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Involvement of lipid transfer proteins in resistance against a non-host powdery mildew in *Arabidopsis thaliana*

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SUMMARY

Non-specific lipid transfer proteins (LTPs) are involved in the transport of lipophilic compounds to the cuticular surface in epidermal cells and in the defence against pathogens. The role of glycophosphatidylinositol (GPI)-anchored LTPs (LTPGs) in resistance against non-host mildews in *Arabidopsis thaliana* was investigated using reverse genetics. Loss of either *LTPG1*, *LTPG2*, *LTPG5* or *LTPG6* increased the susceptibility to penetration of the epidermal cell wall by *Blumeria graminis* f. sp. *hordei* (*Bgh*). However, no impact on pre-penetration defence against another non-host mildew, *Erysiphe pisi* (*Ep*), was observed. LTPG1 was localized to papillae at the sites of *Bgh* penetration. This study shows that, in addition to the previously known functions, LTPGs contribute to pre-invasive defence against certain non-host powdery mildew pathogens.

Keywords: *Arabidopsis thaliana*, lipid transfer proteins, non-host resistance, powdery mildew.

INTRODUCTION

Although plants are constantly exposed to potential pathogens, few plants are ever actually successfully colonized by pathogens. As a result of incompatibilities between the pathogen and the plant, most plants are resistant to most pathogens. This type of resistance, in which all cultivars of a certain plant species are immune to all pathovars of a certain pathogen, is known as non-host resistance (NHR) (Thordal-Christensen, 2003), and depends on both the constitutive barriers of the plant and inducible defences (Lipka *et al.*, 2008). The recognition of so-called microbe-associated molecular patterns (MAMPs), which are conserved molecules present in large groups of microorganisms, leads to defence reactions in plant cells (Tsuda and Somssich, 2015). The interactions between *Arabidopsis thaliana* (hereafter Arabidopsis) and the non-adapted powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) have been established as a model system for NHR (Micali *et al.*, 2008; Thordal-Christensen, 2003). When a powdery mildew conidial spore germinates on the leaf surface, a specialized structure, the appressorium, is used by the fungus to penetrate the cuticle and the underlying cell wall. Many non-host powdery mildews are perceived by the plant as they attempt to penetrate the cell wall, and the epidermal cell responds by defending itself against the attacker. This defence usually comprises the accumulation of the polysaccharide callose, phenolic compounds and other antimicrobial chemicals adjacent to the penetration point, producing a thick reinforcement of the cell wall, known as a papilla, a physical barrier with a built-in chemical defence (Hardham *et al.*, 2007; Hückelhoven, 2007), which successfully stops most spores from infecting. The few spores that successfully penetrate the cell and proceed to produce the haustorial feeding structure will usually trigger additional plant defences. The haustorium may then be encased in callose to prevent it from growing and the epidermal cell wall will ultimately undergo programmed cell death (PCD) (Lipka *et al.*, 2008). Normally, most of the *Bgh* spores will not succeed in penetrating the epidermal cells of Arabidopsis and, in the minority of occasions in which penetration succeeds, the infection is stopped by callose encasement and PCD (Collins *et al.*, 2003; Lipka *et al.*, 2005; Micali *et al.*, 2008; Stein *et al.*, 2006). Forward genetic screens have identified mutations in three different genes that result in a higher ratio of successful penetrations by *Bgh*: the penetration mutants *pen1* (Collins *et al.*, 2003), *pen2* (Lipka *et al.*, 2005) and *pen3* (Stein *et al.*, 2006). These genes are involved in defence against powdery mildews through at least two different pathways (Hückelhoven and Panstruga, 2011), both probably triggered by the recognition of MAMPs. PEN1 is a syntaxin that is involved in the formation of the papilla through the secretion of exosomes (Nielsen and Thordal-Christensen, 2013). PEN2 is believed to produce active substances by the hydrolysis of indole glucosinolates (Bednarek *et al.*, 2009; Lipka *et al.*, 2005), which PEN3, an ABC transporter localized at the plasma membrane (Stein *et al.*, 2006), is thought to deliver into the apoplast to hinder fungal infection.

Lipid transfer proteins (LTPs) belong to a large multigene family present in all land plant species. The Arabidopsis genome contains more than 50 members (Boutrot *et al.*, 2008; Edstam *et al.*, 2011). LTPs are small (7–10.5 kDa) basic proteins with a high content of α-helices stabilized with a common eight-cysteine (Cys) motif, with the general form:

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C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C (Kader, 1997; Liu *et al.*, 2015). All LTPs have a hydrophobic cavity which can bind various lipids and other non-polar compounds (Charvolin *et al.*, 1999). The *in vivo* functions of plant LTPs are generally not well understood, but they are implicated in several important processes in plants. These include seed development and pollen germination (Edstam and Edqvist, 2014), and cutin and wax metabolism (DeBono *et al.*, 2009; Kader, 1997; Liu *et al.*, 2015; de Oliveira Carvalho and Gomes, 2007). The Arabidopsis LTP2 apparently contributes to the overall organization of the cuticle in Arabidopsis hypocotyls (Jacq *et al.*, 2017). In addition, LTPs play a major role in biotic and abiotic stresses (Liu *et al.*, 2015; Yeats and Rose, 2008). Several LTPs are known to have direct antimicrobial activity (Finkina *et al.*, 2016; Segura *et al.*, 1993; Zottich *et al.*, 2011). The LTP DIR1 is linked to long-distance signalling in the establishment of systemic acquired resistance (Champigny *et al.*, 2013; Maldonado *et al.*, 2002). LTPs are synthesized with an N-secretory signalling peptide, targeting them to the plant apoplast (Liu *et al.*, 2015). LTPs have been historically classified into two types: LTP1 type I of approximately 9 kDa and LTP2 type II of approximately 7 kDa. A new classification system introduced by Edstam *et al.* (2011), based on sequence similarity, molecular weight, the presence or absence of a glycophosphatidylinositol (GPI) modification site, the position of a conserved intron and spacing between the Cys residues in the eight-Cys motif, has led to ten subfamilies of proteins (Edstam *et al.*, 2011; Liu *et al.*, 2015; Salminen *et al.*, 2016). Overexpression of antimicrobial LTPs *in planta* often results in enhanced resistance to selected pathogens. A recent study, for instance, has shown that it is possible to modulate the response to clubroot disease in Arabidopsis using mutants or transgenic plants with modified levels of selected LTPs (Julke and Ludwig-Muller, 2015).

GPI-anchored LTPs (LTPGs) represent a subgroup of LTPs (Edstam *et al.*, 2011). The GPI anchor presumably attaches these proteins to the plasma membrane facing the apoplast. Previous studies have indicated that the Arabidopsis LTPG1 (DeBono *et al.*, 2009; Kim *et al.*, 2012; Lee *et al.*, 2009) and LTPG2 (Kim *et al.*, 2012) contribute to the accumulation of cuticular lipids, with LTPG1 also being involved in protection against *Alternaria brassicicola* (Lee *et al.*, 2009). Analysis of the transcriptional regulation of the 34 LTPGs encoded by the Arabidopsis genome placed several of them into three distinct clusters (Edstam *et al.*, 2013).

Given the importance of the outer perimeter defence, it seems reasonable to believe that components involved in cuticle composition, such as LTPGs, could play a role in NHR against powdery mildews. In this study, we investigated the roles of Arabidopsis LTPGs in penetration resistance against the non-host powdery mildews *Bgh* and *Erysiphe pisi* (*Ep*).

RESULTS

Loss of function of LTPG1, LTPG2, LTPG5 and LTPG6 leads to decreased pre-penetration resistance against *Bgh*

As LTPGs are linked to pathogen resistance and processes at the epidermal surface, and all appear to have an apoplastic localization, we decided to test their involvement in NHR in Arabidopsis. To this end, a panel of T-DNA insertion lines for six known Arabidopsis LTPGs was obtained (Fig. S1; Table S1, see Supporting Information). Mutant lines representing at least one LTPG gene found in each distinct transcriptional cluster, as reported previously (Edstam *et al.*, 2013), were selected. The loss of the respective transcripts in these different lines, except the *ltpg4-3* and *ltpg6-3* mutants, has been reported previously (Edstam and Edqvist, 2014; Kim *et al.*, 2012). The *ltpg5-1* line has a T-DNA insertion in the 5′-untranslated region, and expression of the gene is reportedly knocked down to approximately half of that found in the wild-type (Edstam and Edqvist, 2014).

The mutant lines were inoculated with *Bgh* or *Ep*, and the number of germinated spores which successfully penetrated the epidermal cell wall was determined at 3 days after inoculation (Fig. 1). The *ltpg1-1*, *ltpg1-2*, *ltpg2-1*, *ltpg5-1* and *ltpg6-3* mutants all demonstrated an increased frequency of successful penetrations by *Bgh* compared with the wild-type Col-0 (Fig. 1A). This phenotype was strongest for the *ltpg1-1* and *ltpg2-1* mutants, which displayed an almost doubled penetration frequency of *Bgh* compared with the wild-type.

The *ltpg3-1* and *ltpg4-3* knockouts did not display any difference in penetration resistance to *Bgh* when compared with the wild-type. None of the tested mutants displayed any significant difference in penetration resistance to the pea powdery mildew *Ep* (Fig. 1B). As the *ltpg1-1* and *ltpg2-1* mutants displayed the largest decrease in penetration resistance to *Bgh*, a double mutant was tested (Fig. 1C). However, the double mutant *ltpg1-1 ltpg2-1* demonstrated the same increase in *Bgh* penetration compared with the wild-type as the single *ltpg1-1* and *ltpg2-1* mutants. In addition, the *ltpg1-1* mutant was tested with the adapted powdery mildew *Golovinomyces cichoracearum.* The mutant did not display any change in overall disease progress and penetration frequency compared with the wild-type.

Loss of LTPG1 does not have an additive effect on the loss of pre-invasive defence in the *pen1* and *pen3* mutations

To investigate the genetic relationship between LTPG1 and the known mediators of penetration resistance, PEN1 and PEN3, the double mutants *ltpg1-1 pen1-1* and *ltpg1-1 pen3-1* were generated and inoculated with *Bgh*. Neither of the double knockout mutants *ltpg1-2 pen1-1* or *ltpg1-2 pen3-1* demonstrated any

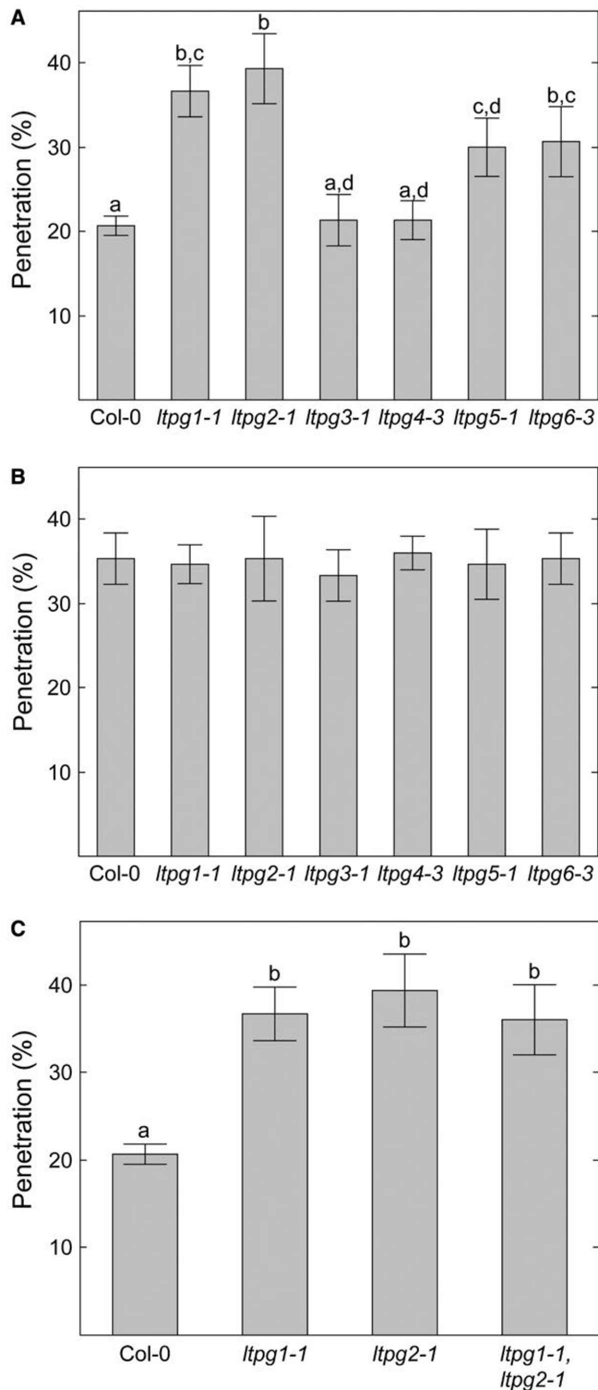

Fig. 1 Effects of loss of glycophosphatidylinositol (GPI)-anchored lipid transfer proteins (LTPGs) on non-host resistance. The indicated lines were inoculated with *Blumeria graminis* f. sp. *hordei* (*Bgh*) (A, C) or *Erysiphe pisi* (*Ep*) (B) and the penetration frequency was determined at 3 days post-inoculation (dpi). The average and standard deviation of three replicates are shown. Letters denote statistically significantly different groups ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) ($n = 6$ for A and B, $n = 3$ for C).

increase in penetration frequency compared with the single *pen1-1* or *pen3-1* mutations alone (Fig. 2).

Large bulk changes in wax load or composition do not affect pre-penetration defence

To test whether the bulk epicuticular wax load and composition had an impact on the penetration resistance, the *cer1* and *cer4* mutants were analysed for their ability to restrict the penetration of *Bgh*. The *CER1* gene encodes a putative fatty aldehyde reductase and the mutant displays severely reduced epicuticular wax load, in particular reductions in alkanes, secondary alcohols and ketones (Hannoufa *et al*., 1993; Samuels *et al*., 2008). The *CER4* gene encodes a fatty acyl-CoA reductase which is involved in the synthesis of the long-chain primary fatty alcohols of the epicuticular wax (Rowland *et al*., 2006). Loss of *CER4* removes almost all formation of primary fatty alcohols in stems and leaves (Rowland *et al*., 2006). The *cer1-1* and *cer4-3* mutants, however, did not show a significantly increased penetration frequency of *Bgh* compared with the wild-type (Fig. 3). This indicates that bulk changes in epicuticular wax load or composition are probably not responsible for the increased penetration frequency found in the *ltpg* mutants. The analysis of leaf wax load and composition in the *ltpg* mutants revealed no specific changes in total wax load or composition that coincided with the penetration phenotype (Fig. S2, see Supporting Information).

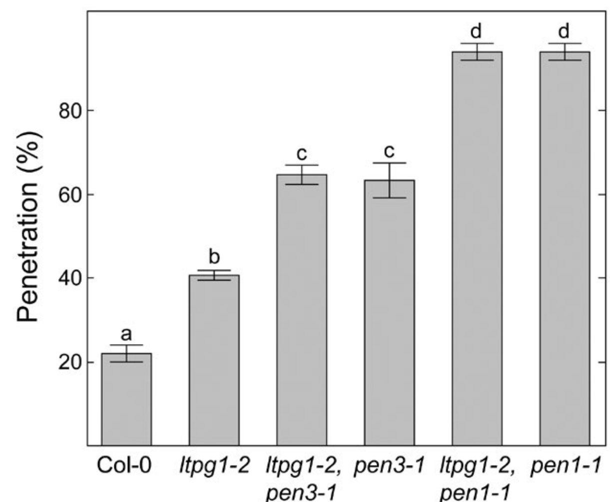

Fig. 2 Effects of combined loss of glycophosphatidylinositol (GPI)-anchored lipid transfer proteins (LTPGs) and PENs on the penetration resistance against *Blumeria graminis* f. sp. *hordei* (*Bgh*). The indicated lines were inoculated with *Bgh* and the penetration frequency was determined at 3 days post-inoculation (dpi). The average and standard deviation of three replicates are shown. Letters denote statistically significantly different groups ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) ($n = 6$).

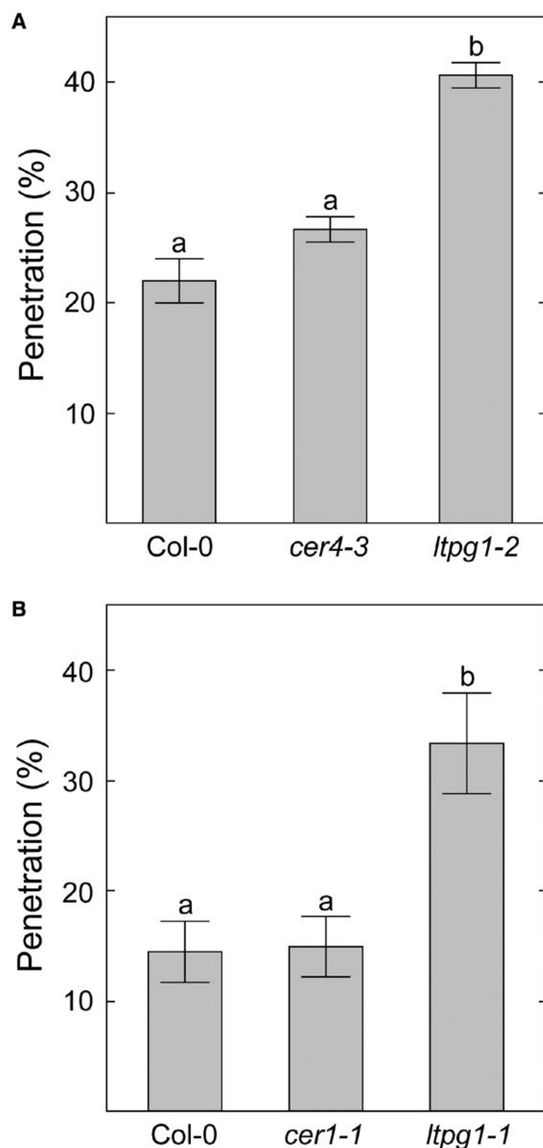

Fig. 3 Effects of loss of CER4 (A) or CER1 (B) on non-host resistance. The indicated lines were inoculated with *Blumeria graminis* f. sp. *hordei* (*Bgh*) and the penetration frequency was determined at 3 days post-inoculation (dpi). The average and standard deviation of three replicates are shown. Letters denote statistically significantly different groups ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) ($n = 6$).

Localization of LTPG1 to sites of interaction with the non-host powdery mildew *Bgh*

To further investigate the role of LTPG1 in penetration defence against *Bgh*, we used a yellow fluorescent protein (YFP)-tagged LTPG1 protein with a native promoter in the *ltpg1-2* background (DeBono *et al.*, 2009). The YFP fusion construct clearly complemented the penetration phenotype of *ltpg1-2* (Fig. 4C). The localization of the YFP fusion construct was investigated using confocal microscopy of uninfected and material infected with *Bgh* (Fig. 4A) or *Ep* (Fig. 4B). YFP-LTPG1 in uninfected cells was predominantly localized next to the borders of adjoining epidermal cells, consistent with previous reports (Ambrose *et al.*, 2013; DeBono *et al.*, 2009). At 1 day post-inoculation (dpi) with *Bgh* or *Ep*, YFP-LTPG1 was found to localize to sites of attempted penetration by the fungal appressoria. Reconstruction in three dimensions of the captured images in *Z*-stacks shows that YFP-LTPG1 localizes around the forming papilla at attempted *Bgh* penetration sites (Fig. 4D).

DISCUSSION

Penetration resistance against non-host powdery mildews in Arabidopsis has been linked previously to at least two different pathways: transcytosis involving the syntaxin PEN1 (Nielsen and Thordal-Christensen, 2013), and the synthesis and secretion of toxic metabolites through the action of PEN2, PEN3 and several other biosynthetic enzymes (Bednarek, 2012). In addition, phospholipase D (Pinosa *et al.*, 2013) has been linked to MAMP recognition inducing penetration resistance and, recently, a potential second exocytotic pathway has been implicated in penetration resistance (Nielsen *et al.*, 2017). This study shows that penetration resistance to *Bgh* is partially conferred by the presence of LTPG1, LTPG2, LTPG5 and LTPG6. Interestingly, although the previously described PEN pathways are active against both *Bgh* and *Ep*, the loss of penetration resistance in the *ltpg* mutants was evident only in defence against *Bgh*. This points to an interesting difference in the defence reactions against the two different non-host mildews. Important differences in the ability of Arabidopsis to resist these two different pathogens have been highlighted previously using the double mutant *pen3 eds1*, in which the double mutant allows *Ep* to complete its life cycle, whereas NHR is maintained against *Bgh* (Stein *et al.*, 2006). Thus, there might be important differences in how Arabidopsis senses and responds to these pathogens during the two superficially similar defence reactions to *Ep* and *Bgh*. Interestingly, loss of LTPG1 has no additive effect to the loss of penetration resistance against *Bgh* in the *pen1* and *pen3* mutants. The most straightforward explanation of this is that LTPGs contribute to activation of both penetration resistance pathways described previously. This needs to be taken into account in hypotheses describing exactly how LTPGs contribute mechanistically to penetration resistance. At this point, a definite mechanistic explanation of how LTPGs in Arabidopsis confer penetration resistance against *Bgh* cannot be provided. However, the presented findings allow a few possible hypotheses to be proposed.

It has been shown that the amount of very-long-chain aldehydes promotes both the germination and differentiation of *Bgh* conidia (Hansjakob *et al.*, 2010). Hexacosanal, found in the epicuticular wax layer of barley, has been found to be the most effective aldehyde in this respect. Several other studies have

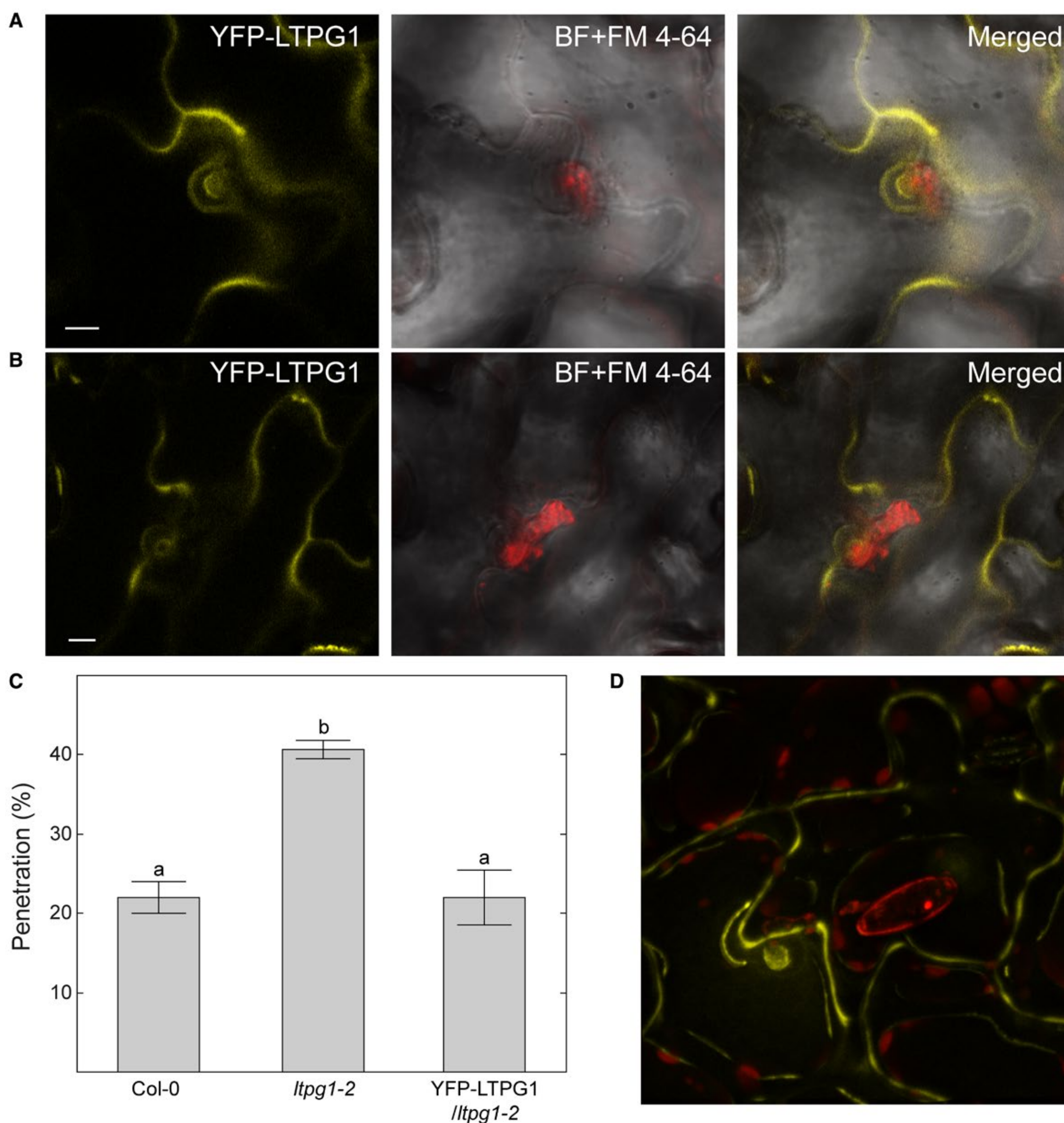

Fig. 4 Localization of glycophosphatidylinositol (GPI)-anchored lipid transfer protein 1 (LTPG1) during penetration resistance. Tissues were investigated by confocal microscopy at 24 h post-inoculation (hpi) with *Blumeria graminis* f. sp. *hordei* (*Bgh*) (A) or *Erysiphe pisi* (*Ep*) (B) to visualize the fungal structures stained with FM 4-64 (red channel), yellow fluorescent protein (YFP) (yellow channel) and bright field (BF). Scale bars denote 5 μm. The indicated lines were inoculated with *Bgh* and the percentage of germinated spores was determined at 3 days post-inoculation (dpi) (C). Bars not annotated with the same letter are significantly different ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) ($n = 3$). Error bars denote the standard deviation from the mean. (D) Three-dimensional reconstruction of YFP-LTPG1 accumulation around the papilla formed as a defence against invading *Bgh* hyphae at 24 hpi. Fungal structures are stained with FM 4-64 (red channel) and YFP fluorescence is shown in yellow. [Colour figure can be viewed at wileyonlinelibrary.com]

reported that such very-long-chain aldehydes are important for the pre-penetration processes of powdery mildews (Hansjakob *et al.*, 2011; Weis *et al.*, 2014). Changes in such compounds could provide an explanation for the penetration phenotype displayed by LTPGs with *Bgh*. The *ltpg1* mutants possess a reduced content of C_{29} alkane (nonacosane) in the epicuticular wax of stems and siliques (DeBono *et al.*, 2009; Lee *et al.*, 2009), but no difference in this or the total wax load has been reported for the leaf epidermis (Kim *et al.*, 2012; Lee *et al.*, 2009). The wax analysis performed in this study did not provide any results that could be connected with the penetration phenotype. Thus, the composition of cuticular wax does not appear to be the primary reason for the penetration phenotype of the *lptg* mutants. In addition, the lack of penetration phenotype of the *cer4* and *cer1* mutants also indicates that the long-chain fatty alcohols and the total wax load are of very minor importance for the penetration

resistance against *Bgh*. Although we found no difference in the wax profile that could explain the penetration phenotype, it cannot be ruled out that subtle differences in minor components might play a role in the penetration resistance processes. Some LTPs have been shown to have direct anti-pathogenic effects *in vitro* (Cammue *et al.*, 1995); this could also be the case for the Arabidopsis LTPGs. However, this hypothesis would be more difficult to reconcile with the very high specificity of *Bgh* penetration resistance shown by the *ltpg* mutants.

The loss of several LTPGs resulted in similar phenotypic effects on the penetration resistance, and the combination of two mutants caused no further increase. It has been suggested that LTPGs can be classified into co-expression modules reflecting genes involved in the same biological processes (Edstam *et al.*, 2013). For example, LTPG1, LTPG2 and LTPG6 form the Arabidopsis module AtI involved in the deposition and biosynthesis of cuticular waxes or cutin. We showed that the penetration resistance to *Bgh* is partially conferred by the presence of LTPG1, LTPG2 and LTPG6. It has been shown previously that the role of LTPG1 in the export of epicuticular wax overlaps with that of LTPG2 (Kim *et al.*, 2012). Thus, higher order mutants or other combinations of loss-of-function mutations in LTPGs might demonstrate a stronger phenotype in penetration resistance. Interestingly, other LTPGs (LTPG3 and LTPG4) that could be linked to sporopollenin biosynthesis in pollen grains (Edstam *et al.*, 2013) did not show any increase in penetration resistance to *Bgh*, suggesting an additional function in defence for LTPG1, LTPG2 and LTPG6 present at the plant–fungus interface. It could also be construed that the phenotype is caused by a threshold effect; a certain amount of LTPGs must be present to confer an effect on penetration resistance, and the loss of just a portion causes the protein level to drop below the threshold. Thus, any additional loss of LTPGs of the same function would cause no additional loss of penetration resistance.

As LTPGs are most probably apoplastic, it is possible that these proteins function by binding to substances in the fungal membrane on the penetration peg, facilitating the transport of other substances to that site. Alternatively, LTPGs could function as reporters or as part of signal transduction when they encounter and bind to fungal membranes or components, which could then be presented to MAMP receptors in the plasma membrane. This hypothesis represents a single explanation most easily compatible with the difference in effect on penetration resistance between *Bgh* and *Ep*, as well as the lack of additive effect of *ltpg* double mutants and double mutants with *pen1* and *pen3*.

A fusion construct of YFP and LTPG1 clearly localizes at the site of formation of papillae during interactions with both *Ep* and *Bgh*. It would also be interesting to investigate the localization of the other LTPGs during the penetration resistance reaction. An interesting question is how LTPG1 accumulates around the papillae. Is this an active process or is it achieved passively by changes in the geometry of the apoplastic space caused by the attempted penetration of the powdery mildew? According to the 'balloon in a box model' (Ambrose *et al.*, 2013), the YFP-LTPG1 proteins could accumulate at locations at which there is a widening between the plasma membrane and cell wall. As the penetration peg pushes down on the cell wall, there would be an increased apoplastic space around the point of attempted penetration, in which YFP-LTPG1 could accumulate. If the penetration peg breaks through, it will push down on the plasma membrane to form a haustorium, creating even more space around itself in the apoplast. An alternative route for the accumulation of LTPG1 at the papillae would be to follow the transcytosis route described for several other proteins (PEN1 for example) to the papillae (Nielsen and Thordal-Christensen, 2013). Further studies are required to investigate this in more detail. It would also be of great interest to investigate the role of the GPI anchor of LTPGs in penetration resistance.

To conclude, it is clear that several closely related Arabidopsis LTPG proteins function in penetration resistance against the non-host powdery mildew *Bgh*, but not against the other non-host mildew *Ep*. Further analysis of the molecular details of the LTPGs and their involvement in other defence processes might shed more light on the details of this.

EXPERIMENTAL PROCEDURES

Plant material and genotyping of mutants

Arabidopsis lines were cultivated in climate chambers (CLF Plant Climatics GmbH, Wertingen, Germany) with short-day conditions (8 h, 120 µE light, 22 °C, 60% humidity, day; 18 °C, night) in soil premixed with perlite (S-jord, Hasselfors Garden, Örebro, Sweden; https://www.hasselforsgarden.se/) with added nutrients ('Blomstra', Cederoth International, Upplands Väsby, Sweden; https://www.cederroth.com/en/), diluted according to the manufacturer's instructions.

Seeds for T-DNA insertion mutants of SALK (Alonso *et al.*, 2003) and SAIL (Sessions *et al.*, 2002) type were acquired from the Nottingham Arabidopsis Stock Centre (NASC) (Scholl *et al.*, 2000), unless stated otherwise (Table S1). DNA extractions were performed using the protocol of Edwards *et al.* (1991), and T-DNA insertions were confirmed by polymerase chain reaction using Platinum Taq polymerase (Clontech Laboratories, Mountain View, CA, USA; https://www.clontech.com/) with T-DNA left border primers for SALK (LBb1.3; 5′-ATTTTGCCGATTTCGGAAC-3′) or SAIL (LB3; 5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′), combined with gene-specific primers (Table S1), according to the manufacturer's instructions, except only one-quarter of the recommended amount of polymerase was used.

The single *ltpg1-1* and *ltpg2-1* mutants, and the double *ltpg1-1 ltpg2-1* knockout mutant, were described in Kim *et al.* (2012), YFP-LTPG1 (in the *ltpg1-2* background) and *ltpg1-2* in

DeBono *et al.* (2009), *pen1-1* in Collins *et al.* (2003), and *pen3-1* in Stein *et al.* (2006). The c*er4-3* line was described in Rowland *et al.* (2006) (Table S1). The *cer1-1* T-DNA insertion mutant line was obtained from NASC (Table S1), and the wax phenotype of this line was verified by epicuticular wax analysis by gas chromatography-mass spectrometry (GC-MS).

Pathogen inoculation

Blumeria graminis f. sp. *hordei* (isolate DH14) was propagated on barley (*Hordeum vulgare* cv. Barbro) and used after 1 week of growth at room temperature from the time of inoculation. *Erysiphe pisi* (isolate CO-01) was propagated on pea plants (*Pisum sativum* cv. Kelvedon Wonder). Four- to five-week-old Arabidopsis plants were infected as described previously (Pinosa *et al.*, 2013). *Golovinomyces cichoracearum* was cultivated on zucchini (*Cucurbita pepo* L. cv. F1 Diamant) and inoculated as described previously (Collins *et al.*, 2003).

Staining and bright-field microscopy

Trypan blue staining was performed as described by Koch and Slusarenko (1990), with some modifications. Briefly, the leaves were collected in Eppendorf tubes, covered in trypan blue staining solution and incubated at 95 °C for 3–5 min (time depending on the amount of material); they were then rinsed once with deionized water before the addition of aqueous chloral hydrate solution for de-staining and clearing. When the leaf material had completely cleared, it was rinsed with water and mounted on microscopy glass slides in 60% glycerol. Penetration and/or cell death was scored as described previously (Pinosa *et al.*, 2013). The average frequency of penetrations from each count was analysed by one-way analysis of variance multiple comparisons, using Tukey's honestly significant difference test and the software package JMP (JMP®, Version 12.0.1, SAS Institute Inc., Cary, NC, USA).

Confocal microscopy

To visualize the mildew spores, 1-dpi leaf tissue was mounted in a solution of 0.1% (v/v) Silwet and 10 μM FM 4-64 (SynaptoRed, Merck Millipore Burlington, MA, USA; https://www.merckmillipore.com/) on microscope glass, and viewed with a Zeiss (Oberkochen, Germany) LSM 700 on an inverted Axio Observer.Z1 microscope with a 40× water-immersion objective (LD C-Apochromat; numerical aperture, 1.1). Excitation was with 488- and 555-nm lasers, and emission was recorded at 518 and 585 nm for YFP and FM 4-64, respectively. The microscope was controlled with ZEN 2010 software (Zeiss Oberkochen, Germany), from which single images and image arrays were saved. Maximum intensity three-dimensional reconstruction and image processing were performed with ZEN 2012 (Zeiss GmbH).

Analysis of leaf cuticular wax

The extraction and analysis method was performed as described by Haslan and Kunst (2013) on 6-week-old plants, using 5 μg tetracosane as internal standard. After extraction, the sample was split into two: one sample was trimethylsilylated using N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Sigma-Aldrich, Saint Louis, MO, USA) in pyridine (1 : 1, v/v), and the other was analysed directly. The samples were analysed using an Agilent (Santa Clara, CA, USA) 7820A GC coupled to an Agilent 5975 mass-selective detector. A DB-5 capillary column (30 m × 0.25 mm; J&W Scientific, Inc., Folsom, CA, USA) was used with helium as carrier gas with a constant flow of 0.7 mL/min. Injector and detector transfer lines were kept at 250 °C and 280 °C, respectively. The oven temperature was increased from 75 °C to 325 °C with a linear increase of 10 °C/min, and held at 325 °C for 10 min. The detector was operated in full scan mode from 50 to 600 *m/z*. Individual wax components were quantified against the peak area of the internal standard and the leaf area.

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AUTHOR CONTRIBUTIONS

M.X.A., P.F. and N.B. designed the study. P.F., N.B. and O.N.J. performed the experiments and calculations. P.F. and M.X.A. wrote the manuscript. N.B. and O.N.J. made manuscript revisions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 Structure of the glycophosphatidylinositol (GPI)-anchored lipid transfer protein (LTPG) genes and T-DNA insertions used in this study.
Fig. S2 Analysis of leaf total cuticular wax content.
Table S1 Mutant lines and primers used for genotyping in this study.