Epidermal chloroplasts are defense-related motile organelles equipped with plant immune components

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In addition to conspicuous large mesophyll chloroplasts, where most photosynthesis occurs, small epidermal chloroplasts have also been observed in plant leaves. However, the functional significance of this small organelle remains unclear. Here, we present evidence that Arabidopsis epidermal chloroplasts control the entry of fungal pathogens. In entry trials, specialized fungal cells called appressoria triggered dynamic movement of epidermal chloroplasts. This movement is controlled by common regulators of mesophyll chloroplast photo-relocation movement, designated as the epidermal chloroplast response (ECR). The ECR occurs when the PEN2 myrosinase-related higher-layer antifungal system becomes ineffective, and blockage of the distinct steps of the ECR commonly decreases preinvasive nonhost resistance against fungi. Furthermore, immune components were preferentially localized to epidermal chloroplasts, contributing to antifungal nonhost resistance in the pen2 background. Our findings reveal that atypical small chloroplasts act as defense-related motile organelles by specifically positioning immune components in the plant epidermis, which is the first site of contact between the plant and pathogens. Thus, this work deepens our understanding of the functions of epidermal chloroplasts.
The epidermis of multicellular organisms acts as a boundary with the environment to protect against a wide variety of biotic and abiotic stresses. Although the plant epidermis is the site of primary pathogen recognition, it can also be attacked by pathogens, resulting in infection. For instance, the anthracnose fungi *Colletotrichum* species and the rice blast fungus *Magnaporthe oryzae* develop a specialized dome-shaped fungal cell called an appressorium, which is highly pigmented with melanin and is used in direct fungal entry into plant epidermis. The melanized appressorium produces a penetration peg, followed by the extension of the invasive hyphae. However, if the plant is a nonhost, a robust and broad-spectrum defense response termed nonhost resistance (NHR) is developed to effectively block the infection of a vast number of nonadapted pathogens. More specifically, epidermal immune responses are of great importance in terminating the entry of nonadapted fungal pathogens.

PEN2 myrosinase is known as a positive regulator of epidermal NHR in *Arabidopsis thaliana* against multiple fungal pathogens, including *Blumeria graminis* f. sp. *hordei*, *Colletotrichum trichome* (*Ctro*), and *Magnaporthe oryzae* (*Moa*). Together with the ATP binding cassette transporter PEN3, PEN2 regulates the tryptophan-derived secondary metabolite-mediated defense pathway. In addition, EDR1 protein kinase has a positive effect on the preinvasive NHR to nonadapted *Ctro*, whereas it has negative effects on immunity to the adapted fungus *Galvinozymes cichoracearum*. However, pen2 and edr1 mutants retain normal resistance against the nonadapted fungus *Colletotrichum orbiculare* 104-T (*Corb*)7,11. Therefore, multiple immune components, including as-yet unknown factors, support the deployment of epidermal NHR against the entry of nonadapted fungal pathogens in *Arabidopsis*.

In plant immunity, chloroplasts play an important role in the biosynthesis of defense-related molecules. Secondary messengers such as reactive oxygen species (ROS) and calcium (Ca^{2+}) are produced and released by chloroplasts, respectively; precursors of phytohormones such as salicylic acid (SA), jasmonic acid, and abscisic acid are also synthesized in chloroplasts. It has been proposed that immune signals triggered by pathogen-associated molecular patterns (PAMPs) are transmitted from the cell surface to chloroplasts via intracellular Ca^{2+} relay, followed by chloroplast-to-nucleus retrograde ROS signaling in *A. thaliana*. In chloroplast-to-nucleus ROS signaling in *Nicotiana benthamiana* in response to viral and bacterial pathogens, the involvement of tubular extensions from the chloroplast (called stromules) has been proposed.

In higher plants, it has been widely recognized that no chloroplasts exist in epidermal cells other than guard cells, with the exception of some plants (e.g., tobacco). A large population of chloroplasts is highly differentiated in leaf mesophyll cells for efficient photosynthesis, while guard cell chloroplasts regulate stomatal movements in response to CO_{2} and light. However, chlorophyll-containing chloroplasts in the epidermal pavement cells of *A. thaliana* have been recognized, although their size and number are smaller than those of mesophyll cells. The number and thickness of grana and the chlorophyll content of epidermal chloroplasts are also low, leading to the plausible assumption that the photosynthetic contribution of these small organelles is lower than that of mesophyll cells. Hence, the functional significance of small epidermal chloroplasts remains unclear.

Here, we report that epidermal chloroplasts dynamically respond to *Colletotrichum* and *Magnaporthe* fungi that are not adapted to *Arabidopsis*. We showed that epidermal chloroplasts emerge at the upper periclinal wall in response to the entry trial of these nonadapted fungi (hereafter referred to as the epidermal chloroplast response, ECR). The frequency of the ECR increased in the absence of the PEN2-based antifungal defense system, suggesting that the ECR is newly activated when PEN2-related defense becomes ineffective. Our data revealed that CHUP1 and JAC1, regulators of chloroplast photorelocation movement in mesophyll cells, also control the ECR. Importantly, blocking the distinct steps of the ECR via mutation or overexpression of the CHUP1 gene significantly reduced preinvasive NHR against nonadapted fungi in epidermal cells. Furthermore, we found that multiple immune components that are preferentially localized to epidermal chloroplasts contribute to preinvasive NHR in the lower layer of PEN2-related immunity. We propose that atypical small chloroplasts in the epidermis function as defense-related motile organelles involved in *Arabidopsis* NHR.

**Results**

**Epidermal chloroplasts emerged on the upper periclinal surface in response to the entry trial by nonadapted *Colletotrichum* fungi.** To investigate the relationship between epidermal chloroplasts and preinvasive NHR against the appressorium-mediated aggression of fungal pathogens in *A. thaliana*, we used three nonadapted *Colletotrichum* strains: *C. oryzae* CC1 (*Cfio*), *C. siamense* MAF1 (*Csia*), and *Corb*, which are cosmos, apple, and cucurbit anthracnose fungi, respectively. We also used the adapted strain *C. higginsianum* Ahr-1-5 (*Chig*), a brassica anthracnose fungus. *Cfio*, *Csia*, or *Corb* did not form any lesions in wild-type *Arabidopsis* (Col-0), whereas *Chig* developed severe lesions (Fig. 1a). Interestingly, microscopic observation revealed that chlorophyll-containing epidermal chloroplasts emerged on the upper periclinal wall (surface) of pavement cells after inoculation with nonadapted *Colletotrichum* fungi, especially *Cfio* and *Csia* (Fig. 1b, c). The degree of this response varied according to the fungal strain, but the response was detected at 1 day post-inoculation (dpi) and tended to increase until 3 dpi. This phenomenon was also observed in a transgenic plant line expressing the plastid-cyan fluorescent protein (CFP) marker (Supplementary Fig. 1). We named this phenomenon the ECR, because the epidermal chloroplasts are usually positioned at the lower periclinal (bottom) and anticlinal walls in a steady state. The ECR against *Chig* was not clearly detectable, suggesting that the ECR is a specific response to nonadapted fungi (Fig. 1c).

Since *Corb* activated the ECR on Col-0 at a lower level compared with *Cfio* and *Csia*, we hypothesized that some preinvasive defenses in NHR may take priority over the ECR. Consistent with this idea, we found that pen2 plants showed increased ECR against *Cfio*, *Csia*, and *Corb* compared to Col-0 plants (Fig. 1c). This suggests that the ECR occurs preferentially in the absence of PEN2-related immunity. Indeed, pen2 increased epidermal invasion (at 4 dpi) and leaf lesion formation (at 7 dpi) by *Cfio* and *Csia*, indicating compromised NHR in pen2 plants (Fig. 1a, d, e). Quantitative real-time PCR (RT-qPCR) analysis also showed that many defense-related genes were induced only in pen2 at 1 dpi (Supplementary Fig. 2). This suggests the presence of a preinvasive defense system that is activated in the absence of PEN2, further supporting that the PEN2-related pathway is a higher-layer preinvasive defense system against *Colletotrichum* fungi. Remarkably, the impact of pen2 mutation on *Corb*-induced ECR was drastic, while *Corb* was not able to invade pen2 (Fig. 1d, e). Since the ECR is not accompanied by fungal invasion, this may imply that the ECR contributes to preinvasive NHR.

Interestingly, we detected only a slight increase in the frequency of the ECR in *edr1* mutants (Fig. 1c). Indeed, unlike the pen2 mutant, the preinvasive NHR of the *edr1* mutant against appressorium-mediated *Cfio* and *Csia* entry did not decrease, with no induction of defense-related genes (Fig. 1e, Supplementary Fig. 2); this result strengthens the link between the
Fig. 1 The ECR occurs at the lower layer of PEN2-related immunity and is critical for NHR against Colletotrichum fungi. a Pathogenicity of Colletotrichum fungi on Arabidopsis mutants. A conidial suspension of adapted Chig and nonadapted Cfi, Csia, and Corb was inoculated onto leaves of wild-type Arabidopsis (Col-0), pen2-1, and edr1-1 and incubated for 7 days. b ECR after Cfi inoculation. The epidermal surface of the Cfi-inoculated cotyledon of Col-0 was investigated at 3 days post-inoculation (dpi). The chloroplasts were visualized based on chlorophyll autofluorescence. The DIC images were captured by confocal microscopy. DW was used as a control. c The ratio of epidermal cells with surface chloroplasts was investigated at 1, 2, and 3 dpi. A total of 100 cells in contact with the melanized appressorium were observed. N.D.: not determined due to damages of epidermal cell by fungal invasion. d, e Fungal invasion of Arabidopsis epidermis. Cfi, Csia, and Corb were inoculated onto cotyledons of the indicated Arabidopsis. The entry ratio was quantified at 4 dpi. A total of 100 melanized appressoria were investigated. The arrowheads and arrows indicate melanized appressoria and invasive hyphae, respectively. Scale bar, 10 µm. The means and SE were calculated from three independent plants. Means not sharing the same letter are significantly different (P < 0.05, two-way ANOVA with Tukey’s HSD test).
breakdown of higher-layer preinvasive defenses such as the PEN2-related pathway and ECR activation.

The ECR was correlated with fungal secretion activity during the entry trial. We focused on the triggers of the ECR. One candidate trigger is pathogen-derived PAMPs. The immune kinases BAK1, BIK1, and PBL1 function in PAMP-triggered immunity. However, bak1-5 and bki pbl1 mutants showed normal ECR (Supplementary Fig. 3). Thus, we analyzed fungal factors other than PAMPs that may induce the ECR. In *Colletotrichum* fungi, the yeast STE12 gene homolog CST1 and tetraspanin gene PLS1 are involved in appressorium-mediated penetration. The csi3 and pls3 mutants could form melanized appressoria, but were defective in penetration peg formation. In contrast, the sterol glucosyltransferase gene ATG26 and isocitrate lyase gene ICL1 were not involved in penetration peg formation, but were involved in subsequent invasive hyphal development in plants. Therefore, atg26 and icl1 mutants were defective in invasive hyphal development. We first inoculated *pen2* plants with these *Corb* mutants (*pls1* was generated in this study, Supplementary Fig. 4) and found that *atg26* and *icl1* could induce the ECR, while *cst1* and *pls1* could not (Fig. 2a). This suggests that fungal penetration peg formation is essential for triggering the ECR (Fig. 2a). We examined the possibility that plant damage caused by penetration peg formation activated the ECR. The wound-induced endogenous peptide Pep1 and its paralogs are recognized by the damage-associated molecular pattern (DAMP) receptors PEPR1 and PEPR2 in *Arabidopsis*. However, we detected normal ECR in *pepr1 pepr2* mutants (Supplementary Fig. 3). Combined with the fact that BAK1 and BIK1 also function in Pep1 DAMP signaling, Pep1 and its paralogs might be not a cue of ECR.

Next, we examined whether fungal secretions from peg triggered the ECR. We inoculated *pen2* plants with the *Corb* expressing mCherry-labeled effector CoDN3 and found that the effector signal at the penetration pore was stronger than that on Col-0 (Fig. 2b). Similar results were observed on *pen3*, implying the possible involvement of tryptophan-derived secondary metabolites in suppressing fungal effector delivery. A gene disruption mutant of the membrane traffic component SEC22 in *Corb*, Δsec22, showed decreased effector secretion, and the effector signals were abnormally retained and dispersed inside fungal cells when Δsec22 was inoculated on host cucumber. As expected, the focal accumulation of the effector at the penetration pore, located in the center at the bottom of the appressorium, was attenuated in Δsec22 when Δsec22 was inoculated on nonhost *Arabidopsis* (Fig. 2b). Additionally, the effector exhibited abnormal retention inside the appressorium (Fig. 2b). Intriguingly, the ECR significantly correlated with fungal secretion activity at the penetration pore (Fig. 2b, c), whereas the *Corb* Δsec22 mutant induced sufficient papillary callose deposition at the entry trial sites of the fungus; papillary callose deposition is one of the defense responses of the plant against fungal entry trials (Fig. 2d). Since penetration peg-defective *cst1* and *pls1* mutants hardly induced papilla (Fig. 2e), these results imply that *A. thaliana* recognizes pathogen-derived secretions from the peg and activates the ECR. However, there is still a possibility that the slight differences in the degree of fungal progression in the penetration attempt, which is not reflected by papilla formation, affects ECR activation.

The ECR shares common regulators with mesophyll chloroplast photorelocation movement. In mesophyll cells, typical large chloroplasts exhibit photorelocation movement. Under weak light, they move toward the periclinal walls to promote photosynthesis (accumulation response). Under strong light, they migrate to the anticlinal wall to reduce photodamage (avoidance response). CHUP1 generates a chloroplast-actin-based motive force through the polymerization of chloroplast-actin; therefore, CHUP1 is essential for both accumulation and avoidance responses. On the other hand, JAC1 plays a role in the appearance and disappearance of chloroplast-actin filaments and is required for the accumulation response.

To gain new insight into the relationship between these components and the ECR, we examined how the levels of CHUP1 and JAC1 proteins affected the ECR (Fig. 3). We generated transgenic plants overexpressing CHUP1 (CHUP1ox) or JAC1 (HA–JAC1ox) and analyzed them with *chup1* and *jac1* mutants. The growth of these plants was normal, except that HA–JAC1ox exhibited a semi-curly leaf phenotype (Fig. 3a, Supplementary Fig. 5). Intriguingly, CHUP1 overexpression completely suppressed the ECR (Fig. 3b, c). In contrast, the *chup1* mutant constitutively displayed epidermal chloroplasts at the surface with or without fungal inoculation (Fig. 3b, c). An inoculum with high concentration of fungal conidia on the *chup1* mutant did not significantly increase the population of ECR-activated cells, but the number of surface chloroplasts was slightly increased (Fig. 3d). Therefore, the *chup1* mutant showed constitutive positioning of the epidermal chloroplasts at the surface, but the mutant is deficient in ECR that is newly triggered by the inoculation of nonadapted *Colletotrichum* fungi (Fig. 3b–d). Thus, both overexpression and mutation of CHUP1 commonly cause impairments in the intracellular movement of epidermal chloroplasts in the ECR. On the contrary, JAC1 overexpression resulted in a *chup1*-like phenotype, although the *jac1* mutant showed normal ECR (Fig. 3b, c).

Mutations in hydrophilic amino acid residues in CHUP1 (*R4A&S12A&R20A*) cause them to change location from the perichloroplasts to the cytosol. We generated CHUP1–R4A&S12A&R20Aox lines and found that this point-mutated CHUP1 exhibited decreased endogenous CHUP1 proteins, thereby resulting in a *chup1*-like dominant negative phenotype; epidermal chloroplasts were constitutively detected at the surface without fungal inoculation, and this phenotype was correlated with the levels of endogenous CHUP1 proteins (Fig. 3a, Supplementary Fig. 5). In brief, the dominant negative effects of these mutant lines were partial; therefore, the ECR still occurred in response to *Cfio* (Fig. 3e). These results further suggest that CHUP1 negatively regulates the ECR.

Chloroplast photorelocation is mediated by the blue light receptors phototropin 1 (phot1) and 2 (phot2). Since phot2 is involved in both accumulation and avoidance responses, we evaluated the ECR of the phot2 and phot1 phot2 mutants. We found that the ECR occurred normally, except for a slight increase in surface chloroplasts in phot1 phot2 without fungal inoculation (Fig. 3f). These findings suggest that chloroplast photorelocation and the ECR have different stimulus recognition systems, although they might share downstream components.

The ECR contributes to NHR against *Colletotrichum* fungi. To clarify whether the ECR is involved in plant immunity, we generated CHUP1ox and *chup1* plants in the *pen2* mutant background (Fig. 4). The fungal entry rates on these generated plants were higher than those on *pen2*, while the CHUP1ox and *chup1* single mutants retained normal resistance (Fig. 4a). Transgenic *pen2 chup1* lines expressing CHUP1 under its own promoter showed complementation of the ECR and antifungal immunity (Fig. 4b–d). Intriguingly, we found that the *edr1* *pen2* double mutant exhibited an increased entry rate compared with the *pen2* single mutant, suggesting that the effect of *edr1* mutation on
preinvasive NHR became apparent in the pen2 background (Fig. 4a). Moreover, the effects of ECR impairment by CHUP1 overexpression and mutation on preinvasive NHR were greater in edr1 pen2 double mutants than in pen2 single mutants. These results demonstrate that the ECR contributes to preinvasive NHR against Colletotrichum fungi at the lower layer of PEN2-related immunity, while the breakdown of additional EDR1-related preinvasive defense has a greater impact on ECR-mediated immunity. RT-qPCR analysis showed a significant decrease or decreasing trend in the expression of some CfiO-induced genes in pen2 CHUP1ox and pen2 chup1 compared to that in pen2, which suggests the possible involvement of the ECR in regulating these defense-related genes (Fig. 4e).

When nonadapted Colletotrichum fungi invade Arabidopsis, postinvasive NHR responses, including plant cell death, are activated. Therefore, we examined the possible involvement of
the ECR in postinvasive NHR. Trypan blue (TB) staining indicated the induction of cell death in the epidermal pavement and palisade mesophyll (not invaded) cells inoculated with Cfo (Fig. 4f), which is consistent with a previous report. The invasive hyphae were confined to the dead epidermal cell, suggesting that postinvasive defense in ECR-defective plants functioned normally (Fig. 4f).

Several preinvasive NHR components, such as PEN1, PEN2, PEN3, and PEN4/PCS1, are known to localize at pathogen entry trial sites. We also detected PEN2-GFP at the entry trial sites of Cfo, Csa, and Corb (Supplementary Fig. 6). However, epidermal chloroplasts did not show specific accumulation at the entry trial sites (Figs. 1b, 3b, Supplementary Fig. 6); instead, we frequently observed rapidly moving chloroplasts on the upper surface (Supplementary Fig. 7, Supplementary Movie 1). Although epidermal chloroplasts were occasionally observed near the appressorium, the majority of the population in the surface chloroplasts was scattered.

Immune components GSH1, EDS5, and CAS preferentially localize to epidermal chloroplasts and stromules in preinvasive NHR. The immune components GSH1, EDS5, and CAS have been reported to localize to small epidermal chloroplasts and palisade mesophyll (not invaded) cells inoculated with Cfo in tobacco mosaic virus (CaMV) 35S promoters. When only expressed under the CaMV 35S promoter, mesophyll chloroplasts had weak EDS5-GFP signals, but the chloroplast periphery-specific pattern was maintained in two types of chloroplasts. CAS-GFP driven by the CaMV 35S promoter was detected in both epidermal and mesophyll chloroplasts, with no difference in their localization patterns. The localization of GSH1-GFP driven by its own promoter in the mesophyll has yet to be confirmed. GSH1 y-glutamylcysteine synthetase contributes to NHR against Ctro via glutathione biosynthesis. EDS5 is a MATE family transporter that is required for the transport of the SA precursor outside the chloroplasts. CAS is a Ca2+-sensing receptor involved in transient Ca2+ signaling in chloroplasts during plant immunity.

To analyze the subcellular localization of these components in ECR-activated cells, we used gsh1 plants expressing GSH1-GFP and generated transgenic Col-0 plants expressing EDS5-superfolder GFP (sfGFP) or CAS-sfGFP under the control of the CaMV 35S promoter. In a steady state, these three components localized to the small epidermal chloroplasts positioned at the bottom of the epidermal cells (Fig. 5 upper), which is consistent with previous reports. At the surface area, we could detect fluorescent signals of these components only on the guard cell chloroplasts. Importantly, after Cfo inoculation, GSH1, EDS5, and CAS localized to ECR-activated surface chloroplasts (Fig. 5 lower). With or without Cfo inoculation, all the fluorescent signals of these components in small epidermal chloroplasts were much stronger than those in the large mesophyll chloroplasts, while CAS-sfGFP was also detected in the epidermal plastid with no chlorophyll (Fig. 5). These localization patterns are different from those of CHUP1, which localizes to both epidermal and mesophyll chloroplasts. Z-stack and sectional images clearly showed preferential localization of GSH1, EDS5, and CAS to the small epidermal chloroplasts (Supplementary Fig. 8, Supplementary Movies 2–7). Altogether, we concluded that the immune components GSH1, EDS5, and CAS preferentially localized to small epidermal chloroplasts in preinvasive NHR.

To further strengthen the link between the ECR and epidermal chloroplast-localized GSH1, EDS5, and CAS, we generated fluorescent marker plant lines of these immune components in the CHUP1ox and chup1 backgrounds (Fig. 6). We found that their localization patterns were strongly correlated with epidermal chloroplasts and the ECR (Figs. 5 and 6, Supplementary Movie 8). These observations suggested that the ECR changes the intracellular locations of the immune components GSH1, EDS5, and CAS.

We also detected fluorescent signals of GSH1-GFP, EDS5-sfGFP, and CAS-sfGFP in stromules (Figs. 5 and 6a), where the plastid-CFP marker was observed, but chlorophyll autofluorescence was not (Supplementary Fig. 1). In the immune response of N. benthamiana, induced stomata are proposed to be a chloroplast-to-nucleus route for ROS signaling and the transport of NRIP1, which is a protein required for the recognition of the tobacco mosaic virus effector p50. N. benthamiana NRIP1, which is exogenously expressed in A. thaliana, also localizes to stromules. Therefore, the stromules possibly link to the regulation of the localization of Arabidopsis immune-related proteins via ECR.

GSH1, EDS5, and CAS contribute to preinvasive NHR against Colletotrichum fungi. To evaluate the contribution of GSH1, EDS5, and CAS to preinvasive NHR against Colletotrichum fungi in epidermal cells, we quantified the entry rates of Cfo, Csa, and Corb. Hiruna et al. reported that gsh1 mutants showed a substantial decrease in preinvasive defense against appressorium-independent atypical entry of Ctro. On the other hand, the gsh1 mutant showed a slight reduction in epidermal NHR against appressorium-mediated Cfo entry, and the effect was weaker than that in pen2. Meanwhile, Csa and Corb barely invaded the gsh1 mutant (Fig. 7a). The eds5 and cas mutants retained normal resistance against these Colletotrichum fungi (Fig. 7a). Furthermore, the gsh1, eds5, and cas mutants exhibited normal ECR (Supplementary Fig. 9). Remarkably, these three mutations significantly reduced the preinvasive NHR against Cfo and Csa in the pen2 background (Fig. 7a). In particular, GSH1 was of paramount importance for preventing fungal entry in the pen2 background. These results demonstrated the contribution of these components to preinvasive NHR and the priority of PEN2-related defense against Colletotrichum fungi.

The Cfo-induced expression of the defense-related genes PR1 and FRK1 in pen2 gsh1, pen2 eds5, and pen2 cas double mutants
showed a greater decrease compared to that in pen2, suggesting the involvement of the epidermal chloroplast-localized proteins GSH1, EDS5, and CAS in the expression of these genes (Fig. 7b). It was reported that PR1 and FRK1 expression is regulated by the SA signaling pathway55,56. Therefore, our data on PR1 and FRK1 expression are consistent with the fact that EDS5 and CAS play a role in SA accumulation17,52,53,57. We also found that a mutation of SID2 involved in SA biosynthesis increased Colletotrichum entry into the epidermis in the pen2 background (Supplementary Fig. 10). Interestingly, Rekhter et al. previously provided images...
Fig. 3 CHUP1 and JAC1 regulate the ECR in a phototropin-independent manner. a Levels of CHUP1 and HA-JAC1 proteins in the indicated Arabidopsis. Protein extracts were analyzed by immunoblot with anti-CHUP1 and anti-HA antibodies. CBB staining was used as a loading control. b, e Effects of CHUP1 and JAC1 on Cfo-induced ECR. Cfo was inoculated onto cotyledons of CHUP1ox and jac1, and the surface of the epidermis was observed. DW was spotted onto chup1, CHUP1-R4A&S12A&R20Aox lines, HA-JAC1ox, and jac1 as a control. The epidermal chloroplasts were visualized based on chlorophyll autofluorescence. The DIC images were captured by confocal microscopy at the indicated time point. Scale bar, 10 µm. c–e Quantification of ECR in CHUP1ox, chup1, HA-JAC1ox, jac1, and CHUP1-R4A&S12A&R20Aox plants. Cfo, Cso, and Corb (5 x 10⁵ conidia/mL) were inoculated onto cotyledons of the indicated Arabidopsis and examined at 1, 2, and 3 dpi (c, e). For more fungal inoculum on the chup1 mutant, Cfo (1.5 x 10⁵ conidia/mL) was inoculated (d). A total of 100 cells in contact with the melanized appressorium were observed. As a control, DW was spotted. f Cfo-induced ECR in phot2 and phot1 phot2 mutants. As a control, DW was spotted. The means and SE were calculated from three independent plants. Means not sharing the same letter are significantly different (P < 0.05, two-way ANOVA with Tukey’s HSD test). n.s. not significant.

showing preferential localization of ICS1/SID2 in small epidermal chloroplasts compared to large mesophyll chloroplasts, although they did not refer to this point in the text.⁵³

Notably, the double and triple mutants tested above retained sufficient preinvasive defense against Corb (Figs. 4a, 7a). To clarify the involvement of these components, we systematically generated multiple mutant plants. Remarkably, the preinvasive NHR against Corb was abolished in correlation with the number of mutations; the invasive hyphae were clearly observed (Fig. 7c, left, 7d upper). Along with the higher entry rates of Cfo and Cso in multiple mutant plants (Supplementary Fig. 11), these results suggest that the epidermal chloroplast-related factors EGR, GSH1, EDS5, and CAS are common NHR determinants that contribute to preinvasive immunity against Colletotrichum fungi in Arabidopsis. On the other hand, many plants with multiple mutations showed enhanced susceptibility to Chig, suggesting that these factors also contribute to basal resistance against adapted Colletotrichum fungus (Supplementary Fig. 12).

Our data also suggested that the ECR had somewhat additive effects with GSH1, EDS5, and CAS on NHR against Corb (Fig. 7d upper). For this reason, we speculate that epidermal chloroplasts of Arabidopsis might carry other components engaged in additional immune pathway(s) that are effective against highly incompatible Corb. It is also possible that GSH1, EDS5, and CAS can partially function in preinvasive NHR against Corb in the absence of the ECR. Nevertheless, we still expected the presence of functional redundancy with GSH1, EDS5, and CAS with the ECR: GSH1, EDS5 and CAS partially have overlapping functions with the ECR for preinvasive NHR, because we here revealed that (i) the ECR regulates the intracellular localization of GSH1, EDS5, and CAS, and (ii) both the ECR and these immune components are required for preinvasive NHR against Corb. The ECR might exhibit functional redundancy with GSH1, EDS5, and CAS on NHR against Cfo and Cso; however, their entry rates on many multiple mutants of Arabidopsis reached 80% or higher (Supplementary Fig. 11). To clarify this point, we searched for additional Colletotrichum fungi and found the Cso strain COC4, which was isolated from the hau tree and had low invasion ability into Arabidopsis mutants. Similar to Corb, this Cso (low-invasive strain) induced lower levels of the ECR in Col-0 (Supplementary Fig. 13), but showed a higher entry rate on multiple mutants than Corb (Fig. 7c, d). Importantly, ECR-deficient mutation exhibited redundancy with gsh1, eds5, and cas mutations in the context of preinvasive NHR against the low-invasive Cso strain (e.g., edr1 pen2 gsh1 eds5 cas vs. edr1 pen2 gsh1 eds5 cas chup1). These results indicate a functional link between the ECR and these immune components in NHR (Fig. 7d lower).

The ECR strongly occurs and contributes to NHR against specific nonadapted Colletotrichum fungi in wild-type Arabidopsis. The ECR was strongly induced and contributed to NHR in the pen2 mutant (Figs. 1c and 4), implying that the ECR ensures preinvasive immunity in NHR when higher-layer preinvasive defenses such as PEN2-related pathway are ineffective. To elucidate the role of the ECR in Arabidopsis under natural condition, we searched for nonadapted Colletotrichum fungi with higher invasion ability than Cfo. We found that C. nymphaeae PL1-1-b (Cnym), isolated from a Japanese flowering cherry, developed small visible lesions on wild-type Arabidopsis Col-0 compared to Cfo, but its progression was tightly restricted (Fig. 8a). Thus, Cnym is not adapted to Arabidopsis in comparison to the pathogenicity of adapted Chig, while Cnym could partly invade epidermal cells (Fig. 8a, b). Consistent with the degree of lesion formation in Col-0, the entry rate of Cnym was higher than that of Cfo (Figs. 1a, e and 8a, c). Importantly, Cnym induced the ECR at a high frequency in Col-0, indicating that the ECR strongly occurs against the nonadapted fungus Cnym in wild-type Arabidopsis (Fig. 8d). Defense-related genes were also induced in Col-0 by Cnym inoculation, which contrasts with the results observed after Cfo inoculation (Supplementary Figs. 2 and 14). Cnym-induced ECR was moderately elevated in the pen2 mutant with increased fungal entry (Fig. 8c, d), suggesting that PEN2-related immunity was still partially working against Cnym. Importantly, CHUP1ox and chup1 single mutants exhibited a significant decrease and decreasing trend, respectively, in preinvasive defense against Cnym compared to Col-0; meanwhile, the ECR-normal phot2 mutant showed resistance comparable to that of Col-0 (Fig. 8c, d). Collectively, these results strongly suggest that the ECR is highly activated and contributes to preinvasive NHR, at least against nonadapted fungus Cnym in wild-type Arabidopsis plants.

The ECR and immune components GSH1, EDS5, and CAS are involved in NHR against M. oryzae. To assess the function of the ECR in plant immunity against appressorium-mediated entry of other fungi, we inoculated A. thaliana with nonadapted M. oryzae (Fig. 9). The ECR also occurred in response to the entry trial of M. oryzae, and this response increased in the pen2 mutant, suggesting a similar epidermal response to that observed for Colletotrichum fungi (Fig. 9a, b). As has been previously reported,⁶ M. oryzae invaded the pen2 mutant to a slight degree, whereas the CHUP1ox plant showed comparable resistance to Col-0 (Fig. 9c). Moreover, both pen2 CHUP1ox and pen2 chup1 plants exhibited a higher reduction in preinvasive defense compared to pen2; the pathogen sufficiently developed invasive hyphae through melanized appressorium but was confined in the dead epidermal pavement cell, both of which were clearly visualized by TB staining (Fig. 9b–d). In contrast, the apoplastic bacterial pathogen Pseudomonas syringae, which cannot penetrate the epidermis but enters through natural openings (e.g., stomata) and multiplies in the apoplastic space, did not induce the ECR (Fig. 9e, Supplementary Fig. 15). These findings further support the idea that the trigger for the ECR is specific to the fungal penetration stage. Consistent with this, the susceptibility to P. syringae was not affected in ECR-defective plants (Fig. 9f). We also investigated the effects of gsh1, eds5, and cas mutations on preinvasive NHR.
against M. oryzae and found that M. oryzae showed a greater increase in entry into all the double mutants with pen2 mutation (Fig. 9c). These results suggest that A. thaliana deploys similar immune responses via epidermal chloroplasts to prevent the appressorium-mediated entry of Colletotrichum and Magnaporthe fungi.

Epidermal chloroplasts support the intracellular movement of other organelles in ECR-activated cells. We frequently observed that several chloroplasts clustered around the presumed nucleus at the surface of ECR-activated cells (Figs. 1b, 3b, 5 and 9a). In the mesophyll and epidermal pavement cells of Arabidopsis, the nuclei move together with chloroplasts in a phototropin-
Fig. 4 The ECR contributes to Arabidopsis NHR against Colletotrichum fungi. a Fungal entry rate into the epidermis of ECR-defective Arabidopsis. A total of 100 melanized appressoria were examined at 4 dpi. b CHUP1 protein levels in the indicated plants. Protein extracts were analyzed by immunoblot with anti-CHUP1 antibody. CBB staining was used as a loading control. c ECR of pen2-1 chup1 and CHUP1-complementation lines. A total of 100 cells in contact with the melanized appressorium were examined at 2 dpi. DW was used as a control. d Entry rate of Cfo and Csa into the epidermis of pen2-1 chup1 and CHUP1-complementation lines at 4 dpi. e Induced gene expression of PAD3, CYP79B2, PDF1.2a, PR1, FRK1, and NHK10 by inoculation of Cfo. Cfo was inoculated onto cotyledons of the indicated Arabidopsis. Gene expression was assayed at 24 hpi by RT-qPCR. DW was used as a control. f Epidermal and mesophyll cell death caused by appressorium-mediated entry of Cfo at 4 dpi. The inoculated plants were subjected to TB staining and observed macroscopically (upper) and microscopically using x40 (middle) and x10 (lower) objective lenses. The arrowhead and arrows indicate melanized appressorium and invasive hyphae, respectively. Scale bar, 20 µm. For all quantification analyses except RT-qPCR, the means and SE were calculated from three independent plants. The means and SE of RT-qPCR results were calculated from three independent experiments. Means not sharing the same letter are significantly different (P < 0.05, two-way ANOVA with Tukey’s HSD test).

Fig. 5 Epidermal chloroplasts preferentially position immune components GSH1, EDSS, and CAS. Localization patterns of GSH1, EDSS, and CAS proteins in Arabidopsis. The transgenic gsh1 mutant expressing GSH1-GFP under its own promoter and Col-0 plants expressing EDSS-sfGFP and CAS-sfGFP under the CaMV 35S promoter were incubated with or without Cfo and observed at 2 dpi. Chloroplasts were visualized using chlorophyll auto-fluorescence. The lower left dashed-line square regions show a magnified image of the dashed box. The white arrowheads and arrows indicate melanized appressoria and stromules, respectively. The orange arrow indicates CAS localized in the epidermal plastid without chlorophyll. Scale bar, 10 µm. Similar results were obtained from three independent experiments.

dependent manner. Moreover, perinuclear clustering of chloroplasts, stromule induction, and stromule-mediated chloroplast-to-nucleus ROS signaling in response to viral effectors have been reported in N. benthamiana. To confirm whether nuclei showed an ECR-like response after fungal attack, we simultaneously visualized the nuclei and chloroplasts of Cfo-inoculated Arabidopsis (Fig. 10). DAPI-stained nuclei with several chloroplasts emerged at the epidermal surface in response to Cfo, whereas no nuclei were observed at the epidermal surface without fungal inoculation (Fig. 10a). We also observed CFP-labeled endoplasmic reticulum (ER) around the presumed nuclei with a cluster of chloroplasts in the ECR-activated cells (Fig. 10b). These
results indicate that epidermal chloroplasts and nuclei surrounded by the ER dynamically respond to the fungal entry trial. It has been reported that perinuclear chloroplast movement is required to generate the motive force of nuclear movement in Arabidopsis epidermis. Consistent with this, the nuclei of the CHUP1ox and chup1 plants that exhibited ineffective ECR showed a similar behavior to that of perinuclear chloroplasts (Fig. 10c, d). Since CHUP1 localizes only to the epidermal and mesophyll chloroplast envelopes and not to the nuclear envelope, it is suggested that CHUP1-dependent ECR repression results in the inhibition of nuclear movement to the epidermal surface with Cfo inoculation. These results indicate that epidermal chloroplasts also function as critical factors supporting the movement of other organelles via the ECR during the antifungal defense response.

**Discussion**

Typical large mesophyll chloroplasts are important plant organelles that are responsible for photosynthesis. Guard cell chloroplasts have recently been reported to regulate stomatal movement in response to CO2 and light. However, although small chloroplasts in the epidermal pavement cells of A. thaliana...
have been recognized\textsuperscript{24,25}, their functional significance remains unknown.

In this study, we discovered a dynamic response of epidermal chloroplasts in response to fungal pathogens (Supplementary Fig. 16). More precisely, the response occurred against appressorium-mediated entry trials of nonadapted Colletotrichum and Magnaporthe fungi. We designated this phenomenon as the ECR. The ECR was activated in the lower layer of PEN2-related immunity (Fig. 1c, Supplementary Fig. 13); therefore, it may not be the first layer of preinvasive NHR. We concluded that the ECR is programmed to be activated when higher-layer preinvasive NHR, such as the PEN2-related pathway, is not effective; this is
because a specific nonadapted fungus, which partly overcomes higher-layer preinvasive defense(s), induces the greatest ECR in the wild-type Arabidopsis background (Fig. 8).

The trigger for the ECR remains unknown. We originally considered PAMPs or DAMPs as candidates; indeed, almost all Cfo-induced genes in pen2 plants (Supplementary Fig. 2) are PAMP- or DAMP-inducible. However, the ECR did not depend on the PAMP and DAMP signaling kinases BAK1, BIK1, and PBL1, nor on the DAMP receptors PEPR1 and PEPR2 (Supplementary Fig. 3). Instead, we found that fungal peg formation and secretion activity were strongly correlated with the ECR, suggesting penetration stage-specific pathogen recognition by A. thaliana (Fig. 2). Colletotrichum effectors are secreted from the penetration pore formed at the base of the appressorium. A specific effector conserved at least in Colletotrichum and Magnaporthe is a candidate trigger for the ECR, although the recognition of pathogen effectors usually induces a hypersensitive response. However, if the ECR is a more universal process against many types of fungal pathogens, plant cell wall damage caused by plant cell wall-degrading enzymes (PCWDEs), which are commonly secreted by fungi, or such PCWDEs themselves might trigger the ECR. Therefore, it is possible that unknown PAMP or DAMP signaling independent of BAK1, BIK1, PBL1, PEPR1, and PEPR2 is involved in this process.

Five nonadapted Colletotrichum strains displayed different abilities to trigger the ECR: Cnym induced the strongest ECR, and Cfo induced the ECR more strongly than Csia (Figs. 1c and 8d). Meanwhile, Csia (low-invasive strain) and Corb almost never induced the ECR, in Col-0 (Fig. 1c, Supplementary Fig. 13). All these fungi induced a rather strong ECR in Arabidopsis. Therefore, it is possible that unknown PAMP or DAMP recognition is involved in this process.

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Fig. 9 The ECR contributes to preinvasive NHR against *M. oryzae*. a) ECR after inoculation of *M. oryzae*. *M. oryzae* was inoculated onto cotyledons of pen2-1 plants, and the ECR was investigated at 2 dpi. The chloroplasts were visualized based on chlorophyll autofluorescence. b) Quantification of ECR against *M. oryzae*. ECR was investigated at 1, 2, and 3 dpi. A total of 100 cells in contact with the melanized appressorium were observed. N.D.: not determined due to damages of epidermal cell by fungal invasion. c) Entry rate of *M. oryzae* into epidermis at 3 dpi. A total of 100 melanized appressoria were investigated. d) Epidermal cell death caused by appressorium-mediated entry of *M. oryzae* at 3 dpi. The inoculated plants were subjected to TB staining and observed microscopically using x40 objective lens. The arrowheads and arrows indicate melanized appressoria and invasive hyphae, respectively. Scale bar, 20 µm. The means and SE were calculated from three independent plants. Means not sharing the same letter are significantly different (*P < 0.05, one-way (c) or two-way (b) ANOVA with Tukey’s HSD test). e) Quantification of ECR against *Pseudomonas syringae* pv. *tomato* (Pst) DC3000. Cotyledons of Col-0 were drop-inoculated with Pst DC3000, and ECR was investigated at 1, 2, and 3 dpi. A total of 100 epidermal pavement cells were observed. The means and SE were calculated from three independent plants. f) Growth of Pst DC3000 in Arabidopsis cotyledons. Cotyledons of indicated Arabidopsis were drop-inoculated with Pst DC3000, and incubated for 4 days. The number of bacteria in eight cotyledons obtained from four independent plants was plotted on a log10 scale. The means and SE were calculated from three independent experiments. n.s. not significant (*P < 0.05, one-way (f) or two-way (e) ANOVA with Tukey’s HSD test).
expression of defense-related genes in pen2 but not in Col-0 at 24 h post-inoculation (hpi) (Supplementary Fig. 2). Most of these genes were also not induced in Col-0 at 72 hpi of Cfo, while Cnym moderately induced these genes at 72 hpi (Supplementary Figs. 2 and 14). These results suggest that the threshold at which the ECR occurs in each epidermal cell is lower than that for the induction of defense-related genes. However, the population of ECR-activated cells gradually increased throughout the pathogen-inoculated leaf during incubation with the pathogen, which might activate the expression of defense-related genes (Fig. 1b, c, Supplementary Fig. 2). Thus, the ECR could be a good indicator to evaluate whether fungal pathogens overcome higher-layer preinvasive defense(s) even in a slight degree.

Importantly, we demonstrated that the ECR is an actual preinvasive NHR determinant against fungal pathogens. The ECR was under the control of CHUP1 and JAC1, which are regulators of chloroplast photorelocation in mesophyll cells (Supplementary Fig. 16). The excess or depletion of CHUP1 proteins resulted in impairments of the ECR (Figs. 3, 8d and 9b). Furthermore, these plants showed decreased preinvasive NHR against the entry of nonadapted fungi in the pen2 background (Figs. 4 and 9c). CHUP1 is a critical factor for the ECR and avoidance and accumulation responses in the mesophyll photorelocation system, whereas the ECR and avoidance response of jac1 were near-normal and normal, respectively (Fig. 3). This indicates that the ECR has a regulatory mechanism similar to the mesophyll chloroplast avoidance response. Since only the photorelocation movement of large mesophyll chloroplasts has been well-studied, our findings revealed that another type of chloroplast in the epidermis also moves under fungal attack.

Fig. 10 The nucleus dynamically responds to fungal entry trial in an ECR-dependent manner. a Simultaneous observation of chloroplasts and nuclei in ECR-activated epidermis. The surface of Cfo-inoculated cotyledons of Col-0 was examined at 3 dpi. The chloroplasts and nuclei were visualized by chlorophyll autofluorescence and DAPI staining, respectively. DW was used as a control. The arrows indicate nuclei. b Simultaneous observation of chloroplasts and ER around nuclei in ECR-activated cells. The surface of Cfo-inoculated cotyledons of the transgenic plant line ER-ck expressing ER-CFP was observed at 3 dpi. The arrows indicate perinuclear ER. c Simultaneous observation of chloroplasts and nuclei in ECR-defective plants at 3 dpi. Cfo-inoculated cotyledons of CHUP1ox plants were investigated. DW was spotted onto chup1 mutant as a control. The arrows indicate nuclei. d The ratio of epidermal cells with surface nuclei was investigated at 3 dpi. A total of 100 cells in contact with the melanized appressorium were observed. Scale bar, 10 µm. The means and SE were calculated from three independent plants. Means not sharing the same letter are significantly different (P < 0.05, two-way ANOVA with Tukey’s HSD test).
implies the versatility of the optical response system for plant immunity. We also aimed to explore the mechanism underlying the involvement of the ECR in antifungal NHR. We showed that the immune components GSH1, EDS5, ICS1/SID2, and CAS contributed to preinvasive NHR (Figs. 7 and 9c, Supplementary Figs. 10 and 11). Importantly, these components were preferentially localized to epidermal chloroplasts, regardless of the fungal entry trials (Fig. 5, Supplementary Fig. 8, Supplementary Movies 2–7)53. Although a previous report has shown that CAS-GFP expressed constitutively also localized to mesophyll chloroplasts, the levels of CAS proteins showed large variation50. Therefore, the expression levels of CAS-sfGFP in our experiment might have been lower than those in the previous study. Alternatively, sfGFP, with its more improved folding efficiency and higher fluorescence intensity in plants than other GFP variants66, might make a difference in the localization pattern of CAS protein in our experiment. Remarkably, gsh1, eds5, sid2, and cas mutations exhibited an increased fungal entry rate, especially in the absence of PEN2-related defense (Fig. 7a, Supplementary Fig. 10). Plants with multiple mutations in these components showed more severe breakdown of preinvasive NHR (Fig. 7d, Supplementary Fig. 11). More importantly, we showed that the ECR-deficient mutation acted redundantly with gsh1, eds5, and cas mutations in the preinvasive NHR against the low-invasive Csia strain (Fig. 7d lower). These results suggest that the movement of these immune components via the ECR is a key factor for epidermis-specific antifungal NHR (Supplementary Fig. 16). In contrast, rice SNARE protein OsVAMP7P14 localizes to both mesophyll chloroplasts and the epidermal vacuolar membrane, and mesophyll localization is more important for the resistance against adapted M. oryzae66. Thus, the functional differences between epidermal and mesophyll chloroplasts in plant immunity remain to be elucidated.

The involvement of CHUP1-mediated chloroplast movement to the host–pathogen interface in immunity to the oomycete pathogen Phytophthora infestans was proposed in N. benthamiana67. Thus, partially similar immune mechanisms involving chloroplast movement may exist in A. thaliana and N. benthamiana. In contrast, we occasionally observed epidermal chloroplasts near the appressorium in ECR-activated cells, and the majority of the population in the surface chloroplasts was scattered. This is slightly different from the observations in N. benthamiana67. Moreover, unlike Arabidopsis ECR (Supplementary Fig. 3), P. infestans-induced stromule formation in N. benthamiana depended on BAK1-mediated signaling67, implying the presence of a different pathogen recognition system from Arabidopsis ECR. In addition, we revealed that chloroplast-associated nuclei surrounded by ER also showed an ECR-like response in an ECR-dependent manner (Fig. 10). It is known that many plant organelles, including the nucleus, move to the site of attempted penetration by fungal pathogens, including Colletotrichum fungii68-71. Although we occasionally observed nuclear translocation associated with epidermal chloroplasts near the fungal appressoria in ECR-activated cells, ECR-dependent intracellular nuclear movement may also be related to antifungal NHR. Meanwhile, there is a possibility that the ECR supports the movement of other epidermal immune-related organelles, in addition to the nuclei and ER. The link between chloroplasts and EDRI has not yet been reported. However, EDRI localizes to the nucleus, ER, cytoplasm, and trans-Golgi network/early endosomes11,72,73; therefore, EDRI might be associated with the ECR through the movement of other organelles.

The immune components GSH1, EDS5, and CAS expanded their location from the epidermal chloroplasts to stomata (Figs. 5 and 6, Supplementary Fig. 8, Supplementary Movies 2–7). Since stomata are considered facilitators of ROS signaling and immune protein transport from chloroplasts to the nucleus in N. benthamiana, the same is probably true for Arabidopsis18. Therefore, our data indicate the potential link between immune-related Arabidopsis proteins and stomata. Stomata have also been reported to be associated with other organelles and subcellular structures74. Further studies are required to investigate how stomata contribute to ECR-mediated immunity by transporting many components. Intriguingly, the Arabidopsis chup1 mutant exhibited constitutive stromule formation and an increased hypersensitive response cell death in response to bacterial effectors as a defense response56. Thus, the decreased preinvasive NHR against Colletotrichum fungi caused by chup1 mutation with constitutive stromule formation further supported the importance of the ECR itself for antifungal immunity (Figs. 4 and 8c, d).

Collectively, our findings shed light on the dynamic responses of atypial small chloroplasts in the plant epidermis. We propose that Arabidopsis epidermal cells deploy an ECR-centered dynamic network, wherein the intracellular traffic of small signaling molecules and immune-related NHR may easily occur via the ECR, and possibly stromules and other organelle movements, to support intracellular signaling and hence antifungal NHR. The plant epidermal cell is the primary stronghold that combats environmental pathogens; therefore, another type of chloroplast in the epidermis may have evolved as a defense-related organelle. Future studies on this system in the ECR-activated epithelium will expand our understanding of plant defense measures at the point of contact with fungal pathogens.

Methods

Fungal strains and media. Colletotrichum higginsianum Abr1-5 (MAFF305635), Colletotrichum nymphaeae PL1-1-b (MAFF40037), Colletotrichum fioriniae CC1 (MAFF365530), Colletotrichum siamense MAFI (MAFF243010), COC4 (MAFF243696), and Magnaporthe oryzae AT79-142 (MAFF101520) were obtained from the Ministry of Agriculture, Forestry and Fisheries GenBank, Japan. The wild-type Colletotrichum orbiculare (syn. C. lagenarium) strain 104-T (MAFF240422) was stored at the Laboratory of Plant Pathology, Kyoto University. C. orbiculare transgenic strains, 104-T expressing CoDNS-CoDN3-3Cherry, and Acse22 expressing CoDNS3-CoDN3-3Cherry58, and the disruption mutants, cmt114, atg625, and ndt117, have been described previously. Cultures of all fungal isolates of Colletotrichum orbiculare were maintained on 3% (w/v) Pla (Nippon (Nihon) or BD Difco) at 24°C in the dark. M. oryzae culture was maintained on oatmeal agar medium at 24°C in the dark. For conidiation, C. nymphaeae, C. fioriniae, and M. oryzae were cultured under a 16 h black light/8 h dark cycle.

Plant lines and growth. Arabidopsis thaliana seeds were sown on rockwool (Grodan, Roermond, The Netherlands) and grown at 22°C with 16 h of illumination per day in nutrient medium. A. thaliana accession Col-0 was used as the wild-type, pen2-1, pen2-1 + PEN2: PEN2-GFP, pen3-1, edr1-1, edr1-1 pen2-1, gsh1-1 + GSH1-GFP, pen2-1 gsh1-1, bkt1-5, bkt1-1 pbl1-1 (SALK_052951, SALK_1236_D07), pep1-1 pep2-1 (SALK_059281, SALK_036542) and sid2-2 mutants have been described previously, gsh1-1 (CS3804), chup1 (SALK_129128), jaih (SAL_57_B09), plot1 (SAL_888441), plot2 (SALK_142725), eds1-1 (CS5375) and cas (SALK_070416) mutants, and ptck (CS16265) and ER ck (CS16250) organelle marker lines were obtained from Arabidopsis Biological Resource Center (Columbus, OH, USA).

Plasmid construction. Escherichia coli strain DH5α was used as the host for DNA manipulation. All primers used for plasmid construction are listed in Supplementary Table 1. All plasmids used for generating A. thaliana transgenic lines were derived from pRI101-AN, a vector for protein expression under the control of the cauliflower mosaic virus (CaMV) 35S promoter (3262, TaKaRa Bio, Japan). The CaMV 35S promoter sequence was removed from pRI1-ACHUP1-p1-ACHUP1 to express CHUP1 under its own promoter. For overexpression of CHUP1 protein, a fragment of CHUP1 amplified from the A. thaliana genome was digested with NdeI and SmaI, and introduced into the corresponding site of pRI101-AN, resulting in pRI101-ACHUP1. To generate CHUP1 with point mutations, the fragment of CHUP1::GFP amplified from the plasmid containing the CHUP1 gene with its upstream region, including the promoter sequence, was amplified from the A. thaliana genome, digested with Pstl

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and Smal, and replaced with the corresponding region including the CaMV 35S promoter in pRI101-AN, resulting in pRI-ACHUp1p-AChUp1. For JAC1 protein overexpression, a fragment of 500 bp (upstream of ATG) was amplified with BamHI and EcoRI, and introduced into the corresponding site of pRI101-AN, resulting in pRI101-HA-JAC1. For P35S-CFP, a fragment of 645 bp was amplified with KpnI-BamHI and introduced into the corresponding site of pRI101-AN, resulting in pRI101-ID3S-CFP, which was used as a control instead of 35S-CFP.

**Chemical treatments**

Col-0 plants were treated with 100 mg/L of hygromycin B and incubated for 4 days. The rate of bacterial growth inside the cotyledons was measured by enumeration of colonies on agar plates.

**RT-qPCR**

RT-qPCR was performed using the primers listed in Supplementary Table 3. The 11-day-old plants were used for fungal inoculation onto cotyledons. The inoculated plants were collected from at least five different plants of each genotype at 24 and 72 hpi. Total RNA was isolated from these plants using an RNEasy Plant Mini Kit (Qiagen) and treated with DNase (Qiagen) to remove any DNA contamination. A TaKaRa PrimeScript RT reagent Kit (Perfect Real Time) was used to obtain cDNA. The primers used for the C. fioreae gene (At3g18780) were designed using Primer3, and the primer sequence contained in the TaKaRa TB Green Premix Ex Taq II (Tli RNaseH Plus) was used for all RT-qPCR. Quantitative analysis of each mRNA was performed using a Thermal Cycler Dice Real Time System III TP970 (TaKaRa).

**Confocal laser-scanning microscopy**

To assess the fluorescent signals in A. thaliana and Colletotrichum fungi, the inoculated cotyledons on a glass slide were observed by confocal laser-scanning microscopy. The fluorescent signals of callose and chlorophyll, respectively. The excitation and emission aperture of calibration slides were used to scan the images. For trypan blue (TB) staining, the inoculated plants were soaked and incubated with TB (10 mL lactic acid, 10 mL glycerol, 10 mL phenol, 10 mL DW, and 10 mg TB) for 3 min at 95°C. The plants were then transferred to a chloral hydrate solution for destaining. To detect the cell death of both epidermal and mesophyll cells stained plants were incubated in chloral hydrate solution for 45 min. For the inoculated cotyledons were mounted on a slide in 50% glycerol and observed under a light microscope (ECLIPSE E100, Nikon).

**Statistical analyses**

All statistical analyses were performed using R version 3.5.2. Levine’s test was applied to check for heteroscedasticity between the treatment groups. Data were arcine-transformed where necessary. To examine the differences among experimental groups, data were analyzed with Tukey’s honestly significant difference (HSD) test. All other data using Dunnett’s test, as appropriate. Differences were considered significant at P < 0.05.
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H.I. designed and performed the research, analyzed the data, and wrote the article. Y.T. analyzed the data and wrote the article. H.I. supervised the project.

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The authors declare no competing interests.

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