RESEARCH PAPER

Identification and utilization of a sow thistle powdery mildew as a poorly adapted pathogen to dissect post-invasion non-host resistance mechanisms in Arabidopsis

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Abstract

To better dissect non-host resistance against haustorium-forming powdery mildew pathogens, a sow thistle powdery mildew isolate designated Golovinomyces cichoracearum UMSG1 that has largely overcome penetration resistance but is invariably stopped by post-invasion non-host resistance of Arabidopsis thaliana was identified. The post-invasion non-host resistance is mainly manifested as the formation of a callosic encasement of the haustorial complex (EHC) and hypersensitive response (HR), which appears to be controlled by both salicylic acid (SA)-dependent and SA-independent defence pathways, as supported by the susceptibility of the pad4/sid2 double mutant to the pathogen. While the broad-spectrum resistance protein RPW8.2 enhances post-penetration resistance against G. cichoracearum UCSC1, a well-adapted powdery mildew pathogen, RPW8.2, is dispensable for post-penetration resistance against G. cichoracearum UMSG1, and its specific targeting to the extrahaustorial membrane is physically blocked by the EHC, resulting in HR cell death. Taken together, the present work suggests an evolutionary scenario for the Arabidopsis–powdery mildew interaction: EHC formation is a conserved subcellular defence evolved in plants against haustorial invasion; well-adapted powdery mildew has evolved the ability to suppress EHC formation for parasitic growth and reproduction; RPW8.2 has evolved to enhance EHC formation, thereby conferring haustorium-targeted, broad-spectrum resistance at the post-invasion stage.

Key words: Callose, encasement of haustorial complex, extrahaustorial membrane, haustorium, jasmonic acid, non-host resistance, post-invasion resistance, powdery mildew, RPW8, salicylic acid.

Introduction

Plants have evolved a multilayered immune system to prevent colonization by most potential pathogens. For non-adapted pathogens, plants exhibit non-host resistance, which is operative and effective in all individuals in a given plant species/genus (Hadwiger and Culley, 1993; Thordal-Christensen, 2003). Non-host resistance in most cases is a manifestation of the innate immune response triggered upon recognition of pathogen-associated molecular patterns (PAMPs) (Chisholm et al., 2006). A successful pathogen, by definition, must have conquered this basal immune system and be able to establish colonization on the defeated plant (then a host). Conceivably, the host plant does not simply yield; rather it still actively fights against the pathogen to limit the severity of the infection. This is achieved through
a defence layer(s) operative in susceptible hosts that is often referred to as basal resistance (Dangl and Jones, 2001). However, for a well-adapted pathogen race, basal resistance is not sufficient to stop the invasive growth and reproduction of the pathogen. In response, the host utilizes a new branch of the immune system controlled by resistance (R) genes often encoding intracellular immune receptors of the nucleotide-binding site and leucine-rich-repeat (NB-LRR) superfamily to trigger stronger and race-specific resistance (Chisholm et al., 2006; Jones and Dangl, 2006). Atypical R genes encoding proteins distinct from NB-LRRs have also been identified. For example, RPW8 from Arabidopsis confers broad-spectrum resistance against well-adapted powdery mildew pathogens (Xiao et al., 2001). Interestingly, basal resistance, RPW8-mediated resistance, and race-specific resistance triggered by a subset of NB-LRR R proteins containing an N-terminal Toll/interleukin-1 receptor (TIR) homology domain share some common essential signalling components such as EDS1 and PAD4 (Aarts et al., 1998; Reuber et al., 1998; Falk et al., 1999; Jirage et al., 1999; Xiao et al., 2003). In addition, the Arabidopsis eds1 mutant plants and plants expressing NahG, the gene encoding a bacterial enzyme that converts salicylic acid (SA) to catechol, were also reported to be compromised in non-host resistance (Parker et al., 1996; Mellersh and Heath, 2003; Zimmerli et al., 2004). Thus, non-host resistance and host resistance may be evolutionarily interconnected and mechanistically interconnected (Nürnberger et al., 2004). However, how these resistance forms are interconnected remains unclear mainly because non-host resistance as a whole is still poorly understood.

Non-host resistance against a biotrophic fungal pathogen is often manifested as the ability of the attacked plant to prevent fungal penetration (thus named penetration resistance), or the ability to terminate the development and/or functioning of the fungal feeding structure such as the intracellular hypha or the haustorium before it extracts enough nutrition from the plant cells for reproduction (thus referred to as post-invasion resistance) (Thordal-Christensen, 2003). Genetic studies of penetration (PEN) resistance using barley powdery mildew (Blumeria graminis Fsp. hordei; Bgh), as a non-adapted pathogen of Arabidopsis, have revealed at least two separate pathways contributing to penetration resistance. One involves an exocytosis pathway controlled by the PEN1 syntaxin and its working partners (Collins et al., 2003; Kwon et al., 2008) and the other requires the PEN2 myrosinase and the PEN3 ATP-binding cassette transporter (Lipka et al., 2005; Stein et al., 2006; Bednarek et al., 2009; Clay et al., 2009). Impairing either or both of the two pathways does not seem to break down the post-invasion resistance, as there is no colonization of barley powdery mildew Bgh and pea powdery mildew (Erysiphe pisi) on pen1 and pen2 single or pen1 pen2 double mutants (Lipka et al., 2005). However, further loss of EDS1 or PAD4/SAG101 in the pen2 or pen3 mutant background results in breakdown of the post-invasion resistance of Arabidopsis to permit microcolonization of Bgh and macro-colonization of E. pisi (Lipka et al., 2005; Stein et al., 2006). These findings suggest that for a non-adapted powdery mildew to adapt a given plant species it has sequentially to overcome multilayered defence barriers of the attacked plant. These findings also imply that for a given plant species there are probably non-host powdery mildews in the natural ecosystem that have conquered penetration resistance but are stopped by post-invasion resistance. Here, the identification of such a powdery mildew isolate relative to Arabidopsis that can largely overcome penetration resistance, but its further haustorium development and function is arrested by post-invasion resistance, is reported. This isolate was thus used as a tool to dissect the genetic mechanisms involved in post-invasion resistance using the Arabidopsis model system.

### Materials and methods

#### Plant lines and growth conditions

Twenty-five Arabidopsis thaliana accessions (Bg-1, Bu-11, Bu-23, C24, Can-0, Col-0, Do-0, Es-0, FL-1, FM3, Gr-0, Hs-1, Kas-1, Knox-1, Ler, Lin, Ms-0, Ob-0, Per-1, Pna10, Sg-1, Sapporo-0, Shadhara, Ta-0, and Wa-1), 13 single mutant lines (edsl-2, pad4-1, sid2-1, eds5-1, ndr1, npr1-1, rlrl-10, sgt1b-1, coil-1, dde2-2, ein2-1, edr2, and pen1-1; all are in the Col-0 background except edsl-2 which is in the Ler background), two double mutant lines (pad4-1/sid2-1 and dde2-2/ein2-2), one quadruple mutant (dde2/ein2/pad4/sid2), and three transgenic lines [Col-0/NahG, Col-0/RPW8.2-YFP (R2Y4), and Col-0/RPW8.1+RPW8.2 (S5)] were used for tests with the sow thistle (Sonchus oleraceus) powdery mildew. The DNA construct RPW8.2-YFP (yellow fluorescent protein) driven by the RPW8.2 promoter (Wang et al., 2009) was introduced into edsl-2 and pad4/sid2-1 via Agrobacterium-mediated transformation. Seeds were sown in Sunshine Mix #1 (Maryland Plant and Suppliers, Baltimore, MD, USA) and cold treated (4 °C for 2 d), and seedlings were kept under 22 ºC, 75% relative humidity, short-day (8 h light at 125 µmol m⁻² s⁻¹, 16 h dark) conditions for 5–6 weeks before pathogen inoculation.

#### Isolation and characterization of the sow thistle powdery mildew isolate Golovinomyces cichoracearum (Gc) UMSG1

The sow thistle powdery mildew was identified in sow thistle (S. oleraceus) plants in the grassland of the University of Maryland Shady Grove campus in the summer of 2007. The fungal isolate was purified by single-colony inoculation of clean sow thistle plants for five consecutive generations. The isolate was then maintained on sow thistle plants at 22 ºC (16 h light, 8 h dark) in a separate growth chamber. Genomic DNA extraction was extracted from fungal spores, and the internal transcribed spacer (ITS) rDNA fragment was amplified using the fungal genomic DNA as template and primers ITS4n, 5′-GAGGCAATAACAGGTCTTATTGATATGC-3′ and NS7, 5′-GCAGCTTCCGCGGTATTGATATGC-3′, described in Adam et al. (1999). The PCR product was cloned into pENTR/D-TOPO (Invitrogen), and sequenced using plasmid DNA prepared from three positive colonies. The ITS rDNA sequence has been deposited in GenBank (accession no. HM449077). Sequence alignment between ITS rDNA sequences from different powdery mildews was performed using the AlignX function of the Vector NTI Suite (Invitrogen) and manually corrected. Phylogenetic analysis was conducted using MEGA version 4.1 (Tamura et al., 2007).

#### Pathogen infection and microscopy analyses

Gc UMSG1 was maintained on live Arabidopsis mutant eds1-2 or pad4-1 plants for generation of fresh inoculum. Bgh isolate 5874
was propagated on *Hordeum vulgare* cv. Manchuria (Haltermann et al., 2001). Inoculation and visual scoring of disease reaction phenotypes were done as previously described (Xiao et al., 2005). Fungal structures and dead cells in inoculated leaves were visualized with trypan blue staining (Xiao et al., 2003). Callose deposited at fungal penetration sites was detected using aniline blue (0.01% in an aqueous solution containing 150 mM KH₂PO₄, pH 9.5) or sirofluor (0.1 mg ml⁻¹; Biosupplies, http://www.biosupplies.com.au/products.htm) (Meyer et al., 2009). In situ detection of H₂O₂ was performed as previously described (Xiao et al., 2003). At least 100 penetration sites per genotype were scored at 24 h post-inoculation (hpi) in each of the three inoculation experiments. Student’s t-test was performed to test statistical significance for fungal penetration rate, hyphal length, and number of conidiophores per colony. χ² test is used to test statistical significance for two categorical variables—that is, cells with an unencased haustorium (i.e. no encasement develops around the haustorium) versus cells with an encased haustorium or undergoing the hypersensitive response (HR)—between Col-0 wild-type and any other genotypes (http://www.atstat.ucla.edu/stat/sppsswhatstat/whatstat.htm). Single or multiple channel epifluorescent images were acquired with a Zeiss Axio microscope coupled with an HBO 100 microscope illumination system. Confocal laser scanning microscopy images were acquired and processed as previously described (Wang et al., 2009).

**Scanning electron microscopy (SEM)**

*Gc UMSG1-*infected sow thistle leaves, *Arabidopsis* (eds1-2) leaves, and *Gc UCSC1-*infected *Arabidopsis* (Col-0) leaves were used for SEM. The infected leaves were cut into 5×5 mm pieces and mounted onto flat specimen holders consisting of 16×30 mm copper plates that contained a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, CA, USA), which acted as the cryoadhesive upon freezing. The samples were frozen conductively, in a styrofoam box, by placing the plates on the surface of a pre-cooled (–96°C) brass bar whose lower half was submerged in liquid nitrogen (LN₂). After 20–30 s, the holders containing the frozen samples were transferred to an LN₂ Dewar for future use or cryotransferred under vacuum to the cold stage in the pre-chamber of the cryotransfer system (Polaron 2000, Quorum Technologies Ltd, East Sussex, UK). Removal of any surface contamination (condensed water vapour) took place in the cryotransfer system by etching the frozen specimens for 10–15 min by raising the temperature of the stage to –90°C. Following etching, the temperature was lowered below –130°C, and a magnetron sputter head equipped with a platinum target was used to coat the specimens with a very fine layer of platinum. The specimens were transferred to a pre-cooled (~140°C) cryostage in the microscope (S4700, Hitachi High Technologies, Pleasanton, CA, USA) for observation. An accelerating voltage of 5 kV was used to observe the specimens. Images were sized and placed together to produce a single figure using Adobe® Photoshop 7.0, visible to the naked eye, indicating that these pathogens are probably adapted to *Arabidopsis* and thus were discarded. Germinated conidia from several other collections, such as those from apples and roses, rarely penetrated the cell wall of Col-0 leaves, similar to those of *Bgh*, indicating that they are non-adapted mildews and their attempted invasion is stopped by penetration resistance. Interestingly, it was found that conidia of two independent collections from common sow thistle were able to penetrate Col-0 at rather high rates (~60%, see later text), suggesting that they may have gained limited adaptation to *Arabidopsis*. Thus further study focused on one of these two collections and this isolate was purified on sow thistle by five consecutive inoculations from single colonies.

The purified isolate is well adapted to the common sow thistle (belonging to *S. oleraceus* as evidenced by the plant morphology, Fig. 1A) biotype from which the original conidia were collected and to several other sow thistles with slightly different leaf morphology (data not shown). The pathogen is so aggressive on sow thistle plants that entire infected leaves could be covered by fungal mass exhibiting a typical 'powdery' look at ~12–14 days post-inoculation (dpi). To see how different this sow thistle powdery mildew is from *Gc UCSC1*, an isolate well adapted to *A. thaliana* (Adam et al., 1999), the two fungi on the infected leaves of their respective hosts were first examined using SEM. The major noticeable morphological difference is the shape of the conidia. The conidia of *Gc UCSC1* have an oval shape (20–28×14–18 μm) (Fig. 1B), with an average length-to-height ratio of 1.53±0.14, whereas the conidia of the sow thistle mildew are oblong or rectangular (20–26×11–13 μm) (Fig. 1C), with an average length-to-height ratio of 1.94±0.13. Also noticeable is that *Gc UCSC1* normally has 5–6 conidia stacking on the conidiophores (Fig. 1B) whereas the sow thistle mildew has only 3–4 (Fig. 1C). Similar morphological features were observed for the sow thistle mildew on *Arabidopsis eds1-2* mutant plants which permitted fungal sporulation of this fungus (Fig. 1D).

Next, the ITS regions of rDNA of the sow thistle mildew were sequenced using primers described by Adam et al. (1999). Blast search showed that the full-length sequence has 98% identity to that of *Gc UCSC1* and *G. orontii*. The 333–951 bp region of this sequence is identical to the rDNA ITS sequences of two *Gc* isolates obtained from sow thistles (*S. oleraceus* and *S. arvensis*). This sequence was then aligned with the rDNA ITS sequences from 17 different powdery mildew isolates and subjected to phylogenetic analysis using MEGA version 4 (Tamura et al., 2007). The sow thistle mildew was placed in subgroup A that contains all *Gc* isolates and *G. orontii*, while *E. pisi* was in subgroup B and *Bgh* in subgroup E (Fig. 2). The sow thistle mildew isolate *Gc UMSG1* was thus tentatively named to reflect the site of its identification (University of Maryland Shady Grove Campus).

To define *Gc UMSG1* further, its host range, along with that of *Gc UCSC1* and *Bgh*, was examined. *Arabidopsis* (Col-0), sow thistle (the progeny of the original sow thistle from which *Gc UMSG1* was isolated), barley (cv. Manchuria), cucumber
(cv. Straight 8), tomato (cv. Moneymaker), and Nicotiana benthamiana were inoculated with the three isolates and the inoculated leaves were examined by trypan blue staining at 7 dpi. The fungal invasiveness was examined by looking at (i) conidium germination and hyphal growth, and (ii) fungal reproduction (sporulation). Based on the observations (Table 1), it is clear that the three isolates have distinct host ranges and Gc UMSG1 is only infectious (i.e. able to reproduce) on sow thistle. However, it was noted that Gc UMSG1 seemed to have some limited hyphal growth on Arabidopsis, and was invasive on cucumber and tobacco. This pattern is similar to that of Gc UCSC1, albeit that Gc UMSG1 is less invasive in cucumber compared with Gc UCSC1.

Post-invasion resistance renders A. thaliana a non-host to Gc UMSG1

To determine if there is genetic variation among Arabidopsis accessions in the resistance response to Gc UMSG1, 25 geographically representative natural Arabidopsis accessions (see Materials and methods) collected from Europe, Asia, or the USA were inoculated with Gc UMSG1 and the inoculated leaves were examined by trypan blue staining. It was found that all accessions permitted very limited hyphal growth as seen in Col-0, but none supported sporulation. This result indicated that A. thaliana is probably a non-host to Gc UMSG1 at the species level and that RPW8 is dispensable for the post-invasion resistance against Gc UMSG1 because accessions lacking RPW8 (Col-0) or containing susceptible (in relation to Gc UCSC1) RPW8 alleles (Bu-11, Bu-23, and FM3) (Orgil et al., 2007) were also resistant.

Next, experiments were conducted to determine if the limited hyphal growth of Gc UMSG1 on Col-0 correlated with a higher penetration rate in comparison with Bgh using a similar method described by Collins et al. (2003). As shown in Fig. 3A and C, Bgh rarely (≤8%) penetrated the epidermal cells of Col-0 under the experimental conditions used here. In contrast, Gc UMSG1 supported a penetration rate of ~62%. This penetration rate, although not as high as that of the well-adapted powdery mildew Gc UCSC1 (93%), is nearly eight times that of Bgh, suggesting that Gc UMSG1 has evolved the ability largely to suppress the penetration resistance of Col-0. The pen1-1 and eds1-2 mutants were also tested with the three mildew isolates. Similar to the results reported earlier (Collins et al., 2003), it was also found that pen1-1 mutant plants supported ~7 times higher penetration for Bgh compared with that of Col-0. However, the penetration rate of Gc UMSG1 in pen1-1 only slightly increased from ~62% to ~71%, suggesting that PEN1-mediated penetration resistance is largely broken down by Gc UMSG1. Interestingly, it was also found that the eds1-2 mutant supported a penetration rate of 43% for Bgh, which is ~5 times that in Col-0, suggesting that EDS1, an essential component engaged in basal and R-gene mediated host resistance (Aarts et al., 1998; Falk et al., 1999; Yun et al., 2003), also contributes to penetration resistance. Similar to pen1-1, eds1-2 had only an ~10% increase in penetration rate for Gc UMSG1, suggesting a large portion...
of EDS1-mediated penetration resistance is also suppressed by Gc UMSG1. As expected, the well-adapted Gc UCSC1 completely overcame both PEN1- and EDS1-mediated resistance because pen1-1 and eds1-2 supported the same penetration rate as Col-0 for Gc UCSC1 (Fig. 3A, C). Interestingly, while Gc UMSG1 failed to colonize on Col-0 (Fig. 4B) or pen1-1 (not shown) as conidiophore formation was never observed, it could successfully complete its life cycle on eds1-2 (Fig. 1D) or Col/NahG plants (not shown), albeit with only a low level of sporulation.

Together, the present observations suggested that Gc UMSG1 has largely overcome penetration resistance of

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**Table 1. Tests for the host range of Golovinomyces cichoracearum UMSG1**

Plants of the indicated species were inoculated with one of the three powdery mildew isolates and maintained in separate growth chambers at 18–24 °C under long-day (16 h light) conditions. Inoculated leaves were collected at 3, 5, and 7 dpi and subject to trypan blue staining followed by microscopy. The amount of fungal hyphal growth and sporulation was estimated using four scales from 0 (not or rarely visible), 1 (little), 2 (some), to 3 (profuse) based on assessment of at least 200 conidia or 20 fungal colonies on leaf surfaces of each plant species at each of the three time points by trypan blue staining followed by microscopy.

| Plant species                        | Hyphal growth | Sporulation |
|--------------------------------------|---------------|-------------|
|                                      | Gc UCSC1 | Gc UMSG1 | Gbh | Gc UCSC1 | Gc UMSG1 | Gbh |
| Arabidopsis thaliana (Col-0)         | 3       | 1        | 0   | 3       | 0        | 0   |
| Sonchus oleraceus (sow thistle)      | 0       | 3        | 0   | 0       | 3        | 0   |
| Hordeum vulgare (barley; cv. Manchuria) | 0       | 0        | 3   | 0       | 0        | 3   |
| Cucumis sativus L. (cucumber; cv. Straight 8) | 3     | 1        | 0   | 2–3     | 0        | 0   |
| Solanum lycopersicum (tomato; cv. Moneymaker) | 0–1   | 0        | 0   | 0       | 0        | 0   |
| Nicotiana benthamiana (tobacco)      | 1       | 0–1      | 0   | 0       | 0        | 0   |
Fig. 3. Gc UMSG1 largely overcomes penetration resistance, but is stopped by post-invasion resistance in Arabidopsis. (A) Frequencies of fungal entries in leaf epidermal cells of the indicated genotypes at 24 hpi. Formation of secondary hyphae and/or haustoria was used to score for successful penetration. Data are mean ± SEM (*P < 0.01, n=3, Student’s t-test), collected from three independent experiments (for each of which >200 interaction sites/genotype were evaluated). (B) Types and frequencies of post-invasion interaction in the leaf epidermal cells of Col-0 infected with three powdery mildew isolates. UH, cells containing an unencased (presumably healthy) haustorium (i.e. without an EHC); EHC, cells containing a haustorium with an EHC; HR, cells undergoing cell death. Data are mean ± SEM, collected from three independent experiments (for each of which >200 interaction sites were evaluated for each isolate). (C) Representative images showing fungal penetration and hyphal growth in the three indicated genotypes at 24 hpi. Conidia and hyphae were stained red by propidium iodide, and callose deposited in papillae and/or EHC was stained blue by aniline blue. Bars, 50 μm. (D) Representative images of three types of post-invasion interaction described in (B). Bars, 20 μm.
Fig. 4. Post-invasion resistance against Gc UMSG1 is controlled by an SA-dependent and SA-independent pathway. (A) Types and frequencies of post-invasion interaction in leaf epidermal cells of the indicated genotypes infected with Gc UMSG1 at 5 dpi. S5 is a Col-0 line transgenic for RPW8.1 and RPW8.2 (Xiao et al., 2003); R2Y4 is a Col-0 line transgenic for RPW8.2-YFP (Wang et al., 2009). Infected leaves were stained with trypan blue and examined by microscopy. Data are mean ± SEM, collected from three independent experiments (for each of which >200 interaction sites/genotype were evaluated). \( \chi^2 \)-test is used to test statistical significance for two categorical variables—that is, cells with an unencased haustorium (UH) versus cells with an EHC or undergoing HR, between Col-0 and any other genotype, \( ^* P < 0.05 \). (B) Representative images showing hyphal growth and sporulation of Gc UMSG1 at 5 dpi (trypan blue-stained leaf sections) or at 10 dpi (whole plant/leaf pictures). Bar, 20 μm. (C and D) Total hyphal length (C) and total number of conidiophores (D) per colony of the indicated genotypes infected with Gc UMSG1 at 4 dpi or 7 dpi. Data are mean ± SEM (for Student t-tests: relative to Col-0, \( {^* P < 0.05, \quad {^*^* P < 0.01, \quad n = 3; \quad between \; pad4/sid2 \; and \; dde2/ein2/pad4/sid2, \; {^* P < 0.05, \quad n = 3}, \quad collected \; from \; three \; independent \; experiments \; (for \; each \; of \; which >20 \; colonies/genotype \; were \; evaluated).} \)
Arabidopsis but its further development is arrested by post-invasion resistance in Arabidopsis controlled by innate immunity components including EDS1.

Encasement of haustorial complex (EHC) and HR cell death contribute to post-invasion resistance in Arabidopsis against Gc UMSG1

As Gc UMSG1 is arrested mainly by post-invasion resistance, it provides an opportunity to examine the possible subcellular responses associated with this layer of non-host resistance using Col-0. To test this idea, plants of Col-0 were first inoculated with Gc UCSC1, Gc UMSG1, and Bgh, and the haustorium development at 2 dpi or 5 dpi was examined by trypan blue staining. Generally there were three types of post-invasion interaction: (i) formation of an unencased, presumably healthy haustorium (i.e. without an EHC); (ii) formation of an encased haustorium (i.e. with an EHC); and (iii) activation of HR cell death. The three representative cell types of Gc UMSG1-Col-0 interaction are shown in Fig. 3D. Consistent with Gc UCSC1 being a well-adapted pathogen of Col-0, ~95% of interaction sites had formation of unencased haustoria, ~4% of penetrated cells had a haustorium with an EHC, and ~1% of attacked cells underwent HR cell death. In contrast, the frequencies of the (i), (ii), and (iii) interaction types found in Col-0 inoculated with Gc UMSG1 were ~8, ~42, and ~49%, respectively. For Bgh, ~88% of the rare post-invasion interaction with Bgh was accompanied by HR cell death, whereas the remaining ~12% contained a distorted haustorium with a partial EHC (Fig. 3B). These data suggest that both EHC formation and the HR appeared to contribute to post-invasion resistance and the well-adapted powdery mildew isolate Gc UCSC1 actively suppresses these defence mechanisms.

Post-invasion resistance against Gc UMSG1 is controlled by both SA-dependent and SA-independent mechanisms

To determine if the known SA-dependent and/or jasmonic acid (JA)/ethylene (JA/ET)-dependent signalling pathways are involved in post-invasion resistance against Gc UMSG1, a panel of Arabidopsis mutants (all in the Col-0 background) that are impaired in SA and/or JA/ET signalling were inoculated and the post-invasion interaction was examined by trypan blue staining at 5 dpi. Consistent with the compromised non-host resistance observed in eds1-2, it was found that the SA pathway plays an important role in post-invasion resistance, as plants containing sid2-1 (a mutation impairing SA biosynthesis), NahG (encoding salicylate hydrolase), or pad4-1 (a mutation impairing signalling upstream of SA) supported significantly higher frequencies of unencased haustoria ($\chi^2$-test relative to Col-0 for two categorical variables: cells with an unencased haustorium versus cells with an encased haustorium or undergoing HR; $P < 0.05$), with an increase of 9, 14, and 19%, respectively, compared with that of Col-0 (Fig. 4A). A very high rate (~60%) of unencased haustoria was found in the pad4-1/sid2-1 double mutant, which is a >7-fold increase compared with that of Col-0 and is significantly higher ($\chi^2$-test; $P < 0.05$) than that of plants containing NahG, pad4-1, or sid2-1 (Fig. 4A). Since sid2-1 is a mutation in the isochorismate synthase gene involved in SA biosynthesis (Wildermuth et al., 2001), it is likely that loss of PAD4 may contribute to the breakdown of the post-invasion resistance via both the SA-dependent and SA-independent pathways. For the JA/ET pathway-related mutants, it was found that dde2-2 (JA synthesis) (von Malek et al., 2002), coi1 (JA pathway) (Xie et al., 1998), and ein2-1 (ET pathway) (Alonso et al., 1999) single mutants, and the dde2/ein2 double mutant (Tsuda et al., 2009) exhibited similar post-invasion interaction patterns to Col-0 (data not shown). However, it was found that the dde2/ein2/pad4/sid2 quadruple mutant consistently supported a higher rate (~68%) of unencased haustoria than the pad4/sid2 double mutant (Fig. 4A), though the $\chi^2$ value of 3.33 is just below $\chi^2_{0.05}=3.84$, indicative of no statistical significance.

To examine if increased formation of unencased haustoria in several tested mutants would lead to more hyphal growth and fungal sporulation, seven representative genotypes (Col-0, Col/NahG, pad4-1, sid2-1, pad4/sid2, dde2/ein2, and dde2/ein2/pad4/sid2) were selected for quantitative assays of the hyphal growth and sporulation by trypan blue staining followed by microscopy at 4 dpi and 7 dpi. It was found that compared with Col-0 plants which supported only limited hyphal growth without formation of any conidiophore, plants of the other six genotypes supported significantly more hyphal growth and weak-to-moderate sporulation (Fig. 4B–D). However, only plants of pad4-1/sid2-1 and dde2/ein2/pad4/sid2 were found to support profuse fungal growth and sporulation with typical mildew disease symptoms visible to the naked eye at 10 dpi (Fig. 4B). Consistent with the difference observed in the rates of unencased haustoria, dde2/ein2/pad4/sid2 supported significantly more hyphal growth and sporulation measured at 7 dpi when compared with pad4/sid2 (Fig. 4B–D).

Taken together, the results showed that the invasive growth of Gc UMSG1 measured by total hyphal growth and sporulation generally correlated positively with the frequencies of unencased haustoria but negatively with the frequencies of EHC formation and HR. Both SA-dependent (via SID2 and PAD4) and SA-independent (probably via PAD4) pathways, and the JA/ET pathway contribute to post-invasion resistance against Gc UMSG1 in Arabidopsis.

Experiments were also carried out to examine if post-invasion resistance is altered due to loss of PEN1. It was found that there were actually fewer cells having an unencased haustorium (~2%) but more cells with HR (~60%) in pen1-1 compared with Col-0 (Fig. 4A). This result was not unexpected given that pen1-1 exhibited enhanced resistance against Gc UCSC1 which was interpreted to be caused by the activation of SA signalling due to loss of PEN1 (Zhang et al., 2007). Therefore, PEN1 did not seem to make a positive contribution to post-invasion resistance against powdery mildew fungi.
RPW8.2’s localization to the extrahaustorial membrane is blocked by the EHC, which may cause excessive HR cell death triggered by Gc UMSG1

Previous studies showed that RPW8.2 is specifically targeted to the extrahaustorial membrane (EHM) induced by Gc UCSC1, and RPW8.2-mediated resistance against this well-adapted pathogen correlates with enhanced EHC formation and HR cell death (Wang et al., 2009). To determine whether RPW8.2 is also targeted to the EHM induced by Gc UMSG1, Gc UMSG1-inoculated Col-0 lines were either transgenic for RPW8.2-YFP (R2Y4) or RPW8.2-YFP at the EHM of deformed haustoria contained by a thick EHC (Fig. 5G). Not surprisingly, pad4-1/sid2-1 plants transgenic for RPW8.2-YFP showed frequencies of EHC formation and HR not significantly different from that of pad4-1/sid2-1 alone (data not shown), which is consistent with previous findings that RPW8.2’s defence activity is SA and/or PAD4 dependent (Xiao et al., 2003, 2005).

Discussion

Arabidopsis—powdery mildew interaction has been successfully used as the model pathosystem to study both host and non-host resistance mechanisms at the molecular level over the past 15 years (Adam and Somerville, 1996; Reuber et al., 1998; Collins et al., 2003; Nishimura et al., 2003; Lipka et al., 2005; Stein et al., 2006). Most studies on this topic were conducted by using four powdery mildews: Bgh and E. pisi, two non-adapted isolates, and Gc UCSC1 and G. orontii, two well-adapted isolates. In this study, a sow thistle powdery mildew isolate Gc UMSG1 was identified as a non-host pathogen of Arabidopsis with some limited, early-stage adaptation: Gc UMSG1 has largely overcome penetration resistance but its haustorium development and/or function is invariably terminated by post-invasion resistance in all tested Arabidopsis accessions. This limited adaptation on Arabidopsis implies that Gc UMSG1 may be able to survive and reproduce on close relatives of Arabidopsis, hence the limited adaption on Arabidopsis. It should be pointed out that many powdery mildew isolates with distinct adaptation on dicots are classified as G. cichoracearum (Garibaldi et al., 2008; Gevens et al., 2009; Troisi et al., 2010). This situation is somewhat reminiscent of formae speciales formation of B. graminis on grasses (Wyand and Brown, 2003). Thus, characterization of G. cichoracearum formae specialis probably requires higher resolution phylogenetic analyses and comparative host-range determination. The resultant high frequency (~43%) of EHC induction by Gc UMSG1 in wild-type Arabidopsis Col-0 (Fig. 4A) makes Gc UMSG1 distinct from Bgh. Therefore, Gc UMSG1 can be a useful, complementary tool to investigate post-invasion resistance which probably represents the last lines of non-host immunity against powdery mildew pathogens in Arabidopsis.

The present data showed that both the formation of the callosic EHC and HR cell death are associated with post-invasion resistance against Gc UMSG1 (Fig. 4). Similar haustorial encasement structures have long been recognized to be associated with host defence in monocot and dicot plants when challenged by non-adapted or adapted fungal and oomycete pathogens (Ehrlich and Ehrlich, 1963; Perumalla and Heath, 1989; Skalamera and Heath, 1995; Starkurnau and Mendgen, 1995; Heath et al., 1996; Silva et al., 1999; Soylu and Soylu, 2003; Soylu et al., 2004). Thus, EHC formation may be a conserved cellular defence mechanism against haustorial invasion in plants.

Earlier studies suggested that papillae contain callose (β-1,3-glucan), phenolic compounds, lignin, and reactive
oxygen species (Aist, 1976; Israel et al., 1980; Thordal-Christensen et al., 1997). However, the physical and chemical nature of the EHC is less characterized (Meyer et al., 2009; Wang et al., 2009) and little is known about the cellular machinery engaged in EHC formation. Donofrio and Delaney (2001) showed that induction of the EHC in Arabidopsis mesophyll cells by an adapted Hyaloperonospora arabidopsidis isolate is compromised by expression of NahG or loss of NIM1 (NPR1), a master regulator of defence signalling downstream of SA. This observation suggested a role for the SA-dependent signalling in EHC formation. From the data in Fig. 4, EHC formation induced by Gc UMSG1 did not seem to be significantly affected in NahG-expressing plants, or in pad4-1 or sid2-1 single mutants, but those plants supported increased (9–19%) frequencies of unencased haustoria, resulting in more hyphal growth and low levels of sporulation. Thus, this partial breakdown of post-invasion resistance seemed to be associated with lower frequencies of HR cells compared with that of wild-type Col-0. However, it should be pointed out that many cells undergoing HR contain a haustorium with an EHC but were scored as the HR type in the present experiments. The classification of EHC formation and HR into two distinct interaction types was an expedient simplification to reflect EHC formation as a subcellular defence response. In reality, an invaded cell having EHC at one time point could be scored as having HR at a slightly later time point. Hence, while the frequency of unencased
haustoria reflects the well-being of the invading fungus. EHC formation together with HR reflects host defence response. The pad4-1/sid2-1 double mutant became almost fully susceptible to Gc UMSG1, suggesting that either PAD4 and SID2 can compensate for each other’s function in post-invasion resistance, similar to their contribution to AvrRpt2-RRPS2-triggered race-specific host resistance (Tsuda et al., 2009), or both SA-dependent (SID2 and PAD4) and SA-independent (PAD4) defence mechanisms are involved in post-invasion resistance. This result is consistent with earlier observations that PAD4 and its homologous partner EDS1 regulate both SA-dependent and SA-independent defence signalling (Bartsch et al., 2006; Venugopal et al., 2009). The contribution of the JA/ET pathway to post-invasion resistance against powdery mildew is rather subtle, because a statistically significant difference in disease phenotypes between single or double JA/ET pathway mutants and Col-0 inoculated with Gc UMSG1 or Gc UCSC1 was not detected (data not shown; Xiao et al., 2005). However, more fungal growth of Gc UMSG1 in dde2l ein2l/pad4l/sid2 than in pad4l/sid2 was observed (Fig. 4B-D), although no significant difference in the growth of Gc UCSC1 was seen between these two genotypes (data not shown). Zimmerli and co-authors found that non-adapted (Bgh) but not host-adapted powdery mildew (Gc UCSC1) induced expression of the defence genes controlled by the JA/ET pathway (Zimmerli et al., 2004). Thus, it seems likely that the JA/ET pathway has a minor contribution to post-invasion resistance against non-adapted/poorly adapted powdery mildew, which can be more readily detectable in plants whose immunity system is already severely compromised. The present results also support the speculation that well-adapted powdery mildew suppresses the JA/ET pathway (Zimmerli et al., 2004) and are compatible with the model that there exist synergistic relationships among different defence signalling pathways in basal resistance (Tsuda et al., 2009).

In a previous study, it was found that both EHC formation and the HR correlated with RPW8.2-mediated host resistance against the adapted powdery mildew isolate Gc UCSC1 (Wang et al., 2009). Thus, EHC formation and the HR may be the common cellular defence mechanisms against haustorial invasion in the post-invasion stage regardless of whether it is from non-adapted or well-adapted pathogens. Combined with earlier studies, the present work supports a co-evolutionary scenario for the interaction between haustorium-forming pathogens such as powdery mildew and their target plants as follows. For non-adapted powdery mildews, their entry into the host cell is stopped by penetration resistance manifested as the formation of papillae (Thordal-Christensen, 2003). When penetration resistance is gradually broken down, haustorium development and/or function is terminated by post-invasion resistance manifested as EHC formation and the HR. Adapted powdery mildew pathogens must have evolved the ability to suppress EHC formation and the HR in order to sustain their haustorium function; in response, host plants have then evolved RPW8.2 or RPW8.2-like proteins to counter this suppression, thereby enhancing post-invasion resistance.

The present examination of RPW8.2:YFP localization in Gc UMSG1-invaded cells (Fig. 5) provided unexpected mechanistic insight into how RPW8.2 might cause excessive HR cell death. In cases where an EHC was rapidly formed, RPW8.2’s targeting to the EHM was then hindered, resulting in accumulation of RPW8.2 peripheral to the EHC or in the trafficking pathway. Inappropriately localized RPW8.2 may trigger whole-cell H2O2 accumulation, leading to HR cell death. This speculation is consistent with earlier observations that RPW8.2 overexpression from its native promoter triggers HR-like cell death (Xiao et al., 2003) and that genetic perturbation of the actin cytoskeleton by overexpression of ADF6 results in RPW8.2 accumulation in punctate spots and subsequent cell death (Wang et al., 2009). This finding also raised interesting questions as to whether the focal accumulation of callose and other components in the EHC and RPW8.2 or RPW8.2-like proteins in the EHM is conducted via the same or two separate polarized trafficking pathways and how these events are spatiotemporally orchestrated. A previous study showed that RPW8.2’s EHM localization does not seem to be affected in the pen1-1 mutant (Wang et al., 2009), whereas the assembly of the callosic papilla is delayed due to loss of PEN1-defined exocytosis (Assaad et al., 2004). Given the blockage of RPW8.2:YFP outside of the EHC induced by Gc UMSG1, it is likely that targeting of RPW8.2 to the EHM may occur at the same or a later stage as for the EHC formation induced by Gc UMSG1 via a PEN1-independent/redundant exocytosis pathway.

Because (i) the EHC appears to have a similar physical structure to papillae and may actually originate from expansion of the papilla via rim growth (Meyer et al., 2009), which is also supported by the present observations (Fig. 5C-E) and (ii) GFP:PEN1 is selectively incorporated into the EHC, even though callose deposition to the EHC is not apparently affected in pen1-1 (Meyer et al., 2009), one may wonder if exocytosis dependent on PEN1 and/or its close homologue SYP122 contributes to deposition of other materials in the EHC. The present result showed that EHC formation induced by Gc UMSG1 was slightly reduced (by ~8%) in pen1-1, but there was an increase in the frequency of HR (by ~14%) in pen1-1 when compared with that of Col-0 (Fig. 4A). It remains unclear whether EHC formation is dependent on PEN1’s function. Future studies using Gc UMSG1 as an EHC-inducible non-adapted pathogen of Arabidopsis and a pen1/syp122 double mutant in a genetic background where HR cell death is suppressed may be able to determine if these two syntaxins contribute to EHC formation as a general cellular defence response in plants.

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