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RESEARCH PAPER

Leaf abaxial immunity to powdery mildew in Arabidopsis is conferred by multiple defense mechanisms

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Abstract

Powdery mildew fungi are obligate biotrophic pathogens that only invade plant epidermal cells. There are two epidermal surfaces in every plant leaf: the adaxial (upper) side and the abaxial (lower) side. While both leaf surfaces can be susceptible to adapted powdery mildew fungi in many plant species, there have been observations of leaf abaxial immunity in some plant species including Arabidopsis. The genetic basis of such leaf abaxial immunity remains unknown. In this study, we tested a series of Arabidopsis mutants defective in one or more known defense pathways with the adapted powdery mildew isolate *Golovinomyces cichoracearum* UCSC1. We found that leaf abaxial immunity was significantly compromised in mutants impaired for both the EDS1/PAD4- and PEN2/PEN3-dependent defenses. Consistently, expression of EDS1–yellow fluorescent protein and PEN2–green fluorescent protein fusions from their respective native promoters in the respective *eds1-2* and *pen2-1* mutant backgrounds was higher in the abaxial epidermal cells than in the adaxial epidermal cells. Altogether, our results indicate that leaf abaxial immunity against powdery mildew in Arabidopsis is at least partially due to enhanced EDS1/PAD4- and PEN2/PEN3-dependent defenses. Such transcriptionally pre-programmed defense mechanisms may underlie leaf abaxial immunity in other plant species such as hemp and may be exploited for engineering adaxial immunity against powdery mildew fungi in crop plants.

Keywords: Abaxial immunity, *Arabidopsis thaliana*, basal resistance, EDS1, PEN2, post-penetration resistance, powdery mildew.

Introduction

Plants have evolved a sophisticated innate immune system that is conceivably deployed and executed in a spatiotemporal manner. As in animals, molecular detection of pathogenic microbes in plants occurs on the cell surface and inside the invaded cells. It is well established that pattern recognition receptors (PRRs) located at the plasma membrane sense the presence of pathogen-associated molecular patterns (PAMPs) and activate defense responses termed PAMP-triggered immunity (PTI). In response to pathogens' deployment of effectors that are delivered to the intracellular space to subvert PTI,

Abbreviations: EDS1, ENHANCED DISEASE SUSCEPTIBILITY 1; ET, ethylene; ETI, effector-triggered immunity; *Gc*, *Golovinomyces cichoracearum*; JA, jasmonic acid; NLR, nucleotide-binding and leucine-rich-repeat; PAD4, PHYTOALEXIN-DEFICIENT 4; PAMP, pathogen-associated molecular pattern; PEN1, PENETRATION1; PEN2, PENETRATION2; PEN3, PENETRATION3; PRR, pattern recognition receptor; PTI, PAMP-triggered immunity; SA, salicylic acid; SID2, salicylic acid induction-deficient 2.

plants have evolved nucleotide-binding leucine-rich repeat (NLR) receptors to detect the presence or activity of pathogen effectors, resulting in effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Jones *et al.*, 2016; Ngou *et al.*, 2022). Recent studies demonstrate that PTI and ETI share common signaling modules (Pruitt *et al.*, 2021) and mutually potentiate each other (Ngou *et al.*, 2021; Yuan *et al.*, 2021). Thus, at the molecular level, PTI and ETI are spatiotemporally coordinated and executed in a cell-autonomous manner.

At the phenotypical or symptomatic level, spatiotemporally distinctive immunity or susceptibility to pathogens is commonly observed in plants. For example, different tissues/organs of many plant species show differential levels of resistance/susceptibility to a particular pathogen and, conversely, many pathogens have preferred plant tissues/organs for colonization (Hermanns *et al.*, 2003). Leaf pathogens may not infect stems, flowers, or roots, while root pathogens may not affect aboveground parts of the plant (Marcel *et al.*, 2010; Strugala *et al.*, 2015). An elegant study by Zhou and colleagues showed that root PTI is configured differently compared with that in leaves, and activation of immunity in root cells requires both PRR detection of PAMPs and local cell damage signals (Zhou *et al.*, 2020). This finding suggests that plants have evolved mechanisms to discriminate beneficial microbes from pathogens that cause damage to root cells in the soil environment (rhizosphere) that is very different from the phyllosphere (Zhou *et al.*, 2020; Tsai *et al.*, 2023). Another more recent elegant study by Wang *et al.* identified a broad-spectrum clubroot resistance gene from Arabidopsis whose transcription is induced by the root pathogen *Plasmodiophora brassicae* specifically in the pericycle and prevents colonization of the pathogen in the stele (Wang *et al.*, 2023). On the other hand, the age of a plant or an organ (e.g. leaf) is a major factor influencing host resistance or susceptibility to various pathogens (Hu and Yang, 2019), including bacterial pathogens such as *Pseudomonas syringae* (Hu *et al.*, 2023) and fungal pathogens such as powdery mildew. For the latter, resistance of many plants to adapted powdery mildew fungi significantly increases as leaves mature. Such a tendency has been described in grapevine (Calonnec *et al.*, 2021), strawberry (Asalf *et al.*, 2014), and rubber tree (Cao *et al.*, 2021). These phenomena indicate that plant immunity is spatiotemporally programmed, which at least partially explains why host adaptation of pathogens often occurs in a tissue/cell-specific manner (Lacaze and Joly, 2020). However, the molecular bases of cell- and tissue/organ-specific immunity and age-related resistance of plants remain poorly characterized in general (Hu and Yang, 2019; Lacaze and Joly, 2020).

Powdery mildew diseases are caused by ascomycete fungi in the order of Erysiphales that only invade host epidermal cells (Micali *et al.*, 2008). There are two sides (i.e. adaxial and abaxial) of each plant leaf. In Arabidopsis, the organ-specific BTB-POZ domain protein BOP1 functions as a transcriptional activator to drive the expression of *ASYMMETRIC LEAVES2* (*AS2*) to promote leaf cell fate specification and adaxial polarity (Jun *et al.*, 2010). A few published studies showed that powdery mildew pathogens establish infection in both the adaxial and abaxial leaf surfaces of strawberry (Asalf *et al.*, 2014), California poppy (Camacho-Tapia *et al.*, 2018), pumpkin (Wyenandt *et al.*, 2008), and gerbera daisy (Kloos *et al.*, 2005). A few studies also showed that in some species, while the leaf adaxial surface is susceptible, the abaxial surface is resistant. For example, an examination of *Hordeum chilense*, a species of wild barley native to Chile and Argentina, showed that compared with the adaxial surface of this wild barley, the abaxial leaf surface exhibited higher levels of penetration resistance to three formae speciales of cereal powdery mildew fungi (Rubiales and Carver, 2000). Similarly, the abaxial leaf surface of perennial ryegrass is rarely infected by adapted *Blumeria graminis*, while the adaxial surface is very susceptible (Carver *et al.*, 1990). In contrast, powdery mildew infection is more prominent in the abaxial side of leaves of some strawberry genotypes compared with their respective adaxial side (Sombardier *et al.*, 2009; Asalf *et al.*, 2014; Wang *et al.*, 2022).

Investigating organ/tissue-specific resistance of Arabidopsis against powdery mildew, Inada and Savory (2011) examined rosette leaves, stems, fruits (siliques), and roots of the Arabidopsis Col-0 accession for the spore germination and hyphal growth of the sporelings at 2 days post-inoculation (dpi) with an adapted powdery mildew isolate *Golovinomyces orontii* (Inada and Savory, 2011). They claimed that both adaxial and abaxial surfaces of mature rosette leaves supported *G. orontii* growth, whereas fungal growth was much reduced in cauline leaves and almost completely inhibited on stems, fruits, and roots. However, the authors did not show whether the abaxial surface of the rosette leaves could support fungal growth (after 2 dpi) and sporulation (after 5 dpi) of *G. orontii* similar to those on the abaxial surface.

In this study, we focused on our investigation on the genetic/molecular mechanisms that contribute to leaf abaxial immunity of Arabidopsis using various Arabidopsis mutants. We found that leaf abaxial immunity of Arabidopsis is primarily due to higher levels of basal resistance that probably relies on more active glucosinolate metabolism and EDS1/PAD4-dependent defenses in the abaxial epidermal layer compared with those in the adaxial epidermal layer. We also examined both the leaf adaxial and abaxial surfaces of seven other plant species for their infection phenotypes upon inoculation with the respectively adapted powdery mildew fungi. Similar leaf abaxial immunity against powdery mildew may exist in other plant species such as hemp.

Materials and methods

Plant lines and growth conditions

The Arabidopsis accessions Per-1 and Sg-1 were obtained from the ABRC (https://abrc.osu.edu), and their powdery mildew infection phenotypes

were described in a previous report (Orgil *et al.*, 2007). Mutant lines *pen1-1* (Collins *et al.*, 2003), *pen2-1* (Lipka *et al.*, 2005), *pen3-1* (Stein *et al.*, 2006), *eds1-2* (Bartsch *et al.*, 2006), *pad4-1* (Jirage *et al.*, 1999), *sid2-2* (Dewdney *et al.*, 2000), *dde2-2* (von Malek *et al.*, 2002), *ein2-1* (Alonso *et al.*, 1999), *pad4-1sid2-2* (Tsuda *et al.*, 2009), *eds1-2pad4-1* (Kim *et al.*, 2014), *eds1-2pad4-1sid2-2* (Zhang *et al.*, 2018), *adr1* triple, and the helperless *adr1-nrg1* sextuple mutants (Wu *et al.*, 2019) have been described previously. The higher order mutants *eds1pad4sid2pen1*, *eds1pad4sid2pen2*, *eds1pad4sid2pen3*, and *eds1pad4sid2pen1pen2pen3* were generated via clustered regularly interspaced palindromic repeats (CRISPR)-targeted mutagenesis using the pHEE401E plasmid containing CRISPR-associated protein 9 (Cas9) under control of the egg cell-specific promoter (Wang *et al.*, 2015) and the single guide (sg) RNAs designed for targeting *PEN1* (At3g11820), *PEN2* (At2g44490), and *PEN3* (At1g59870). Primers for making sgRNAs and for genotyping targeted mutations are listed in Supplementary Table S1. The Col-*gl* line transgenic for *RPW8.2*^{D116G}*-YFP* under control of the *RPW8.2* promoter was reported in an earlier study (Wang *et al.*, 2013). The *EDS1-YFP/eds1-2* transgenic line (Cui *et al.*, 2018) and *PEN2-GFP/pen2-1* line (Fuchs *et al.*, 2016) were kindly provided by Jane Parker and Volker Lipka, respectively. Plant growth conditions for Arabidopsis were the same as reported (Zhang *et al.*, 2018). Seeds of sow thistle (*Sonchus oleraceus*) (Wen *et al.*, 2011) were generated in the lab, seeds of tomato (*Solanum lycopersicum*, Moneymaker), tobacco (*Nicotiana tabacum*), strawberry (*Fragaria iinumae*, a diploid), industrial hemp (*Cannabis sativa* L.), squash (*Cucurbita pepo*, Black beauty), and barley (*Hordeum vulgare*) were either purchased from a local Home Depot store or obtained from collaborators.

Microscopy and image data analysis

The expression and localization of the EDS1–yellow fluorescent protein (YFP) and PEN2–green fluorescent protein (GFP) fusions were examined by confocal microscopy using a Zeiss LSM710 microscope (Wang *et al.*, 2009). Confocal images were processed using the ZEN software (blue edition) from Carl Zeiss (https://ctsi.umn.edu/sites/ctsi.umn.edu/files/2021-03/zen_lite_blue_edition_quick_guide.pdf) and Adobe Photoshop CC. For quantification of YFP or GFP signal intensity in abaxial and adaxial epidermal cells, five leaves from each genotype were cut into halves, and a leaf disc (1 × 1 cm) away from the midrib in the middle of the leaf was cut from each half. One disc was used for imaging the adaxial epidermal cells and the other was used for imaging the abaxial epidermal cells. Six images were captured from different fields of a leaf disc (totaling 30 images for each genotype) using LSM710 with the exact same parameters. ImageJ (Schneider *et al.*, 2012) was used to measure the strength of the fluorescent signal across the plasma membrane region (60 cells measured for each genotype) where the signal was relatively strong and homogeneous.

Powdery mildew species, spore inoculation, disease phenotyping, and quantification

Arabidopsis-adapted PM isolate *Golovinomyces cichoracearum* (*Gc*) UCSC1 was maintained on *eds1-2* plants. Plant inoculation, scoring of disease reaction phenotypes, and visualization of fungal structures were done as previously described (Zhang *et al.*, 2018; Wu *et al.*, 2021). For quantification of disease susceptibility in both abaxial and adaxial sides of inoculated leaves, 6–8 fully expanded leaves from each Arabidopsis genotype or other plant species were flipped and their position was fixed with tape to allow even inoculation with powdery mildew spores. The individual leaves were collected at 10–12 dpi, weighed, and used for spore quantification immediately or stored at −80 °C for late spore counting. A spore suspension of each leaf sample was made by vortexing the leaves for 1 min in 2 ml or 4 ml of H_2O with 0.025% Silwet L-77 (further dilution can be made if necessary for super-susceptible genotypes or other plant species with big leaves) for spore counting using an Automated Cell Counter (Luna™) (https://www.youtube.com/watch?v=Cg4rZ1nwKXg). Spore counts were normalized to the fresh weight of the corresponding leaf samples. All statistical analyses were done in R (R Core Team, 2017), and graphics were generated using 'ggplot2' (Wickham, 2016). Powdery mildew isolates that are infectious on squash, sow thistle, tomato, and tobacco were described in a previous report (Wu *et al.*, 2018), barley powdery mildew isolate *Blumeria graminis* f. sp. *hordei* 5874 was obtained from the laboratory of Dr Roger Wise, and new powdery mildew isolates infectious on strawberry and industrial hemp were recently identified and purified by the Xiao lab.

Results

Abaxial immunity of Arabidopsis restricts hyphal growth and sporulation of powdery mildew

For a long time, we have observed that the midrib area of mature Arabidopsis leaves of susceptible accession Col-0 often exhibits resistance against adapted powdery mildew isolate *Gc* UCSC1. The midrib immunity is compromised in *pad4-1*, and almost completely abolished in the *eds1-2* single mutant and the *eds1pad4* double mutant (Zhang *et al.*, 2018) (Supplementary Fig. S1), indicating that leaf midrib immunity is primarily due to the enhanced basal resistance via the EDS1/PAD4-dependent signaling pathway. We have also observed that the leaf abaxial side of Col-0 plants lacks powdery mildew infection visible to the naked eyes (Fig. 1A, B) even if we flipped the leaves and fix their new position by taping (Supplementary Fig. S2A) to enable even inoculation of the abaxial surface by our recently published method (Wu *et al.*, 2021). To determine if the abaxial immunity observed in Col-0 is conserved in other Arabidopsis accessions, we tested Per-1 and Sg-1, two accessions susceptible to *Gc* UCSC1 (Orgil *et al.*, 2007) and found similar leaf abaxial immunity in these two accessions (Supplementary Fig. S3). To investigate possible genetic mechanisms involved in leaf abaxial immunity, we first monitored the fungal development using trypan blue staining to determine when the fungal development is arrested. There was no significant difference in spore germination on the two leaf surfaces (Supplementary Fig. S2B, C); however, the hyphal growth in the first 24 h was significantly less in the abaxial layer compared with that in the adaxial layer (Fig. 1C, D). Consistently, the fungal colonial network is much less in the abaxial layer compared with the adaxial layer at 4 dpi (Fig. 1E, F). More importantly, at this time point, very few (7%) colonies produced one or two small immature conidiophores in the abaxial layer, whereas most colonies (95%) in the adaxial side developed up to six conidiophores, with ~40% of them producing close-to-mature spores (arrows in Fig. 1E). One likely cause of poor fungal growth and very rare sporulation in the abaxial epidermal cells could be due to formation of fewer functional haustoria. To test this, we inoculated either the abaxial or the adaxial side of leaves of a Col-*gl* line stably expressing RPW8.2^{D116G}–YFP from the *RPW8.2* promoter. RPW8.2^{D116G} is an RPW8.2 mutant that

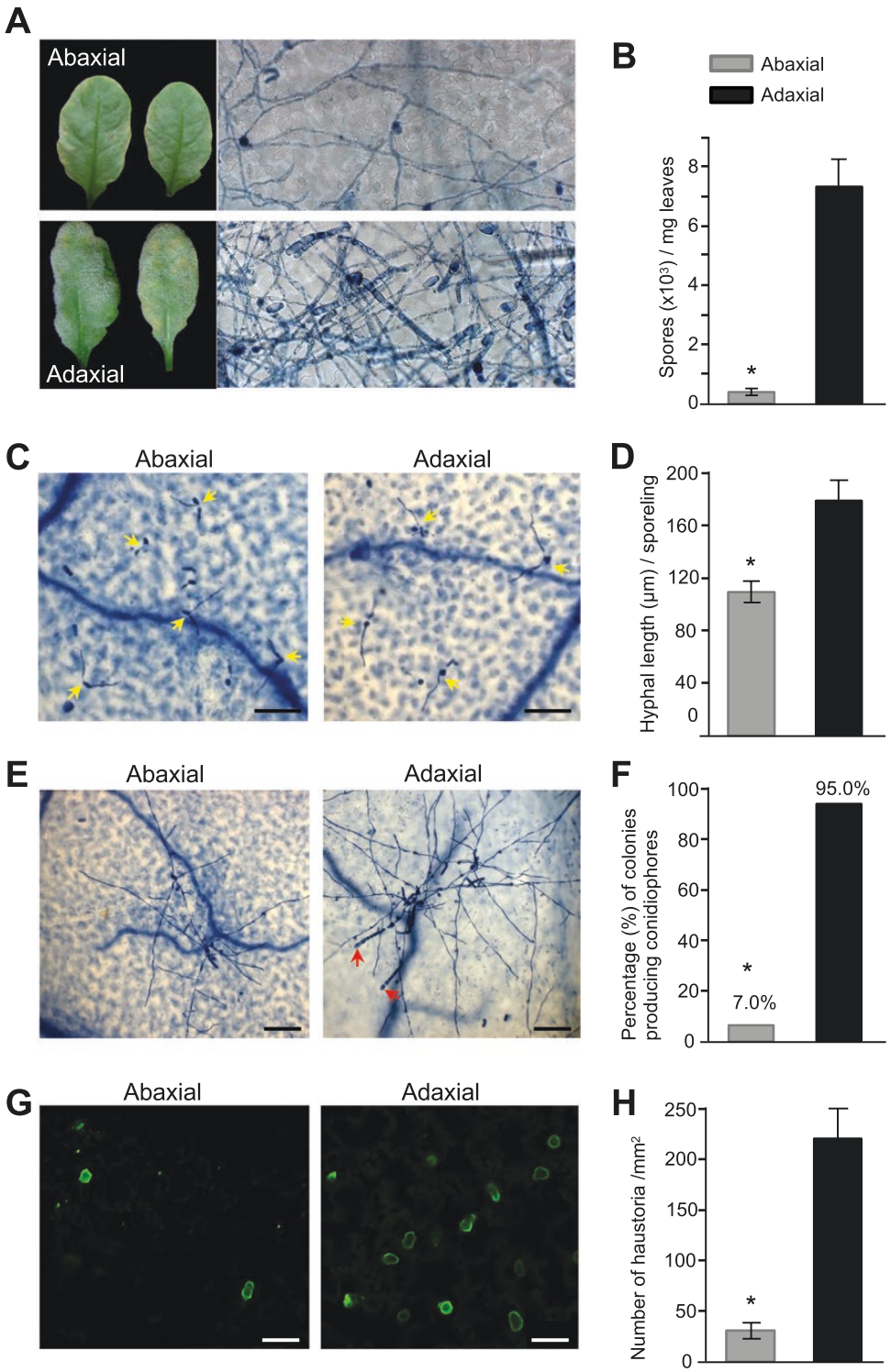

Fig. 1. Abaxial immunity to adapted powdery mildew in Arabidopsis accession Col-0. Leaves of 8-week-old wild-type Col-0 or Col-*gl* expressing RPW8.2^{D116G}–YFP from *the RPW8.2* native promoter were inoculated with *Golovinomyces cichoracearum* (*Gc*) UCSC1 on either the adaxial side (natural) or the abaxial side (taped as shown in Supplementary Fig. S2) and subjected to photography and/or trypan blue staining and/or microscopy. (A–F) Leaves of Col-0 were examined for infection phenotypes at 11 dpi (A, B), hyphal growth at 24 hpi (C, D), or conidiophore formation at 4 dpi (E, F). Yellow arrowheads point to germinated sporelings in (C), and red arrowheads indicate conidiophores in (E). (G and H) Leaves of Col-*gl* expressing RPW8.2^{D116G}–YFP were examined at 4 dpi by confocal microscopy for the formation of haustoria encased by the extra-haustorial membrane labeled by RPW8.2^{D116G}–YFP. Bar=200 μm in (C) and (E); 50 μm in (G). Asterisk indicates significant difference (P<0.01; unpaired Student t-test for B, D, and H or χ^2 test for F) between abaxially inoculated leaves and adaxially inoculated leaves. This experiments in (A–F) were repeated three times with similar results.

does not activate resistance but retains its specific localization to the extra-haustorium membrane encasing the haustorium (Wang *et al.*, 2013). We found that the leaf abaxial side supported far fewer (~16%) haustoria compared with the adaxial side (Fig. 1G, H) at 4 dpi. Taken together, the above observations suggest that abaxial immunity in Col-0 is mostly attributable to post-penetration resistance at an early stage before or around haustorium biogenesis.

EDS1/PAD4-dependent pathway contributes to abaxial immunity

To test the above speculation, we inoculated both leaf sides of plants from a panel of Arabidopsis mutants defective in salicylic acid (SA) signaling and/or SA biosynthesis. They are *eds1-2*, *pad4-1*, and *sid2-2* single; *eds1pad4* double; and *eds1pad4sid2* (*eps*) triple mutants that exhibited various degrees of enhanced susceptibility to powdery mildew isolate *Gc* UCSC1 (Zhang *et al.*, 2018). As shown in Fig. 2A, fungal mass was visible to the naked eye in the leaf abaxial surface of the *eds1-2* and *pad4-1* single mutants, and the e*ds1pad4* double and *eds1pad4sid2* triple mutants, but not from Col-0 and the *sid2-2* single mutant. Results from spore quantification supported the visible infection phenotypes; that is, there was a significant increase of sporulation in the abaxial side of the leaves of all tested mutants except *sid2-2* when compared with that in Col-0 (Fig. 2B). The genetic data indicate that similar to midrib immunity, leaf abaxial immunity is compromised when the EDS1/PAD4-dependent signaling pathway is defective. However, it is necessary to point out that the level of infection in the abaxial side is still significantly lower than that in the adaxial side in all genetic backgrounds tested. Recent studies have shown that the EDS1–PAD4 heterodimer forms a protein complex with the helper NLRs to promote PTI (basal resistance) and ETI (Wu *et al.*, 2019; Pruitt *et al.*, 2021; Sun *et al.*, 2021; Huang *et al.*, 2022). We thus also tested if ADR1s also contribute to abaxial immunity. We found that like the *eds1-2* and *pad4* mutants (Fig. 2A), abaxial immunity was also similarly compromised in the *adr1* triple and *adr1nrg1* sextuple mutants (Supplementary Fig. S4). Collectively, our genetic data described above indicate that the EDS1–PAD4–ADR1-mediated post-penetration resistance can partially explain leaf abaxial immunity against powdery mildew in Arabidopsis.

The glucosinolate metabolism pathway also contributes to abaxial immunity

To search for other defense pathways that may also contribute to leaf abaxial immunity, we examined the infection phenotypes of both leaf surfaces in two additional Arabidopsis mutants *dde2-2* and *ein2-1* that are defective in jasmonic acid (JA) biosynthesis (von Malek *et al.*, 2002) and ethylene signaling (Guzman and Ecker, 1990; Alonso *et al.*, 1999), respectively. We found that leaf abaxial immunity is not compromised in these two mutants (Supplementary Fig. S5), indicating that the JA- or ET-dependent defense mechanism makes no or an insignificant contribution to leaf abaxial immunity.

Previous studies have revealed two major pathways for cell wall-based resistance against non-adapted powdery mildew in Arabidopsis. One is the secretory membrane trafficking pathway regulated by the PEN1 (SYP121) syntaxin, which is evidenced by the enhanced cell wall penetration of non-adapted barley powdery mildew in the *pen1-1* mutant (Collins *et al.*, 2003). The other pathway is defined by PEN2 (an atypical myrosinase) (Lipka *et al.*, 2005; Sanchez-Vallet *et al.*, 2010) and PEN3 (an ATP-binding cassette transporter) (Stein *et al.*, 2006) that are respectively involved in the production and secretion of antimicrobial compounds via tryptophan-derived glucosinolate metabolism (Bednarek *et al.*, 2009; Clay *et al.*, 2009). We inoculated both the abaxial and adaxial leaf surfaces of Col-0, *pen1-1*, *pen2-1*, *pen3-1*, and *pen2pen3* mutants with *Gc* UCSC1 and found that except for *pen1-1*, leaf abaxial immunity was significantly breached in the mutants, as evidenced by visible (albeit a small amount of) fungal mass on the leaf abaxial surface (Fig. 3A) and spore quantification (Fig. 3B). This observation suggests that defense molecules produced via the glucosinolate metabolic pathway also contribute to abaxial immunity while the PEN1-dependent defense mechanism does not or the effect is insignificant.

To evaluate the combined effect of the EDS1/PAD4-dependent and PEN2/PEN3-dependent defense mechanisms on abaxial immunity against powdery mildew, we generated five higher order mutants in the *eds1pad4sid2* (*eps*) background by CRISPR-targeted mutagenesis of *PEN1* (At3g11820), *PEN2* (At44490), and *PEN3* (At1g59870) individually (Supplementary Fig. S6) and combined the mutations in *PEN1*, *PEN2*, and/or *PEN3* by genetic crossing. These higher order mutants are *eps-pen1*, *eps-pen2*, *eps-pen3*, *eps-pen1pen2*, and *eps-pen1pen2pen3*. Infection tests showed that the abaxial side of inoculated leaves of the *eps-pen1* mutant had similar susceptibility to that of *eps* (Fig. 4A), agreeing with the earlier result concerning *pen1-1* (Fig. 3). Interestingly, the abaxial side of inoculated leaves of all the other four mutants appeared fully susceptible to *Gc* UCSC1 with whitish fungal mycelia covering the entire surface similar to what was seen in the adaxial surface (Fig. 4A). However, spore quantification showed that the abaxial side of inoculated leaves of *eps-pen1pen2* and *eps-pen1pen2pen3* supported only a slightly higher level of sporulation than that of *eps-pen2* and *eps-pen3* (Fig. 4B) despite the fact that sporulation in the adaxial surface of the former two higher order mutants was significantly higher than in that of the latter two mutants (Fig. 4B). Notably, the level of sporulation on the abaxial surface was significantly lower compared with that on the adaxial surface in all genetic backgrounds (Fig. 4B). Taken together, our genetic data demonstrate important and additive roles for both EDS1/PAD4-based and PEN2/PEN3-based defense in abaxial immunity against powdery mildew, and further imply the existence of other factors influencing the disease susceptibility of the two leaf epidermal layers.

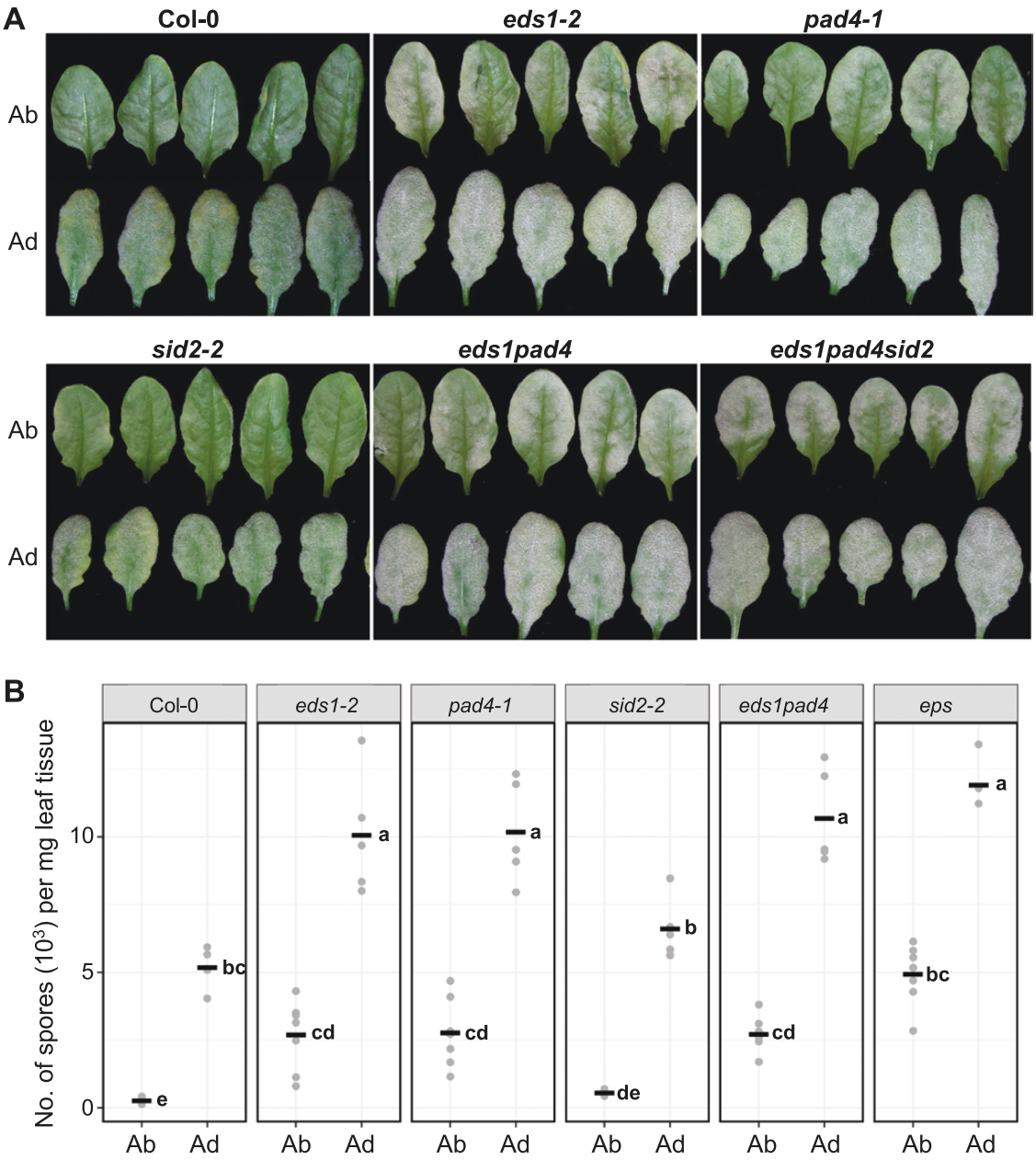

Fig. 2. Abaxial immunity is compromised in *EDS1/PAD4* pathway mutants. Leaves of 8-week-old Col-0 and the indicated mutants were inoculated with *Gc* UCSC1 on either the adaxial side (Ad) or the abaxial side (Ab). (A) Five representative leaves of each genotype were photographed at 11 dpi and are shown. (B) Spore quantitation for the indicated genotypes at 11 dpi. Five to seven leaves were used for spore counting for each leaf side. A gray dot represents spore density of one leaf and a dark bar represents the mean value. Different letters indicate statistically significant differences ($P<0.05$) between different genotypes, as determined by multiple comparisons using one-way ANOVA followed by Tukey's HSD test. This experiment was repeated three times with similar results.

Both EDS1 and PEN2 proteins accumulate at a higher level in abaxial epidermal cells

One may question why the leaf abaxial surface, compared with the susceptible adaxial surface, is highly resistant to *Gc* UCSC1 in Arabidopsis Col-0 wild-type plants where both the EDS1/PAD4-dependent and the PEN2/PEN3-dependent defenses are intact. One possibility is that one or both of these defenses are constitutively more active in the abaxial epidermal cells. To test this speculation, we obtained an *eds1-2*/Col-0 line in which the loss of the endogenous *EDS1* is complemented by *EDS1-YFP* expressed from the *EDS1* promoter (Garcia *et al.*, 2010) and a *pen2-1*/Col-0 line in which the loss of the endogenous *PEN2* is complemented by *PEN2-GFP* driven by the *PEN2* promoter (Fuchs *et al.*, 2016). We assessed the expression

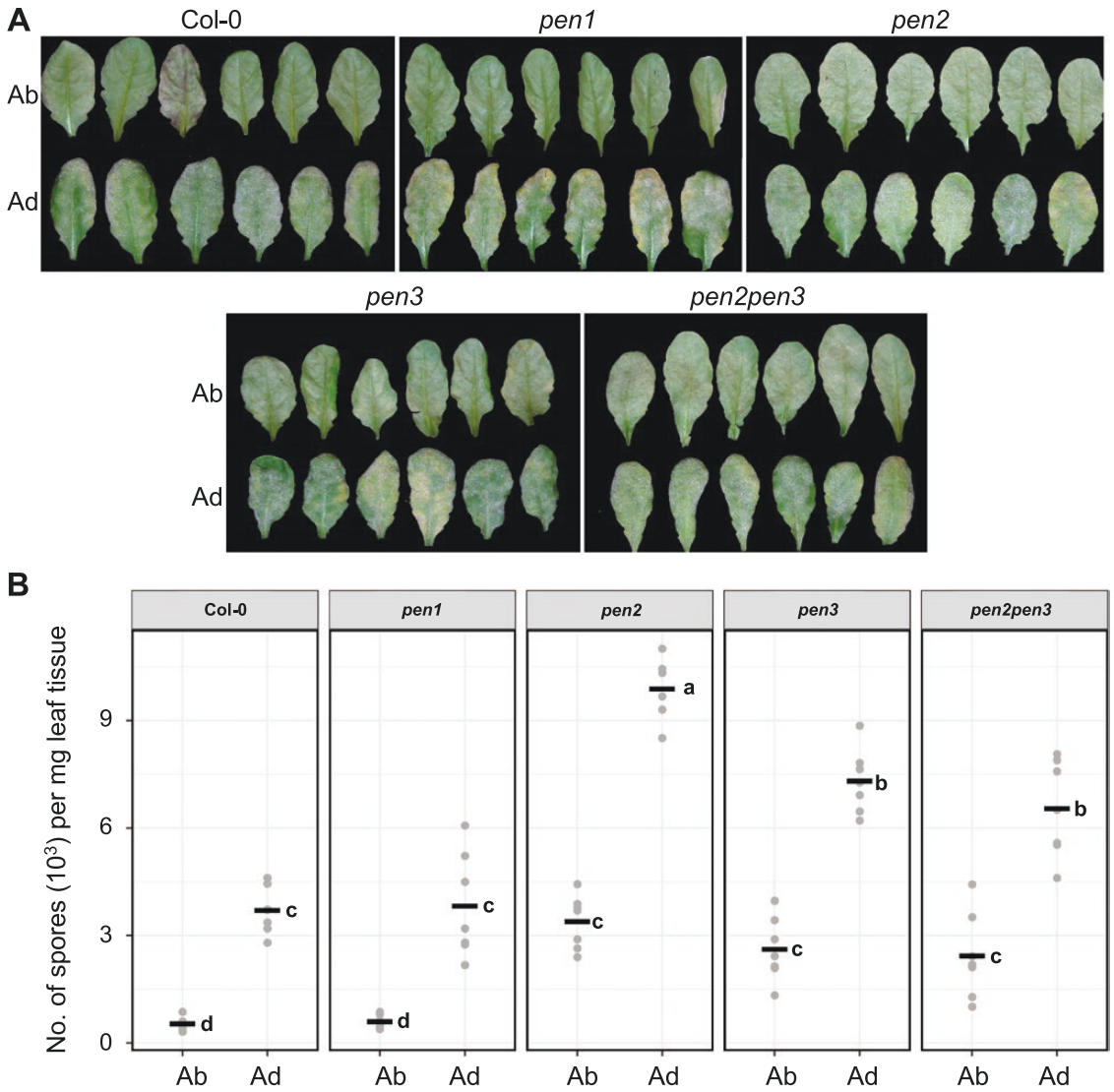

Fig. 3. Abaxial immunity is compromised in mutants defective in glucosinolate metabolism. Leaves of 8-week-old Col-0 and the indicated mutants were inoculated with *Gc* UCSC1 on either the adaxial side or the abaxial side. (A) Six representative leaves of each genotype were photographed at 9 dpi and are shown. (B) Spore quantitation for the indicated genotypes at 9 dpi. Five to seven leaves were used for spore counting for each leaf side. A gray dot represents spore density of one leaf and a dark bar represents the mean value. Different letters indicate statistical differences ($P<0.05$) between different genotypes, as determined by multiple comparisons using one-way ANOVA followed by Tukey's HSD test. This experiment was repeated three times with similar results.

levels of EDS1–YFP and PEN2–GFP in both sides of the same mature, fully expanded leaves of 8-week-old uninfected plants by confocal microscopy. As shown in Fig. 5A and B, EDS1–YFP signal was observed in the nucleus, the plasma membrane, and the cytoplasm of epidermal cells in both sides, and the YFP signal intensity in the abaxial epidermal cells was significantly higher than that in the adaxial epidermal cells. Likewise, PEN2–GFP signal observed in the plasma membrane and cytoplasm (as puncta) of abaxial epidermal cells was significantly higher than that in the adaxial epidermal cells (Fig. 5C, D). These observations suggest that compared with the adaxial epidermal cells, the abaxial epidermal cells of Arabidopsis leaves may possess stronger constitutive EDS1-dependent and PEN2-dependent defenses, resulting in enhanced abaxial immunity to an adapted powdery mildew pathogen.

Abaxial immunity varies in other plant species

Our laboratory maintains seven different powdery mildew species in their respective host plants, permitting us to investigate whether abaxial immunity exists in other plant species. Interestingly, we found that strong leaf abaxial immunity as seen in Arabidopsis was only observed in hemp (*Cannabis sativa*) (Fig. 6G), while the leaf abaxial surfaces of tomato, tobacco,

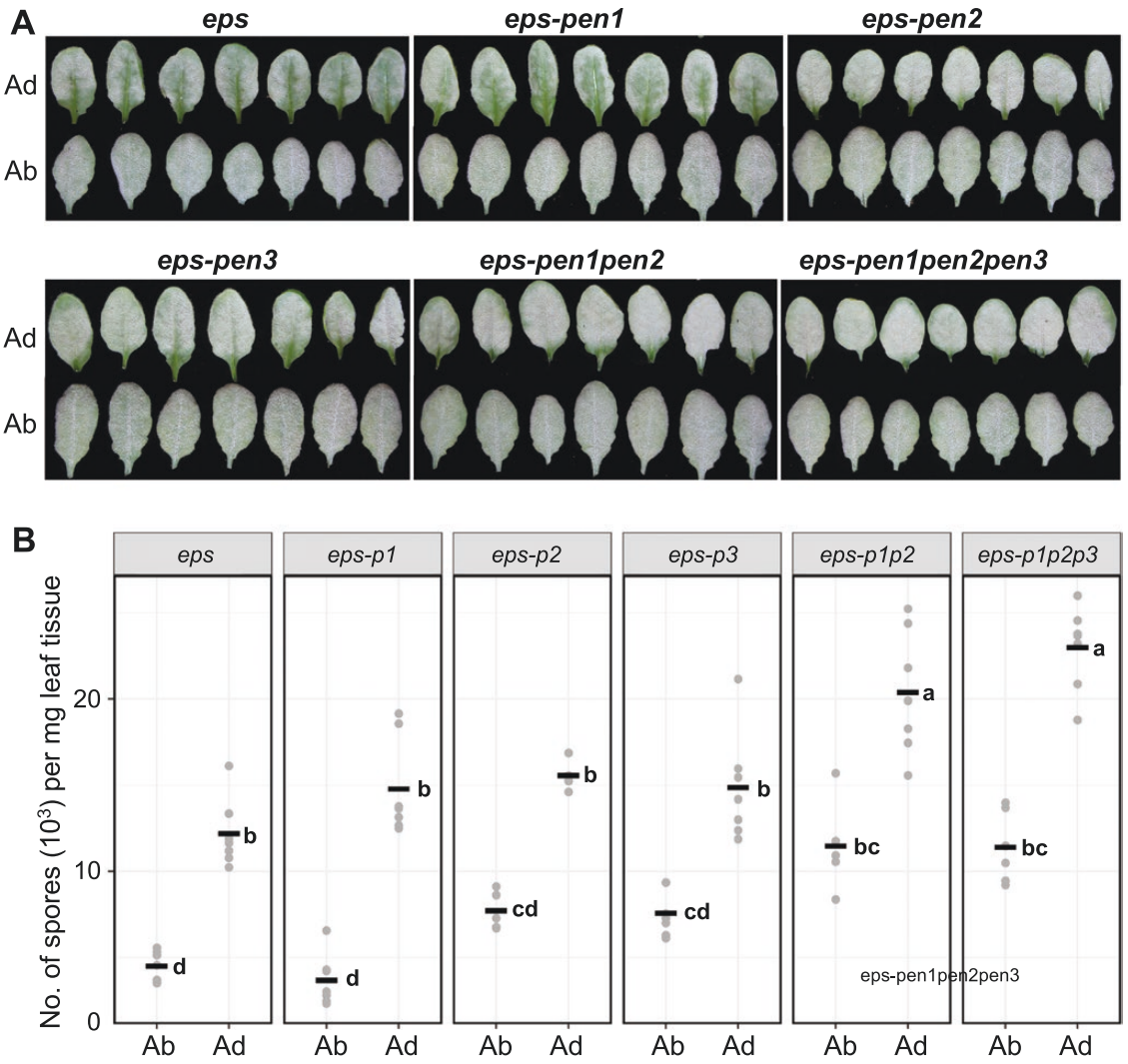

Fig. 4. Abaxial immunity is further compromised when both EDS1/PAD4- and the glucosinolate metabolic pathway are impaired. Leaves of 8-week-old *eps* and the indicated mutants were inoculated with *Gc* UCSC1 on either the adaxial side or the abaxial side. (A) Seven representative leaves of each genotype were photographed at 11 dpi. (B) Spore quantitation for the indicated genotypes at 11 dpi. Seven leaves were used for spore counting for each leaf side. A gray dot represents spore density of one leaf and a dark bar represents the mean value. Different letters indicate statistical differences ($P<0.05$) between different genotypes, as determined by multiple comparisons using one-way ANOVA followed by Tukey's HSD test. This experiment was repeated three times with similar results.

sow thistle, strawberry, squash, and barley all supported clearly visible and, in some cases, significant sporulation of their respective adapted powdery mildew fungi, despite the fact that they were not as susceptible as their respective adaxial layers except in the case of tomato (Fig. 6A–F). These observations suggest that different plant species may have different spatial configurations for the expression of defense genes in the epidermal cells of both sides of their leaves.

Discussion

Plant–microbe interactions are intrinsically spatiotemporal in most, if not all, cases because microbes are small organisms that only encounter and dwell in their host plants locally at a given time. Conceivably, long-term co-evolution between a host and a pathogen under such a context must have led to organ/tissue-specific adaptation of the pathogen and corresponding spatiotemporal resistance mechanisms in the host. For the latter, differential transcriptional programming of defense pathways due to or along with interconnected developmental programs in different organs/tissues of plants over time probably underlies age-related and organ/tissue-specific disease resistance or susceptibility to pathogens (Hu and Yang, 2019; Lacaze and Joly, 2020).

In this study, we investigated the genetic basis of abaxial immunity against an adapted powdery mildew pathogen in Arabidopsis. We found that Arabidopsis abaxial immunity is at

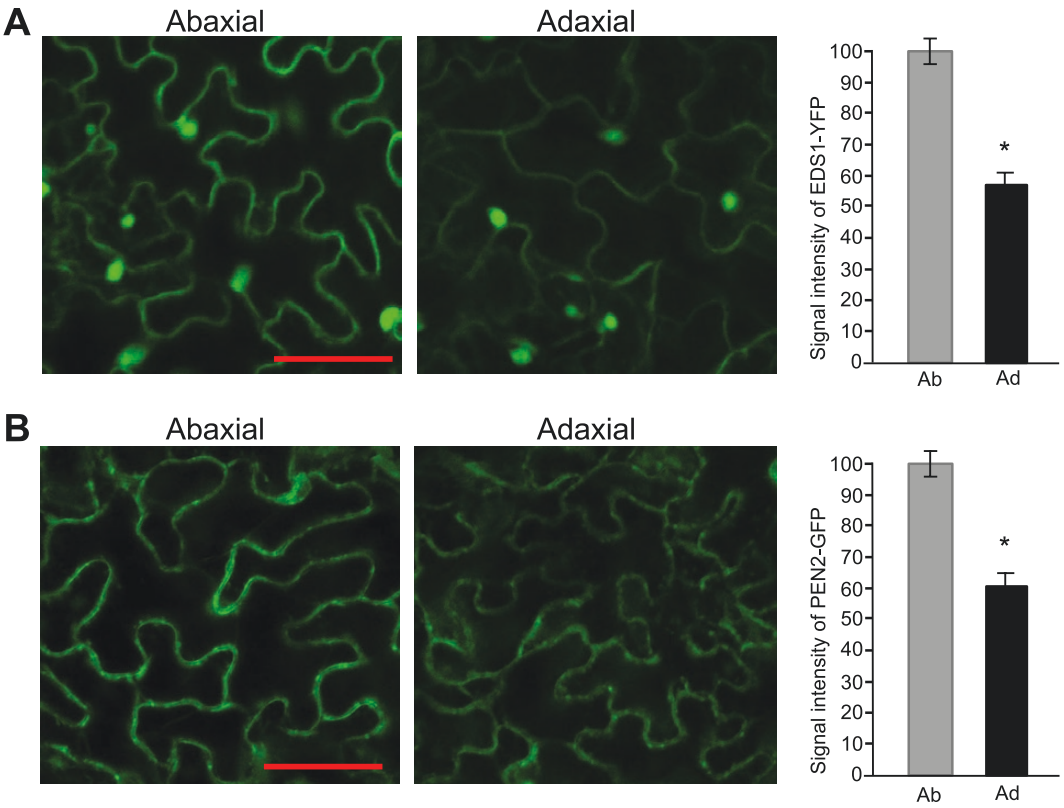

Fig. 5. Both EDS1 and PEN2 are more highly expressed in abaxial epidermal cells compared with adaxial epidermal cells. Mature leaves of 7-week-old *eds1-2* plants expressing EDS1–YFP from the *EDS1* promoter and those of 7-week-old plants expressing PEN2–GFP from the *PEN2* promoter were cut into two halves from the midrib and used for confocal microscopy with the same imaging setting. YFP or GFP signal intensity across the plasma membrane region of 30 epidermal cells from five leaves for each genotype was quantified by Image J. (A) Representative confocal images showing EDS1–YFP expression and quantification of relative YFP signal intensity in abaxial (Ab) or adaxial (Ad) epidermal cells. (B) Representative confocal images showing PEN2–GFP expression and quantification of relative GFP signal intensity in abaxial (Ab) or adaxial (Ad) epidermal cells. Asterisks indicates significant differences (P<0.01; unpaired Student t-test) between the two leaf sides. Bar=50 μm.

least partially attributable to two distinct defense mechanisms (i.e. EDS1/PAD4-dependent and PEN2/PEN3-dependent) that may be programmed to be more active in the abaxial epidermal cells compared with the adaxial epidermal cells. It is worth noting that we observed increased protein accumulation of EDS1–GFP and GFP–PEN2 in epidermal cells of both leaf surfaces infected by *Gc* UCSC1 as expected (not shown). However, it is difficult to determine if the enhanced expression is higher in the invaded abaxial epidermal cells than in the invaded adaxial epidermal cells. While similar abaxial immunity may exist in other plant species such as hemp (Fig. 6G) or ryegrass (Carver *et al.*, 1990), six out of seven other plant species tested in this study do not show strong abaxial immunity against powdery mildew fungi.

Conceivably, there are multiple factors influencing the infection phenotypes of the two sides of a host leaf when challenged by a pathogen. First, the two leaf surfaces may be anatomically very different in the presence/absence of specialized cells such as trichomes (leaf hairs) and density of stomata. For example, trichomes on the adaxial surface may facilitate the adhesion of the fungal spores/hyphae, and genetic mutation reducing trichome number in the adaxial leaf surface of Arabidopsis plants results in increased tolerance against the necrotrophic fungus *Botrytis cinerea* (Calo *et al.*, 2006). A study of 98 bread wheat genotypes showed that the higher density of trichomes on the adaxial surface of flag leaves entrapped pathogen spores and prevented direct contact between the spores and the leaf epidermis, and hence was negatively associated with the development of the spot blotch disease caused by *Bipolaris sorokiniana* (Gupt *et al.*, 2021). In the same study, the authors showed that stomatal density was positively associated with the development of the disease, which is expected since the fungus enters leaf tissues via stomata (Gupt *et al.*, 2021). In this study, we tested leaves of an Arabidopsis mutant line Col-*gl* that does not have trichomes in the leaf adaxial side with *Gc* UCSC1. However, we found no significant difference in disease susceptibility in the adaxial surface compared with that in Col-0 (data not shown). Also, the higher stomatal density on the leaf abaxial surface of Col-0 plants (Hetherington and Woodward, 2003) apparently cannot explain the abaxial immunity against powdery mildew fungi.

Beside the anatomical difference, the two leaf surfaces may also be different in chemical composition of their epicuticular

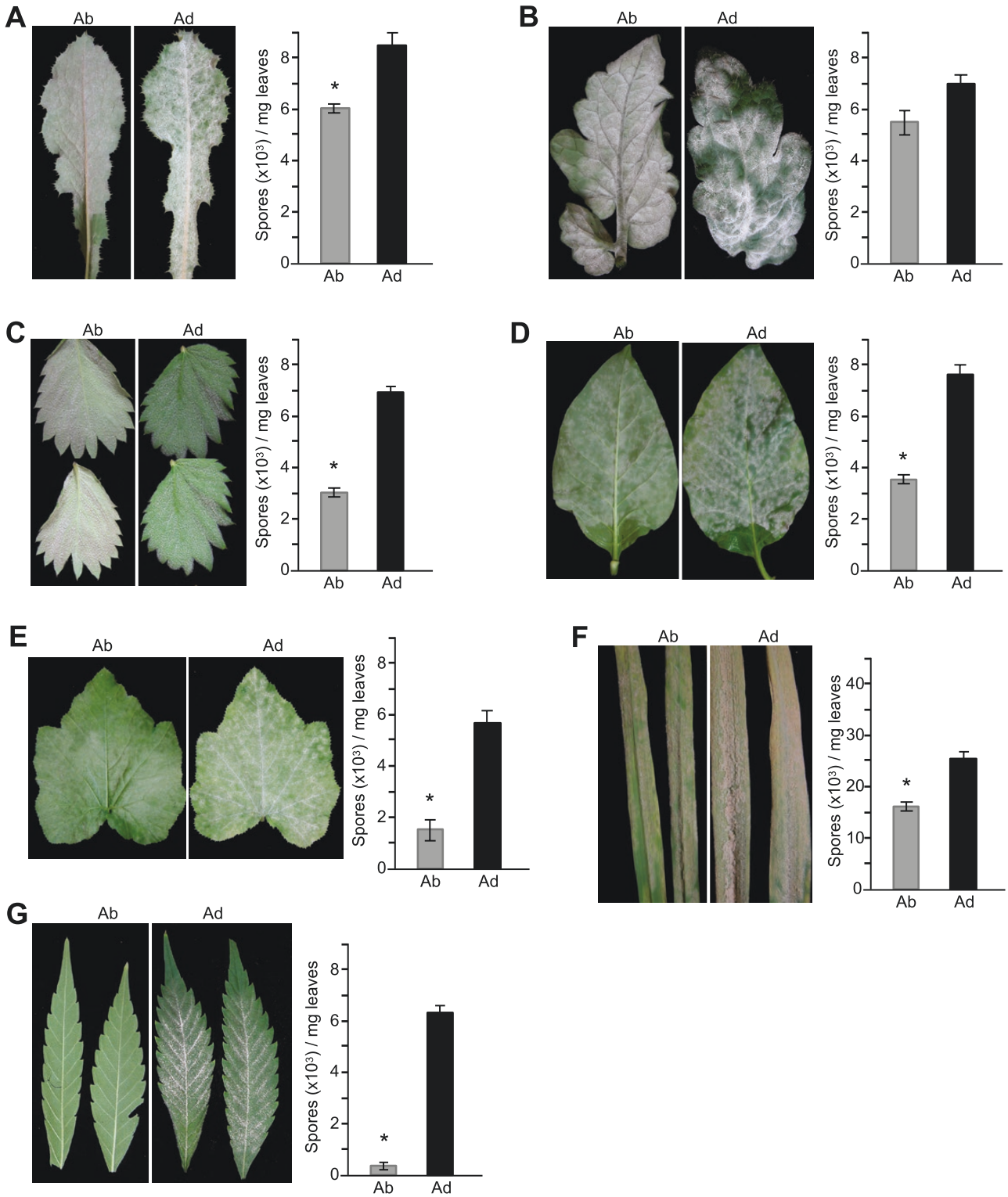

Fig. 6. Powdery mildew infection phenotypes of both leaf sides in seven other plant species. Fully expanded leaves of mature plants of the indicated species were inoculated on the adaxial (Ad) or abaxial (Ab) surface with their respective adapted powdery mildew isolates. Infected leaves were photographed at ~10 dpi, and disease susceptibility was determined by quantification of spores per mg of leaf tissue. (A) Sow thistle (*Sonchus oleraceus*) infected by *G. cichoracearum* UMSG1. (B) Tomato (*Solanum lycopersicum*, Moneymaker) infected with *Oidium neolycopersici* UMSG2. (C) Diploid strawberry (*F. iinumae*) infected with *Podosphaera aphanis* UMSG4. (D) Tobacco (*Nicotiana tobacum*) infected with *G. cichoracearum* UMSG3. (E) Squash (*Cucurbita pepo*, Black beauty) infected with *G. cichoracearum* UCSC1. (F) Barley (*Hordeum vulgare*) infected with *Blumeria graminis hordei*. (G) Hemp (*Cannabis sativa*) infected with *G. ambrosiae* UMSG5. Asterisks indicates significant differences ($P<0.01$; unpaired Student *t*-test) between the two leaf sides.

waxes that influences resistance or susceptibility to a pathogen. For example, leaf abaxial immunity against powdery mildew was also observed in ryegrass (*Lolium* spp.) (Carver *et al.*, 1990). Interestingly, the epicuticular waxes on the abaxial side of ryegrass differ from those on the adaxial side, and removal of the waxes from the abaxial side resulted in improved differentiation of the fungal structures (Carver *et al.*, 1990). This observation suggested that the epicuticular wax layer on the abaxial surface contributes to resistance against powdery mildew in ryegrass (Carver *et al.*, 1990). However, a follow-up study showed that the C-26-aldehyde *n*-hexacosanal, the major compound that induces powdery mildew spore germination and differentiation in the adaxial epicuticular waxes, is absent from the abaxial epicuticular waxes, and supplying a synthetic *n*-hexacosanal improved germination and differentiation rates of the powdery mildew fungus on wax-coated glass slides (Ringelmann *et al.*, 2009). More convincing genetic evidence supporting a role for epicuticular wax in fungal pathogenesis was provided by Uppalapati *et al,* (2012) who found that loss of abaxial epicuticular wax in *Medicago truncatula* due to the impairment of a Cys(2)His(2) zinc finger transcription factor, PALM1, resulted in reduced spore differentiation of anthracnose and non-host rust pathogens (Uppalapati *et al.*, 2012). A further study in barley also suggested a conserved role for leaf epicuticular wax as a 'plant' signal to be sensed by powdery mildew spores for germination and differentiation (Weidenbach *et al.*, 2014). Given that *Gc* UCSC1 spores can germinate and differentiate hyphae on the abaxial surface as on the adaxial surface (Fig. 1C; Supplementary Fig. S2B), the differences in late hyphal development and sporulation in the two leaf surfaces is unlikely to be due to compositional differences in their respective epicuticular waxes.

Additionally, differences in micro-environmental conditions which the two leaf surfaces encounter may also influence their infection phenotypes. Local relative humidity in the leaf abaxial side is often higher compared with that in the adaxial side, which may facilitate development of downy mildew caused by oomycete pathogens (Purayannur *et al.*, 2021; Nirwan *et al.*, 2023). Differences in temperature and light intensity between the two leaf surfaces may conceivably also affect the severity of diseases caused by bacterial and fungal pathogens, but there are no reports that demonstrate causal relationships. Deployment of wearable micro-leaf sensors can help conduct correlative studies to assess the impact of microenvironmental conditions on disease development in the laboratory or field (Lee *et al.*, 2023). In our study, because we flipped those leaves and fixed their position to examine abaxial infection (Supplementary Fig. S2A), there should be no or little difference in micro-environmental conditions between the inoculated abaxial and adaxial surfaces.

Therefore, the above-discussed factors do not seem to explain the strong abaxial immunity we observed in Arabidopsis. Instead, the genetic evidence we obtained in this study strongly suggests that the relatively higher constitutive expression of both EDS1/PAD4-based and PEN2/PEN3-based defense mechanisms in the abaxial epidermal cells (Fig. 5) at least partially explains the abaxial immunity to powdery mildew fungi. Impairment of both defense pathways in Col-0 not only severely compromised its abaxial immunity, but also leads to enhanced disease susceptibility in the adaxial side to the well-adapted powdery mildew isolate *Gc* UCSC1 (Figs 2–4). Interestingly, along with the diminishment of abaxial immunity, midrib immunity in the adaxial side is also abolished in these higher order mutant backgrounds (Fig. 4; Supplementary Fig. S1), suggesting that the immunity observed in the midrib of adaxial epidermal cells is probably also attributable to the heightened EDS1/PAD4- and PEN2/PEN3-based defenses relative to those in the peripheral adaxial epidermal cells. Our genetic data also support the notion that PEN2 (hence the glucosinolate pathway) is involved in both penetration resistance and post-penetration resistance against a broad range of non-adapted (Lipka *et al.*, 2005; Nakao *et al.*, 2011; Okawa *et al.*, 2013) and adapted pathogens (Johansson *et al.*, 2014; Frerigmann *et al.*, 2016; Xu *et al.*, 2016) (this study; Figs 3, 4). We also noticed that compared with *pen2*, the *pen3* single and *pen2pen3* double mutants supported significantly less sporulation in the leaf abaxial surface (Fig. 3B). This may be attributable to more leaf chlorosis (which can limit powdery mildew proliferation) in *pen3* and *pen2pen3* mutants (Fig. 3A) probably due to overaccumulation of PEN2-dependent (in *pen3*) and PEN2-indepndent (in *pen2pen3*) antimicrobial compounds as substrate of the PEN3 ABC transporter within the infected cells at a late infection stage (i.e. 9 dpi). It is also necessary to point out that despite the full coverage of *Gc* UCSC1 mycelia on the abaxial surface of the most susceptible quintuple (*eps-pen1pen2*) or sextuple (*eps-pen1pen2pen3*) mutants, the level of fungal sporulation in the abaxial side of these mutants was still significantly lower than that in the adaxial side (Fig. 4). This difference may be due to the presence of residual defense independent of EDS1/PAD4- and PEN2/PEN3-based mechanisms in the abaxial epidermal cells. Currently, it is not known why the immunity levels in abaxial and adaxial midrib epidermal cells are pre-programmed to be higher than that in the adaxial peripheral epidermal cells. It is possible that, due to structural specificity, abaxial epidermal cells (and perhaps also adaxial midrib epidermal cells) are preferentially targeted by other types of pathogens in plant species such as Arabidopsis and hemp; hence, the heightened abaxial and/or midrib immunity could be a result of a co-evolutionary adaptation in these plants. Finally, given that the ultimate goal of the fungus is simply to extract nutrients from host cells for its reproduction and our earlier observation that overexpression of a host sugar transporter in Arabidopsis resulted in enhanced disease susceptibility to *Gc* UCSC1 (Liu *et al.*, 2021), the lack of sporulation on the abaxial surface of Col-0 or relatively lower fungal sporulation on the abaxial surface of higher order mutants examined may also be partially attributable to a possible lower sugar level in abaxial epidermal cells relative to that in adaxial epidermal cells.

In conclusion, our work in this study has revealed the main (epi)genetic basis of leaf abaxial immunity to powdery mildew in Arabidopsis; that is, higher constitutive expression of both the PEN2/PEN3- and EDS1/PAD4-dependent defenses renders the abaxial epidermal cells more resistant to powdery mildew. Apparently, this spatial immunity is one of the manifestations of the intrinsic leaf abaxial–adaxial polarity formed during leaf development in Arabidopsis (Jun *et al.*, 2010). Similar mechanisms may also explain abaxial immunity and other tissue-/organ-specific resistance against powdery mildew in other plant species. Our results also suggest that simultaneously augmenting the PEN2/PE3- and EDS1/PAD4-dependent defense mechanisms may create effective resistance to powdery mildew fungi in crop plants.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. Primers used for CRISPR-targeted mutagenesis of *PEN1*, *PEN2*, and *PEN3*.

Fig. S1. Leaf midrib immunity against powdery mildew in Col-0 is abolished in *eds1-2* and *eds1-2pad4-1* mutants.

Fig. S2. The abaxial leaf surface supports normal powdery mildew spore germination.

Fig. S3. Abaxial immunity in two additional Arabidopsis accessions susceptible to *Gc* UCSC1.

Fig. S4. Abaxial immunity is also compromised in the *adr1*-triple mutant and the *adr1nrg1* sextuple mutant.

Fig. S5. Abaxial immunity is not affected in JA/ET pathway mutants *dde2-2* and *ein2-1*.

Fig. S6. CRISPR-targeted mutagenesis of *PEN1*, *PEN2*, and *PEN3* in the *eds1pad4sid2* mutant background.

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Author contributions

Ying W, CW, and SX: conceptualization and design; Ying W, WKS, QZ, DB, Yan W, CH, FC, AV, and SX: helping maintain the powdery mildew isolates and/or collecting the data; Ying W, WKS, and SX: data analysis and writing the manuscript.

Conflict of interest

No conflict of interest declared.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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