumulation of callose in response to *G orontii*- (48 h). Open- arrows and
772 arrow heads point to the developing IPS and secondary hyphae, respectively. Bars = 20 μ m.

773 **Figure 3-figure supplement 2. PEN1/SYP122 are required for papilla responses to *Bgh*.** (A-D)
774 Accumulation of callose in response to *Bgh* attack at non-penetrated (arrowheads) and penetrated
775 (arrows) attack sites. Bars = 20 μ m.

776 **Figure 4: PEN1/SYP122 are required for encasement responses towards *Bgh*.** (A-B, D)
777 Accumulation of callose (A-B) or H₂O₂ (D) in response to *Bgh* attack (arrows) at penetrated attack
778 sites. Open arrows point to the developing IPS. Bars = 10 μ m. (C, E) Frequency of encasements in
779 response to *Bgh* in penetrated cells as detected by staining for callose (C) or H₂O₂ (E). (F)
780 Frequency of penetrations by *Bgh*. (C, E-F) All values are mean $\pm$ SD (n = 5 leaves per genotype).
781 Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic
782 regression.

783 **Figure 4-figure supplement 1. PEN1/SYP122 are required for encasement responses to *Bgh*.**
784 (A-D) Accumulation of callose in response to *Bgh* attack at non-penetrated (arrowheads) and
785 penetrated (arrows) attack sites. Open arrows point to the developing IPS. * marks cells with cell
786 death response. Bars = 20 μ m.

787 **Figure 4-figure supplement 2. PEN1/SYP122 are required for papilla and encasement**
788 **responses to *Bgh*.** (A-F) Accumulation of callose in response to (A-B) *G. orontii*- and (C-F) *Bgh*
789 (48 hours) attack at (C-D) non-penetrated and (A-B, E-F) penetrated attack sites (arrows). Open
790 arrows point to the developing IPS. Open- arrows and arrow heads point to the developing IPS and
791 secondary hyphae, respectively. Bars = 20 μ m.

792 **Figure 4 – Video 1.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
793 *orontii* in *fmo1*.

794 **Figure 4 – Video 2.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
795 *orontii* in *fmo1 pen1 syp122*.

796 **Figure 4 – Video 3.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
797 *orontii* in *pad4 sid2 pen1 syp122*.

798 **Figure 4 – Video 4.** Z-stack (callose and bright field overlay) of successful penetration by *G.*
799 *orontii* in *amsh3 pen1 syp122*.

800 **Figure 4 – Video 5.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
801 *fmo1*.

802 **Figure 4 – Video 6.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
803 *fmo1 pen1 syp122*.

804 **Figure 4 – Video 7.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
805 *pad4 sid2 pen1 syp122*.

806 **Figure 4 – Video 8.** Z-stack (callose and bright field overlay) of successful penetration by *Bgh* in
807 *amsh3 pen1 syp122*.

808 **Figure 5: PEN1/SYP122 are required for pre-invasive immunity towards *C. destructivum*.**
809 (A,B and D,E) Accumulation of callose in response to attack by melanized *C. destructivum*
810 appressoria (arrows) at (A and B) non-penetrated and (D and E) penetrated attack sites. Open
811 arrows point to the developing IPS. Bars = 10 μ m. (C) Frequency of papillae in response to *C.*
812 *destructivum* in non-penetrated cells. (F) Frequency of penetrations by *C. destructivum*. (C, F) All
813 values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different
814 values at $P \leq 0.001$ estimated using logistic regression.

815 **Figure 5-figure supplement 1. PEN1/SYP122 are required for pre-invasive immunity towards**
816 ***C. destructivum*.** (A-C) Localisation of GFP-PEN1 (A), GFP-SYP122 (B) and TET8-GFP (C) in
817 response to attack by *C. destructivum*. (D-E) Accumulation of callose in response to attack by *C.*
818 *destructivum* in (D) non-penetrated cells and (E) at penetration sites (arrows). Open arrows point to
819 the developing IPS. * marks cell with cell death response. (F) Frequency of penetration by *C.*
820 *destructivum*. (G) Macroscopic phenotype at 5 dai with *C. destructivum*. Bars = 10 μ m. (F) All
821 values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different
822 values at $P \leq 0.001$ estimated using logistic regression.

823 **Figure 6: PEN1/SYP122 are required for pre-invasive immunity towards *P. infestans*.** (A-B,
824 D-E) Accumulation of callose in response to *P. infestans* attack (arrows) at (A and B) non-
825 penetrated and (D and E) penetrated attack sites. Open arrows point to the developing IPS. Bars =
826 10 μ m. (C) Frequency of papillae in response to *P. infestans* in non-penetrated cells. (F) Frequency
827 of penetrations by *P. infestans*. (C, F) All values are mean $\pm$ SD (n = 4 leaves per genotype).

828 Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic
829 regression.

830 **Figure 6-figure supplement 1. PEN1/SYP122 are required for pre-invasive immunity towards**
831 ***P. infestans*.** (A-C) Localisation of GFP-PEN1 (A), GFP-SYP122 (B) and TET8-GFP (C) in
832 response to attack by *P. infestans*. (D) Frequency of penetration by *P. infestans*. Bars = 10 μ m. (D)
833 All values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly
834 different values at $P \leq 0.001$ estimated using logistic regression.

835 **Figure 7. Responses in *Marchantia polymorpha* to filamentous pathogens.** (A-B) Accumulation
836 of callose in *Marchantia* in response to attack (arrows) by (A) *P. infestans* or (B) *C. destructivum* in
837 non-penetrated cells. Note that the frequency of *P. infestans* spores that attack a host-cell is very
838 low. (C-F) mCitrine signal of MpSYPs in response to attack (arrows) by *C. destructivum* in non-
839 penetrated cells. We did not observe any successful penetrations in *Marchantia* by *C. destructivum*.
840 Bars=10 μ m.

841 **Figure 7-figure supplement 1. Responses in *Marchantia polymorpha* to filamentous pathogens.**
842 (A) On *M. polymorpha*, spores from *Bgh* (dotted line) were mis-differentiated with no or wrongly
843 orientated appressoria that did not attack the host-cell. (B) Accumulation of callose in *M.*
844 *polymorpha* in response to attack by *P. infestans* (arrows) in penetrated cells. Open arrow points to
845 the developing IPS. Note that the frequency of *P. infestans* spores that attack a host-cell is very low.
846 Bars=10 μ m.

847 **Figure 8: *Marchantia* syntaxins rescue papilla and encasement responses in *Arabidopsis*.** (A,
848 B) Accumulation of callose in response to attack (arrow) by *Bgh* in (A) non-penetrated- and (B)
849 penetrated cells. Open arrow points to the developing IPS. (C, D) Localization of MpSYP12A and
850 PEN1 in response to attack (arrows) by *Bgh* in (C) non-penetrated- and (D) penetrated cells. Bars =
851 10 μ m. (E ,F) Frequency of penetration by (E) *Bgh* and (F) *P. infestans*. All values are mean $\pm$ SD (n
852 = 4 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$
853 estimated using logistic regression.

854 **Figure 8-figure supplement 1. *Marchantia* syntaxins are functional in *Arabidopsis*.** (A) Plants at
855 five weeks, expressing SYP12 clade members from either *Arabidopsis* or *Marchantia polymorpha*.
856 (B) Localization of *Marchantia* SYP12 clade members in the epidermis of leaves from plants of
857 four weeks. Bars=10 μ m.

858 **Figure 8-figure supplement 2. *Marchantia* syntaxins rescue papilla responses.** (A)
859 Accumulation of callose in response to *Bgh* attack at non-penetrated (arrows) attack sites. Bars = 10
860 μm . (B) Frequency of papillae in response to *Bgh* in non-penetrated cells. All values are mean $\pm$ SD
861 ($n = 5$ leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$
862 estimated using logistic regression.

863 **Figure 8-figure supplement 3. *Marchantia* syntaxins rescue encasement responses.** (A)
864 Accumulation of callose in response to *Bgh* attack at successful penetration sites. Open arrows point
865 to the developing IPS. Bars = 10 μm . (B) Frequency of encasements in response to *Bgh* haustoria in
866 penetrated cells. All values are mean $\pm$ SD ($n = 5$ leaves per genotype). Different letters indicate
867 significantly different values at $P \leq 0.001$ estimated using logistic regression.

868 **Figure 8-figure supplement 4. Localization of SYP12 clade members at *Bgh* attack sites.**
869 Comparative papilla localization of SYP12 members from *Arabidopsis* or *Marchantia* in response
870 to attack by *Bgh* in non-penetrated cells. Bars = 10 μm .

871 **Figure 8-figure supplement 5. Localization of SYP12 clade members at *Bgh* penetration sites.**
872 Comparative encasement localization of SYP12 members from *Arabidopsis* or *Marchantia* in
873 response to haustoria by *Bgh* in penetrated cells. Bars = 10 μm .

874 **Figure 8-figure supplement 6. *Marchantia* SYP12s restore immunity in *Arabidopsis* towards *C.***
875 ***destructivum*.** (A) Macroscopic phenotype at 5 dai with *C. destructivum*. (B-D) Frequency of
876 penetration by *C. destructivum* (B), *G. orontii* (C) and *C. higginsianum* (D). (B-D) All values are
877 mean $\pm$ SD ($n = 4$ leaves per genotype). Different letters indicate significantly different values at $P \leq$
878 0.001 estimated using logistic regression.

879 **Figure 8-figure supplement 7. Localization of *Marchantia* SYP12 clade members at attack**
880 **sites.** (A-D) Localization of GFP-MpSYP12A (A, C) and GFP-MpSYP12A (B, D) in response to
881 attack by *P. infestans* (A-B) and *C. destructivum* (C-D) in non-penetrated cells. Bars = 10 μm .

882 **Fig. 9. Simplified model of syntaxin mediated immunity.** In response to attack by filamentous
883 pathogens, cargo for the papilla/encasement is received at the plasma membrane (PM) by PEN1 or
884 SYP122 and secreted in between the plant cell wall (CW) and the PM. For pre-invasive immunity
885 against powdery mildew fungi, PEN1 is required at the TGN to enable recycling of cargo that
886 facilitates a fast papilla response (shown in red). The general papilla/encasement response (shown
887 in green) does not require PEN1. Instead, this response likely involves a specialization of the

888 conventional secretory pathway or an alternative recycling pathway that is not dependent on PEN1.
889 Likely, the fast papilla response pathway also contributes to formation of the encasement resulting
890 in a mixture of cargo (such as exosomes and antimicrobial metabolites) transported by the two
891 pathways.

892

893 **Source files:**

894 **Figure 3 Source Data File:** Source data for the graphs in figure 3C and F.

895 **Figure 4 Source Data File:** Source data for the graphs in figure 4C, E and F.

896 **Figure 5 Source Data File:** Source data for the graphs in figure 5C and F.

897 **Figure 6 Source Data File:** Source data for the graphs in figure 6C and F.

898 **Figure 8 Source Data File:** Source data for the graphs in figure 8E and F.

899

900

Figure 1

Figure 1. PEN1/SYP122 are required for papilla and encasement responses to *Bgh*. (A, B) Spontaneous callose depositions in leaves of 3 week old plants. (C-G) Accumulation of callose in response to *Bgh* attack at appressoria (arrows) and primary germ tube (arrowheads) in (C-D) non-penetrated cells and (E-G) and cells following a successful penetration. Open arrows point to the developing IPS. (G) Same as F, but optical section taken below the point of entry. * marks cells with cell death response. Bars = 20 μm.

Figure 2

Figure 2. PEN1/SYP122 are required for accumulation of TET8-GFP in response to penetration by *Bgh*. (A-D) Localization of TET8-GFP in (A, C) resting epidermal cells and in (B, D) response to successful penetration by *Bgh* (stained with propidium iodide, PI) and initiation of the IPS (arrows). Bars = 10 μ m.

Figure 3

Figure 3: PEN1/SYP122 are required for papilla responses towards *Bgh*. (A-B, D-E) Accumulation of callose (A-B) or H_2O_2 (D-E) in response to *Bgh* attack (arrows) at non-penetrated attack sites. Bars = 10 μ m. (C, F) Frequency of papillae in response to *Bgh* in non-penetrated cells as detected by staining for callose (C) or H_2O_2 (F). (C, F) All values are mean $\pm$ SD (n = 5 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 4

Figure 4: PEN1/SYP122 are required for encasement responses towards *Bgh*. (A-B, D) Accumulation of callose (A-B) or H_2O_2 (D) in response to *Bgh* attack (arrows) at penetrated attack sites. Open arrows point to the developing IPS. Bars = 10 μ m. (C, E) Frequency of encasements in response to *Bgh* in penetrated cells as detected by staining for callose (C) or H_2O_2 (E). (F) Frequency of penetrations by *Bgh*. (C, E-F) All values are mean $\pm$ SD (n = 5 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 5

Figure 5: PEN1/SYP122 are required for pre-invasive immunity towards *C. destructivum*. (A,B and D,E) Accumulation of callose in response to attack by melanized *C. destructivum* appressoria (arrows) at (A and B) non-penetrated and (D and E) penetrated attack sites. Open arrows point to the developing IPS. Bars = 10 μ m. (C) Frequency of papillae in response to *C. destructivum* in non-penetrated cells. (F) Frequency of penetrations by *C. destructivum*. (C, F) All values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 6

Figure 6: PEN1/SYP122 are required for pre-invasive immunity towards *P. infestans*. (A-B, D-E) Accumulation of callose in response to *P. infestans* attack (arrows) at (A and B) non-penetrated and (D and E) penetrated attack sites. Open arrows point to the developing IPS. Bars = 10 μ m. (C) Frequency of papillae in response to *P. infestans* in non-penetrated cells. (F) Frequency of penetrations by *P. infestans*. (C, F) All values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 7

Figure 7. Responses in *Marchantia polymorpha* to filamentous pathogens. (A-B) Accumulation of callose in *Marchantia* in response to attack (arrows) by (A) *P. infestans* or (B) *C. destructivum* in non-penetrated cells. Note that the frequency of *P. infestans* spores that attack a host-cell is very low. (C-F) mCitrine signal of MpSYPs in response to attack (arrows) by *C. destructivum* in non-penetrated cells. We did not observe any successful penetrations in *Marchantia* by *C. destructivum*. Bars=10 μ m.

Figure 8

Figure 8: *Marchantia* syntaxins rescue papilla and encasement responses in *Arabidopsis*. (A, B) Accumulation of callose in response to attack (arrow) by *Bgh* in (A) non-penetrated- and (B) penetrated cells. Open arrow points to the developing IPS. (C, D) Localization of MpSYP12A and PEN1 in response to attack (arrows) by *Bgh* in (C) non-penetrated- and (D) penetrated cells. Bars = 10 μ m. (E, F) Frequency of penetration by (E) *Bgh* and (F) *P. infestans*. All values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 9

Fig. 9. Schematic model of immunity against filamentous pathogens mediated by PEN1 and SYP122. In response to attack by filamentous pathogens, cargo for the papilla/encasement is received at the plasma membrane (PM) by PEN1 or SYP122 and secreted in between the plant cell wall (CW) and the PM. For pre-invasive immunity against powdery mildew fungi, PEN1 is required at the TGN to enable recycling of cargo that facilitates a fast papilla response (shown in red). The slower, general papilla/encasement response (shown in green) does not require rapid recycling at the TGN using PEN1. Instead, this response likely involves a specialization of the conventional secretory pathway or an alternative recycling pathway where either PEN1 or SYP122 are required at the PM to receive vesicular cargo for the papilla/encasement formation. Likely, the fast papilla response pathway also contributes to formation of the encasement resulting in a mixture of cargo (such as exosomes and anti microbial metabolites) transported by both pathways.

Figure 1-figure supplement 1

Figure 1-figure supplement 1. Pre- and post-invasive immunity. (A) Attack by non-adapted filamentous pathogens such as *Bgh* on *Arabidopsis*, is met by a localized apposition (papilla) formed between the plant cell wall (CW) and the plasma membrane (PM), containing callose, phenolic compounds, reactive oxygen species, and exosomes, that likely prevents penetration (Zeyen et al., 2002; Assaad et al., 2004; An et al., 2006). (B) Upon successful penetration, the host cell forms an encasement, similar in composition to the papilla, that eventually encloses the developing haustorium (H) and prevents nutrient uptake. Similar to *Bgh*, spores of *C. destructivum* and *P. infestans* attempt to penetrate and form an intracellular pathogenic structure (IPS, haustoria, biotrophic hyphae and infection vesicle, respectively). In the case of an ineffective encasement response, the host cell initiates a cell death response that prevents pathogen growth. (C-D) Accumulation of callose in response to successful penetration by *G. orontii* (24 h). Open arrows point to the developing IPS. Bars = 20 μ m.

Figure 3-figure supplement 1

Figure 3-figure supplement 1. Loss of FMO1 attenuates *pen1 syp122* autoimmunity. (A) Plants at 4 weeks, showing the growth phenotypes of the mutant lines used in these studies. (B) Relative transcript levels of PR1 in four week old plants. (F) Values are mean $\pm$ SD (n = 3). ***, $P < 0.001$, Student's t test. (C-D) Accumulation of callose in response to *G. orontii*- (48 h). Open- arrows and arrow heads point to the developing IPS and secondary hyphae, respectively. Bars = 20 μ m.

Figure 3-figure supplement 2

Figure 3-figure supplement 2. PEN1/SYP122 are required for papilla responses to *Bgh*. (A-D) Accumulation of callose in response to *Bgh* attack at non-penetrated (arrowheads) and penetrated (arrows) attack sites. Bars = 20 μ m.

Figure 4-figure supplement 1

Figure 4-figure supplement 1. PEN1/SYP122 are required for encasement responses to *Bgh*. (A-D) Accumulation of callose in response to *Bgh* attack at non-penetrated (arrowheads) and penetrated (arrows) attack sites. Open arrows point to the developing IPS. * marks cells with cell death response. Bars = 20 μ m.

Figure 4-figure supplement 2

Figure 4-figure supplement 2. PEN1/SYP122 are required for papilla and encasement responses to *Bgh*. (A-F) Accumulation of callose in response to (A-B) *G. orontii*- and (C-F) *Bgh* (48 hours) attack at (C-D) non-penetrated and (A-B, E-F) penetrated attack sites (arrows). Open arrows point to the developing IPS. Open arrowheads point to the developing IPS and secondary hyphae, respectively. Bars = 20 μ m.

Figure 5-figure supplement 1

Figure 5-figure supplement 1. PEN1/SYP122 are required for pre-invasive immunity towards *C. destructivum*. (A-C) Localisation of GFP-PEN1 (A), GFP-SYP122 (B) and TET8-GFP (C) in response to attack by *C. destructivum*. (D-E) Accumulation of callose in response to attack by *C. destructivum* in (D) non-penetrated cells and (E) at penetration sites (arrows). Open arrows point to the developing IPS. * marks cell with cell death response. (F) Frequency of penetration by *C. destructivum*. (G) Macroscopic phenotype at 5 dai with *C. destructivum*. Bars = 10 μ m. (F) All values are mean $\pm$ SD (n = 4 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 6-figure supplement 1

Figure 6-figure supplement 1. PEN1/SYP122 are required for pre-invasive immunity towards *P. infestans*. (A-C) Localisation of GFP-PEN1 (A), GFP-SYP122 (B) and TET8-GFP (C) in response to attack by *P. infestans*. (D) Frequency of penetration by *P. infestans*. Bars = 10 μ m. (D) All values are mean $\pm$ SD ($n = 4$ leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 7-figure supplement 1

Figure 7-figure supplement 1. Responses in *Marchantia polymorpha* to filamentous pathogens. (A) On *M. polymorpha*, spores from *Bgh* (dotted line) were mis-differentiated with no or wrongly orientated appressoria that did not attack the host-cell. (B) Accumulation of callose in *M. polymorpha* in response to attack by *P. infestans* (arrows) in penetrated cells. Open arrow points to the developing IPS. Note that the frequency of *P. infestans* spores that attack a host-cell is very low. Bars=10 μ m.

Figure 8-figure supplement 1

Figure 8-figure supplement 1. *Marchantia* syntaxins are functional in *Arabidopsis*. (A) Plants at five weeks, expressing SYP12 clade members from either *Arabidopsis* or *Marchantia polymorpha*. (B) Localization of *Marchantia* SYP12 clade members in the epidermis of leaves from plants of four weeks. Bars=10 μ m.

Figure 8-figure supplement 2

Figure 8-figure supplement 2. *Marchantia* syntaxins rescue papilla responses.

(A) Accumulation of callose in response to *Bgh* attack at non-penetrated (arrows) attack sites. Bars = 10 μ m. (B) Frequency of papillae in response to *Bgh* in non-penetrated cells. All values are mean $\pm$ SD (n = 5 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 8-figure supplement 3

Figure 8-figure supplement 3. *Marchantia* syntaxins rescue encasement responses. (A) Accumulation of callose in response to *Bgh* attack at successful penetration sites. Open arrows point to the developing IPS. Bars = 10 μ m. (B) Frequency of encasements in response to *Bgh* haustoria in penetrated cells. All values are mean $\pm$ SD (n = 5 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 8-figure supplement 4

Figure 8-figure supplement 4. Localization of SYP12 clade members at *Bgh* attack sites. Comparative papilla localization of SYP12 members from *Arabidopsis* or *Marchantia* in response to attack by *Bgh* in non-penetrated cells. Bars = 10 μ m.

Figure 8-figure supplement 5

Figure 8-figure supplement 5. Localization of SYP12
clade members at *Bgh* penetration sites. Comparative
encasement localization of SYP12 members from
Arabidopsis or *Marchantia* in response to haustoria by *Bgh* in
penetrated cells. Bars = 10 μ m.

Figure 8-figure supplement 6

Figure 8-figure supplement 6. *Marchantia* SYP12s restore immunity in *Arabidopsis* towards *C. destructivum*. (A) Macroscopic phenotype at 5 dai with *C. destructivum*. (B-D) Frequency of penetration by *C. destructivum* (B), *G. orontii* (C) and *C. higginsianum* (D). (B-D) All values are mean $\pm$ SD ($n = 4$ leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 8-figure supplement 7

Figure 8-figure supplement 7. Localization of *Marchantia* SYP12 clade members at attack sites. (A-D) Localization of GFP-MpSYP12A (A, C) and GFP-MpSYP12A (B, D) in response to attack by *P. infestans* (A-B) and *C. destructivum* (C-D) in non-penetrated cells. Bars = 10 μ m.