Title:

Plant SYP12 syntaxins mediate evolutionarily conserved general immunity to filamentous pathogens

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Abstract:

Many filamentous fungal and oomycete plant pathogens invade by direct penetration through the leaf epidermal cell wall and cause devastating plant diseases. In response to attack, plants form evolutionarily conserved cell autonomous defense structures, named papillae and encasements, that are thought to block pathogen ingress. Previously, the syntaxin PEN1 in *Arabidopsis*, like its orthologue ROR2 in barley, was found to mediate pre-invasive immunity towards powdery mildew fungi, where it assures the timely formation of papilla defense structures. However, this powdery mildew-specific function of PEN1 in papilla timing, thought to take place at the *trans*-Golgi network, does not explain how plants generally ward off other filamentous pathogens. In the present study, we found that PEN1 has a second function, shared with its closest homologue SYP122, in the formation of papillae, as well as encasements. This second function provides pre-invasive immunity towards highly diverse non-adapted filamentous pathogens, underlining the versatility and efficacy of these defense structures. PEN1 and SYP122 belong to the broadly conserved land plant syntaxin clade SYP12, suggested to function in specialized forms of polarized secretion. In support of this, complementation studies using SYP12s from the basal plant, *Marchantia polymorpha*, showed that the SYP12 clade immunity function has survived 450 My of independent evolution. As saprophytic filamentous land fungi predate plant terrestrialization, we suggest ancestral land plants evolved the SYP12 clade to provide a durable immunity to facilitate their life on land.

Introduction:

In response to attack by filamentous pathogens (fungi and oomycetes), plant cells protect themselves by assembling localized pre-invasive papillae and post-invasive encasements at sites of attack (Fig. S1) (1). These conserved defense structures are thought to provide effective and durable resistance against diverse filamentous pathogens, and fossil evidence suggests they appeared very early in the evolution of land plants (2-4). The discovery that the secretory syntaxin PEN1 is required for the timely formation of papillae to defend against non-adapted powdery mildew fungi, highlighted the key role played by membrane trafficking in plant immunity (5-7). PEN1 (also referred to as syntaxin of plants 121 or SYP121) is primarily located at the plasma membrane. On the other hand, the ARF-GEF, GNOM, regulates recycling of PEN1 between the plasma membrane and the *trans*-Golgi network, where it is needed for a fast papilla response (6). Accordingly, PEN1 is thought to mediate

membrane fusion both at the plasma membrane and the *trans*-Golgi network. The ROR2 syntaxin in barley is orthologous to PEN1 (5), suggesting that the pre-invasive immunity dependent on PEN1/ROR2 was present in early angiosperms before the divergence of monocots and dicots. Loss of PEN1/ROR2 delays, but does not prevent, the papilla response after attack by the barley powdery mildew *Blumeria graminis* f.sp. *hordei* (*Bgh*) (8, 9), while haustorial encasements are unaffected (10, 11). Moreover, although many filamentous pathogens penetrate directly through the host cell wall, the PEN1/ROR2-specific immunity appears dedicated towards the haustorium-forming powdery mildew- and rust fungi (5, 12-14). Thus, the molecular mechanisms underlying papilla/encasement formation, as well as their role in general plant invasive immunity towards other filamentous pathogens, remain obscure.

PEN1/ROR2 belongs to the SYP12 clade of secretory syntaxins, which first appears in embryophytes and is conserved in all land plants (15). Consequently, the appearance of the SYP12 clade has been proposed to play an important role in the terrestrialization of plants due to a specialization in the secretory pathway (16). This is supported by the fact that four of the five SYP12 clade members in *Arabidopsis* have specialized roles in polarized secretion during pre-invasive immunity towards powdery mildew fungi (PEN1), root hair tip growth (SYP123), and pollen tube growth (SYP124/SYP125) (5, 15, 17). Yet, the function of SYP122, which is the closest homologue of PEN1, is currently unresolved (8). Interestingly, the *pen1 syp122* double mutant is autoimmune, probably due to NLR-protein activation. This shows that the two syntaxins are functionally redundant and that the shared function of PEN1 and SYP122 is likely to be related to defense (8, 18, 19).

Here we show that formation of papillae and encasements in *Arabidopsis* requires either of the two clade SYP12 members, PEN1 and SYP122, as plants lacking both these syntaxins are completely devoid of the two defense structures. We show that PEN1/SYP122 provide invasive immunity not only against the powdery mildew *Bgh* but also against distantly related filamentous pathogens that invade host cells, namely the fungus *Colletotrichum destructivum* and the oomycete *Phytophthora infestans*. Complementation studies show that SYP12a from *Marchantia polymorpha* functions and behaves like SYP122 in *Arabidopsis*, thereby highlighting a conserved ancestral mechanism. Our findings thus indicate that early land plants evolved the SYP12 clade of plant secretory syntaxins to provide an immunity that is effective against a broad range of invasive filamentous pathogens.

Results:

The role of PEN1 in timely papilla deposition in response to *Bgh* attack prompted us to investigate the involvement of other syntaxins in invasive immunity, notably SYP122 that shares an as-yet unidentified functional role with PEN1. This is apparent from the autoimmune lesion-mimic phenotype of the *pen1 syp122* double mutant, which depends on several immunity signaling components and is thus expected to result from activation of immune receptors (8, 18-21). To overcome the difficulties of working with this lesion-mimic mutant, we made use of the partially rescued triple mutant, *fmo1 pen1 syp122* (19). In response to attack by *Bgh*, we found that plants lacking PEN1 and SYP122 were unable to form papillae at sites of unsuccessful attack as visualized by detection of callose, a major constituent of papillae and encasements (Fig. 1a, b, e and Fig. S2a-d). Moreover, when penetrated by *Bgh*, the host cells were unable to create an encasement around the developing haustoria (Fig. 1c, d and Fig. S2e-i). To avoid the possibility that the lack of these defense structures is due to a host cell death response, such dead cells were excluded from our observations based on Trypan blue staining. We found a significant increase in the fungal penetration frequency in *fmo1 pen1 syp122* plants when compared to *pen1* and *fmo1 pen1* plants (Fig. 1f). This supports our previous finding of a minor, but significant PEN1-independent contribution to pre-invasive immunity (11). Moreover, in the non-rescued lesion-mimic *pen1 syp122*, attempted penetration by *Bgh* appressoria did not result in papilla formation. Surprisingly however, we observed large papillae induced by the non-invasive primary germ tube, which was not observed in any other genetic background (Figs. S1, S3a-d). Thus, the *pen1 syp122* mutant remains capable of generating focused depositions of callose and seems to be highly sensitive to signals released by *Bgh*, despite being unable to form a papilla at the site of attempted entry. Successful penetration of *pen1 syp122* epidermal cells by *Bgh* resulted in a cell death response. However, unlike unsuccessful penetrations, the successful penetration of *pen1 syp122* cells often induced a strong accumulation of callose different from normal papillae formed at the site of fungal entry, but without formation of an encasement (Fig. S3e-g). These observations show that the *Bgh*-induced formation of papillae and encasements at attack sites is completely dependent on a functional overlap of these two syntaxins.

To further dissect the overlapping functions of PEN1 and SYP122 in immunity, we tested responses to two highly diverged, non-adapted pathogens *P. infestans* (the oomycete potato blight pathogen) and *C. destructivum* (a fungal pathogen causing alfalfa anthracnose). Despite an invasion strategy very similar to that of *Bgh*, invasive immunity towards *P. infestans* and *C. destructivum* is highly effective in *Arabidopsis* and does not require PEN1 (12, 22). Remarkably, however, we found plants

lacking both PEN1 and SYP122 showed a dramatic increase in penetration frequency by both pathogens (Fig. 2a, b and Figs. S4a, S5a). Similarly to *Bgh*, the loss of invasive immunity towards *P. infestans* and *C. destructivum* correlated with a lack of papilla formation at sites of unsuccessful penetration (Figs. S4b-d, S5b, d, e). Meanwhile, upon successful penetration by *P. infestans*, host cells expressing PEN1 and/or SYP122 initiate a strong accumulation of callose and form an encasement around the haustorium, often followed by host cell death. These encasements were absent in the *fmo1 pen1 syp122* triple mutant (Fig. 2c, e). In the case of *C. destructivum,* the host cell response in *fmo1 pen1 syp122* varied from no detectable callose to a large accumulation of callose at the penetration site (Fig. 2d, f and Fig. S5f, g). Thus, PEN1 and SYP122 are required for papilla and encasement responses, which mediate immunity towards these phylogenetically distant non-adapted filamentous pathogens.

PEN1 and SYP122 belong to the SYP12 clade of secretory syntaxins, which evolved during plant terrestrialization and is conserved in all land plants (15, 16). Moreover, papilla- and encasement-like responses are conserved even in early divergent land plants such as *Marchantia polymorpha* (Fig. S6) and *Physcomitrella patens* (23, 24). Consequently, we considered whether the immunity function of the SYP12 clade is also evolutionarily conserved among land plants. Thus, we introduced GFP-fused versions of the two *M. polymorpha* SYP12s (*i.e.* MpSYP12A and MpSYP12B) into the *Arabidopsis pen1 syp122* mutant. GFP-MpSYP12A fully suppressed the necrotic and dwarfed phenotype of *pen1 syp122* plants, while GFP-MpSYP12B only partially suppressed these phenotypes (Fig. 3a). This suggests that MpSYP12A shares the PEN1/SYP122 overlapping function and that MpSYP12B does so incompletely. Although GFP-MpSYP12A is partially retained at the ER, both *Marchantia* GFP-MpSYP12s localized at the plasma membrane. However, unlike GFP-PEN1, neither fusion protein co-localized with internalized FM4-64 (Fig. 3b and Fig. S7), suggesting that the MpSYP12s are stable at the plasma membrane, similar to SYP122, but in contrast to PEN1, which is thought to continuously recycle between the plasma membrane and *trans*-Golgi network (6). We tested this by applying the fungal toxin brefeldin A (BFA) that in *Arabidopsis* blocks endocytosed plasma membrane proteins from being recycled back to the plasma membrane (25). As previously described, GFP-PEN1 was highly sensitive to BFA, with all the GFP signal accumulating in BFA compartments. In contrast, we found GFP-SYP122 and both GFP-MpSYP12s were less sensitive to BFA, with most of the GFP signal remaining at the plasma membrane (Fig. 3c and Fig. S8).

Finding that MpSYP12A fully complements *pen1 syp122* at the macroscopic level, we next analyzed how these plants reacted to attack by *Bgh*. We found that MpSYP12A completely, and MpSYP12B

partly, restored both papilla and encasement responses (Fig. 4a, b and Figs. S9, S10). Unlike GFP-PEN1, neither GFP-SYP122 nor the two GFP-MpSYP12s accumulated in papillae at sites of attempted penetration (Fig. 4c and Fig. S11). However, all the tested SYPs accumulated in the encasement matrix at sites of successful penetration (Fig. 4d and Fig. S12). Interestingly, pre-invasive immunity towards *Bgh* was not restored by GFP-MpSYP12A. Instead, the penetration frequency of *Bgh* was similar to that of plants expressing GFP-SYP122 (Fig. 4e). Thus, MpSYP12A resembles SYP122, and not PEN1, both in its localization and functionality. In support of this, GFP-MpSYP12A fully restored immunity towards both *P. infestans* and *C. destructivum* (Fig. 4f and Fig. S13). Accumulation of GFP at attack sites was not observed for either of these two pathogens. Taken together, these results reveal a conserved SYP12 clade functionality that has survived 450 My of independent evolution. On the other hand, our findings also suggest that the PEN1-specific immunity towards powdery mildew fungi evolved at a later stage of land plant evolution.

Discussion:

Our findings have revealed a highly conserved role of the SYP12 clade of plant syntaxins in mediating pre- and post-invasive immunity to highly diverse filamentous pathogens. *Arabidopsis* plants lacking both PEN1 and SYP122 were incapable of forming papillae and encasements in response to attack by the non-adapted *Bgh*, *C. destructivum* and *P. infestans*, resulting in highly elevated penetration frequencies. It is noteworthy that although the *pen1 syp122* double mutant lacks these defense structures, epidermal cells are still able to respond to pathogen-derived signals in a polarized fashion. This underlines that neither the general ability to secrete callose, nor the underlying mechanism needed for polarized secretion is dependent on PEN1/SYP122. Instead, we suggest that PEN1/SYP122-dependent papillae and encasements are defense structures specifically aimed at blocking the ingress of filamentous pathogens. In support of this, knock-down of StSYR1 in potato, the single homolog of PEN1, results in reduced papilla formation in response to *P. infestans* (26).

Previous studies on the interaction between *Arabidopsis* and *Bgh* have revealed that pre-invasive immunity is dependent on the timely formation of papillae. This fast response likely requires the recycling of papilla material in a process involving PEN1 and GNOM (6). In contrast, although SYP122 also locates to the plasma membrane and interacts with the same SNARE partners as PEN1, *syp122* mutants show a wild-type response to *Bgh* (8). Based on the work presented here, it is now evident that PEN1 and SYP122 functionally overlap in the formation of papillae and encasements. In contrast to SYP122, PEN1 therefore serves two functions in pre-invasive immunity. The first

mediates a fast recycling of papilla material in response to powdery mildew attack and the other being a more general papilla/encasement response to filamentous pathogens. As suggested previously, the powdery mildew-specific function is likely related to the ability of PEN1 to recycle between the plasma membrane and *trans*-Golgi network, which is less prominent for SYP122 (4, 7, 27). Consequently, PEN1 could function at the *trans*-Golgi network to mediate the GNOM-dependent recycling of papilla material that wards off the powdery mildew fungus. We envisage that the second function, takes place at the plasma membrane, at which PEN1 or SYP122 receives membrane material for the papilla/encasement formation.

The SYP12 clade of plant secretory syntaxins is thought to have evolved during colonization of the land and seems conserved in all land plants (15). Because terrestrialization must have presented enormous challenges to plants, the SYP12 clade was suggested to have acquired specialized roles of secretion needed to facilitate this transition (16). This is consistent with the previously identified functions of PEN1 (powdery mildew resistance), SYP123 (root hair tip growth) and SYP124/SYP125 (pollen tube growth), all of which are associated with specialized forms of polarized secretion (5, 15, 17). However, this gives little insight into the original function of the SYP12 clade in plant terrestrialization. Our discovery that SYP12a from Marchantia can complement SYP122, but not PEN1, in *Arabidopsis* reveals a conserved syntaxin functionality spanning 450 My of independent evolution. In accordance with this, MpSYP12a showed a localization behavior identical to that of SYP122. Therefore, the functionality of the original SYP12 clade syntaxins is likely to have been similar to that of MpSYP12a/SYP122. At a later time point in plant evolution, PEN1 has evolved an additional specialized function in powdery mildew resistance, which could explain its distinct altered subcellular localization (7, 27). Interestingly, MpSYP12b locates to oil bodies, unique to liverworts, and could represent yet another functional specialization of the SYP12 clade (28).

We suggest that MpSYP12A/SYP122 represent the original SYP12 clade functionality in land plants which provides a highly conserved and effective broad-spectrum immunity towards filamentous pathogens. Coinciding with plant terrestrialization, the appearance of SYP12s likely provided important specializations in the secretory pathway in response to the change of environment (15, 16). Before plant terrestrialization, the land was already colonized by filamentous fungi that were able to degrade cell walls and to utilize living or dead plant tissue as a nutrient source (29). Recent findings

suggest that even before terrestrialization, plants used nucleotide-binding leucine-rich repeat receptors (NLRs) to activate their defense responses (30). Activation of plant NLRs leads to a fast, programmed cell death response, which does not provide immunity against fungi living on dead or dying plant tissue (31-34). Instead, the papilla/encasement defense structures would have provided an effective defensive barrier to attack by the early saprophytic filamentous fungi already present on the land. Moreover, it is broadly accepted that plant colonization of land was aided by, or even dependent on, mycorrhiza-like fungi (35-37). These mutualistic interactions are thought to have resulted from ancestral land plants being able to 'tame' early saprophytic filamentous fungi (29, 38). We propose that the SYP12 syntaxins enabled early land plants to regulate fungal entry, leading to the mutually beneficial interactions that facilitated terrestrialization.

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Figure 1

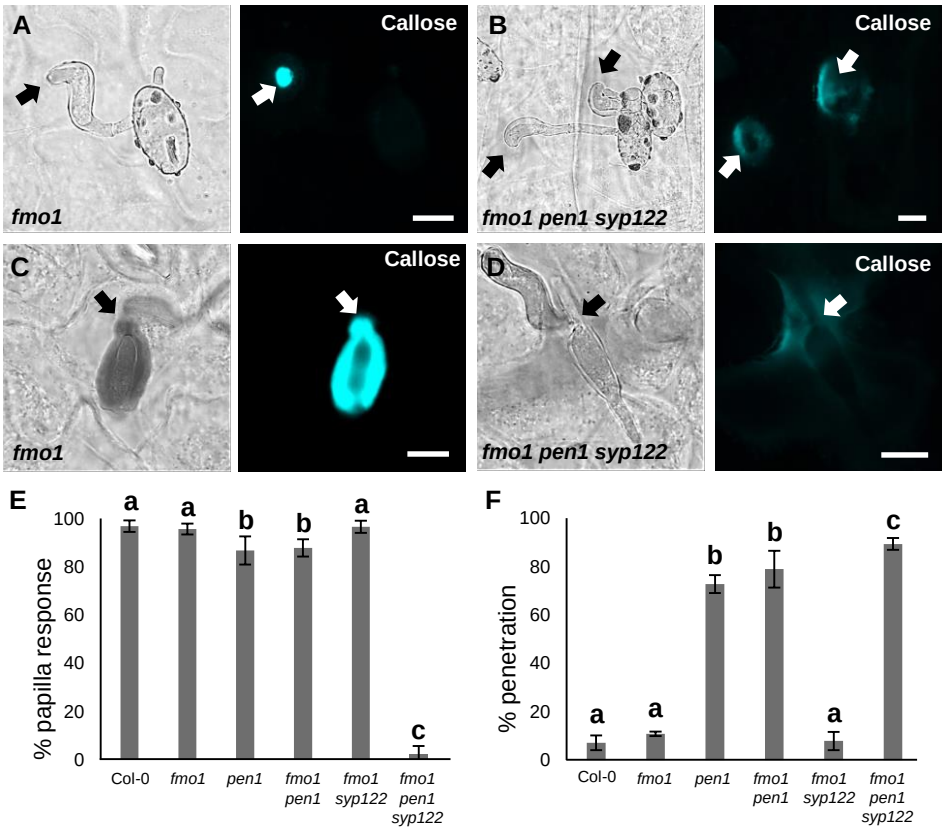

Figure 1: PEN1/SYP122 are required for papilla and encasement responses towards *Bgh*. (A-D) Accumulation of callose in response to *Bgh* attack (arrows) at (A and B) non-penetrated and (C and D) penetrated attack sites. Bars = 10 µm. (E) Frequency of papillae in response to *Bgh* in non-penetrated cells. (F) Frequency of penetrations by *Bgh*. (E-F) All values are mean ±SD (n = 5 leaves per genotype). Different letters indicate significantly different values at $P \leq 0.001$ estimated using logistic regression.

Figure 2

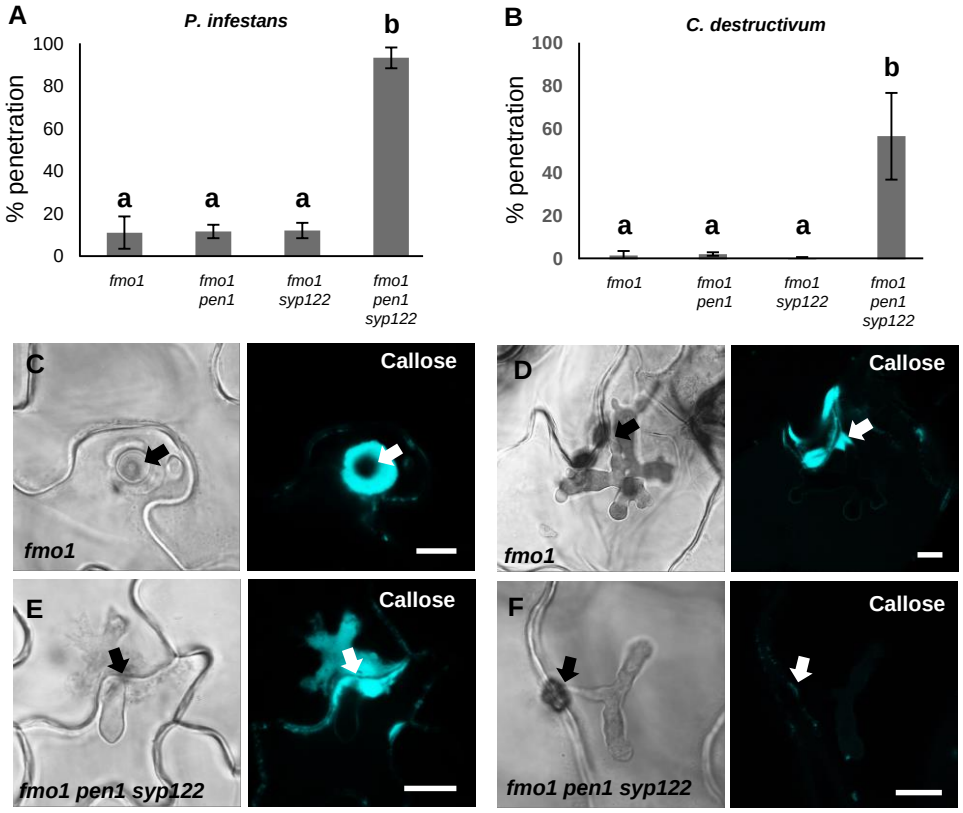

Figure 2: PEN1/SYP122 are required for pre-invasive immunity towards *P. infestans* and *C. destructivum*. (A, B) Frequency of penetration by (A) *P. infestans* and (B) *C. destructivum*. (C-F) Accumulation of callose in response to penetration (arrows) by (C, E) *P.infestans* and (D, F) *C. destructivum*. Bars = 10 µm. (A-B) All values are mean ±SD (n = 4 leaves per genotype). Different letters indicate significantly different values at P ≤ 0.001 estimated using logistic regression.

Figure 3

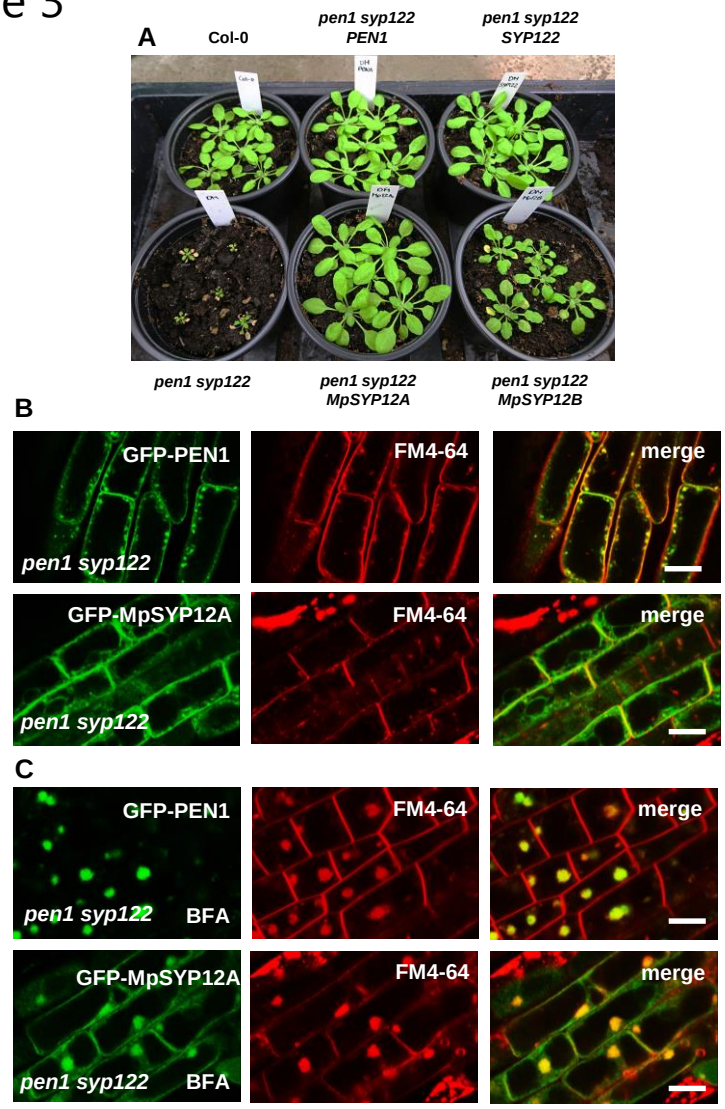

Figure 3: SYP12 clade members from *Marchantia polymorpha* are functional in *Arabidopsis*. (A) Plants at five weeks, expressing SYP12 clade members from either *Arabidopsis* or *Marchantia polymorpha*. (B) Roots of plants expressing either PEN1 or MpSYP12A fused to GFP and counter stained with FM4-64. (C) Same as B, but after treatment with 50 µM BFA for 1 h. Bars = 10 µm.

Figure 4

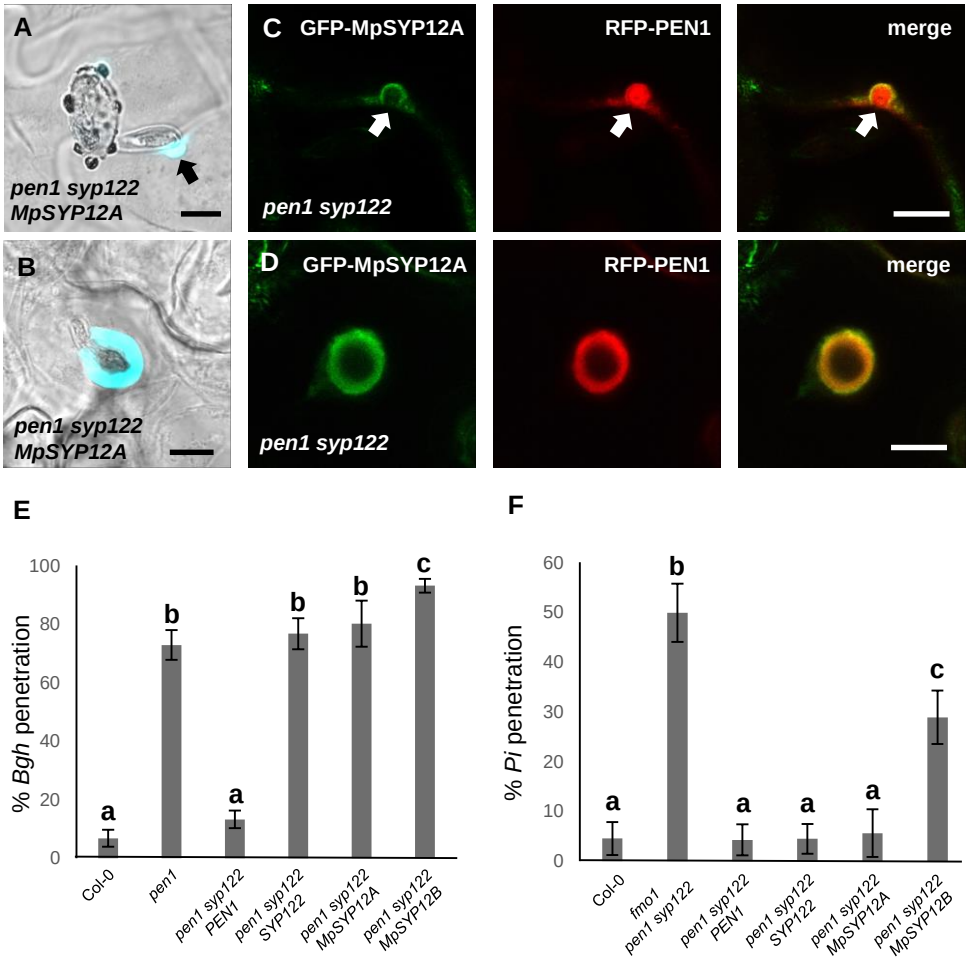

Figure 4: *Marchantia* syntaxins rescue papilla and encasement responses in *Arabidopsis*. (A, B) Accumulation of callose in response to attack (arrow) by *Bgh* in (A) non-penetrated- and (B) penetrated cells. (C, D) Localization of MpSYP12A and PEN1 in response to attack (arrows) by *Bgh* in (C) non-penetrated-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