Perturbation of Arabidopsis Amino Acid Metabolism Causes Incompatibility with the Adapted Biotrophic Pathogen Hyaloperonospora arabidopsidis

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Reliance of biotrophic pathogens on living plant tissues to propagate implies strong interdependence between host metabolism and nutrient uptake by the pathogen. However, factors determining host suitability and establishment of infection are largely unknown. We describe a loss-of-inhibition allele of ASPARATE KINASE2 and a loss-of-function allele of DIHYDRODIPICOLINATE SYNTHASE2 identified in a screen for Arabidopsis thaliana mutants with increased resistance to the obligate biotrophic oomycete Hyaloperonospora arabidopsidis (Hpa). Through different molecular mechanisms, these mutations perturb amino acid homeostasis leading to overaccumulation of the Asp-derived amino acids Met, Thr, and Ile. Although detrimental for the plant, the mutations do not cause defense activation, and both mutants retain full susceptibility to the adapted obligate biotrophic fungus Golovinomyces orontii (Go). Chemical treatments mimicking the mutants’ metabolic state identified Thr as the amino acid suppressing Hpa but not Go colonization. We conclude that perturbations in amino acid homeostasis render the mutant plants unsuitable as an infection substrate for Hpa. This may be explained by deployment of the same amino acid biosynthetic pathways by oomycetes and plants. Our data show that the plant host metabolic state can, in specific ways, influence the ability of adapted biotrophic strains to cause disease.

INTRODUCTION

Plant-infecting microbes have evolved diverse lifestyles to survive changing environments and host selection pressures. The success of many pathogens depends on a high degree of specialization to a particular plant host which, at the same time, can restrict their host range (Kämper et al., 2006; Tyler et al., 2006). Non-host resistance, defined as immunity of an entire plant species against all genetic variants of a pathogen species, is one major host range-limiting factor. In Arabidopsis thaliana, non-host resistance has been shown to depend on the rapid mobilization of vesicles and defense-associated proteins to attempted invasion sites (Kwon et al., 2008; Wang et al., 2009).

Also, chemical responses involving the Trp-derived phytoalexin camalexin and indolic glucosinolates protect cells against attempted infection by biotrophic and necrotrophic pathogens (Bednarek et al., 2009; Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010). As a consequence, mutant plants defective in two redundant CYP79B monooxygenases catalyzing the conversion of Trp to indole-3-acetaldoxime, and thus lacking Trp-derived secondary metabolites, support increased growth of normally nonadapted pathogen isolates (Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010).

Host-adapted pathogens have evolved the ability to overcome non-host defenses and successfully colonize plant tissues. Effector molecules produced by infectious microbes are known to suppress plant defenses or manipulate host development in favor of pathogen growth (reviewed in Bent and Mackey, 2007). In a coevolutionary conflict with the host, some effectors become recognized by intracellular nucleotide binding site–leucine-rich repeat–containing immune sensors (NLRs). As a pathogen effector-driven process, receptor-mediated (race-specific) resistance depends on the genetic repertoire of the host and adapted pathogen (Bent and Mackey, 2007). NLR activation upon direct or indirect recognition of an effector molecule induces a signaling cascade leading to pathogen containment often involving host programmed cell death (the hypersensitive response [HR]) and accumulation of the systemic resistance signaling hormone salicylic acid (SA) (Bent and Mackey, 2007; Vlot et al., 2009). Many NLRs additionally require HSP90 and its
cochaperones SG1 and RAR1 for stability and/or activation (Shirasu, 2009; Zhang et al., 2010).

The obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsis* (Hpa) has become specialized to infect *Arabidopsis*, causing downy mildew disease. Coadaptation within this natural pathosystem is reflected by the extensive genetic variation in responses of different *Arabidopsis* accessions to *Hpa* isolates (Holub et al., 1994). Oomycetes are distinct from true fungi in that they possess a cellulose-based cell wall with little or no chitin and are phylogenetically close to brown algae and diatoms within the Stramenopile (heterokont) lineage (reviewed in Coates and Beynon, 2010). On susceptible *Arabidopsis* accessions, *Hpa* asexual conidiospores can germinate on leaves to produce a ramifying intercellular network of hyphae with haustorial invaginations of individual host cells believed to serve as specialized feeding structures (Coates and Beynon, 2010). As an obligate biotrophic parasite, *Hpa* is adept at maintaining host cell viability and integrity, most likely through the collective activities of protein effectors, some of which are translocated across the haustorial and host cell membranes into the plant cytoplasm (Whisson et al., 2007; Kale et al., 2010). The *Hpa* life cycle is completed by formation of conidiospores on the leaf surface and generation of longer lived sexual oospores inside leaves (Coates and Beynon, 2010). NLR recognition of specific *Hpa* effectors on resistant *Arabidopsis* accessions leads to a typical HR and accumulation of SA (Vlot et al., 2009; Coates and Beynon, 2010).

The absolute dependence of obligate biotrophs such as *Hpa* on their hosts and the intimate physical and molecular interactions between host and pathogen structures implies that additional host factors besides the dedicated defense machinery may be decisive for infection. Plant factors required for host colonization by symbiotic nitrogen-fixing bacteria have been identified (e.g., Hakoyama et al., 2009). Plant susceptibility determinants for biotrophic pathogen colonization have also been described but are not well understood. For example, successful entry of powdery mildew fungal germinating spores into host epidermal cells depends on functional MILDWE RESISTANCE LOCUS O (MLO) protein in barley (*Hordeum vulgare*) and *Arabidopsis*, although the precise function of MLO is unclear (Büschges et al., 1997; Consonni et al., 2006). Genetic screens of *Arabidopsis* identified a range of recessive powdery mildew resistant and downy mildew resistant (dmr) mutants that affect host cell wall composition and/or stress metabolite status (reviewed in O’Connell and Panstruga, 2006). *dmr1*, mediating strong and specific resistance to *Hpa*, encodes homoserine kinase, a chloroplast enzyme involved in the biosynthesis of the Asp-derived amino acids Met, Thr, and Ile (van Damme et al., 2009). *dmr1* mutants accumulated high levels of homoserine (HS), and treatment of plants with HS induced resistance to *Hpa* but did not interfere with *Hpa* spore germination or radial growth of the hemibiotrophic oomycete *Phytophthora capsici* in vitro (van Damme et al., 2009). The mechanism underlying increased downy mildew resistance through accumulation of HS in the chloroplast was not defined (van Damme et al., 2009).

Here, we describe the characterization of two nonallelic *Arabidopsis* mutants that display enhanced resistance to host-adapted *Hpa*. These mutants were identified in a screen for genetic suppressors of susceptibility to *Hpa* (isolate Noco2) caused by a *rar1* mutation disabling RPP5 (NLR) recognition (Muskett et al., 2002). The *rar1* suppressor (*rsp*) mutations cause perturbations of plant amino acid homeostasis by different molecular mechanisms, leading to strong and specific resistance to *Hpa*. In contrast with the induced defense programs triggered by *Hpa* effector recognition, we find that suppression of *Hpa* growth on *rsp* mutants is a result of Thr accumulation rendering host tissues unsuitable as a growth substrate. The metabolic state provoked by the *rsp* mutations is also detrimental for the plant, and specific loss of susceptibility to adapted *Hpa* isolates in the *rsp* mutants might be explained by deployment of the same metabolic pathway in the phylogenetically related oomycete. Our data suggest that host metabolic status can influence the range of biotrophic pathogens causing disease.

**RESULTS**

**Mutants with Altered Responses to *Hpa***

Complete resistance in *Arabidopsis* accession Landsberg erecta (Le) to *Hpa* isolate Noco2 mediated by RPP5 (Parker et al., 1993) depends on *RAR1*, encoding an HSP90 cochaperone (Muskett et al., 2002; Zhang et al., 2010). We used the intermediate resistance phenotype of a partially defective *rar1* mutant, *rar1-15*, as a sensitized background for a forward genetic screen to identify mutants with an altered response to Noco2 infection. Seeds of *rar1-15* were ethyl methanesulfonate mutagenized, and ~2600 M2 families derived from single M1 plants screened for enhanced (rar1 enhancer [ren]) or reduced (rsp) susceptibility to *Hpa* Noco2. A number of *ren* and *rsp* mutants were selected for further characterization. Here, we describe analysis of two nonallelic *rsp* mutants, *rsp1* and *rsp2*. Increased resistance of *rsp1* and *rsp2* to *Hpa* Noco2 was maintained after crossing into the *rar1-13* null mutant background, indicating that the resistance does not depend on a partially functional *rar1-15* protein. We used the *rar1-13* *rsp1* and *rar1-13* *rsp2* mutants for further characterization of *rsp1* and *rsp2* phenotypes. When infected with *Hpa* isolate Noco2, pathogen growth and sporulation were seen on *Ler* *rar1-13*, contrasting with the complete resistance associated with an HR in wild-type *Ler*, visualized microscopically after trypan blue (TB) staining of leaves (Figure 1A). The *rar1-13* *rsp1* and *rar1-13* *rsp2* double mutants exhibited strong resistance to *Hpa*. Whereas limited pathogen growth was occasionally observed in leaves of *rar1-13* *rsp1*, there were no symptoms of disease or host cell death in *rar1-13* *rsp2* plants (Figure 1A). The absence of HR lesions in *rar1-13* *rsp2* was especially evident when infected plants were observed under UV light (see Supplemental Figure 1A online). To test whether the increased resistance of *rar1-13* *rsp1* and *rar1-13* *rsp2* was dependent on RPP5, we infected the mutants with the virulent *Hpa* isolate Cala2, which is not recognized by RPP5. As expected, enhanced Cala2 sporulation was observed on *rar1-13* and *eds1-2* compared with wild-type *Ler* (Figure 1B), *eds1-2* is defective in downstream signaling after activation of NLRs containing an N-terminal Toll/interleukin-1 receptor domain (Aarts et al., 1998; Wirthenmueller et al., 2007) and was included in pathogenicity assays as a mutant with
they may be expressing constitutive resistance, which is normally accompanied by growth retardation, increased steady state accumulation of SA, and expression of Pathogenesis-Related (PR) genes leading to broad spectrum resistance (e.g., Lu et al., 2003; Zhang et al., 2003). We therefore tested for hallmarks of constitutive resistance. Free and total SA levels were reduced in rar1-13 compared with the wild type. In rar1-13 rsp1 and rar1-13 rsp2, SA accumulation was intermediate between wild-type Ler and rar1-13 (Figure 2A) and therefore not characteristic of constitutive resistance mutants. We then tested whether the enhanced Hpa resistance of the rsp mutants might correlate with increased basal expression or accelerated PR gene induction upon infection that would reflect priming of defenses (Conrath et al., 2002). Plants were infected with Hpa isolate Noco2. Samples were taken up to 72 h after inoculation and expression of the SA-responsive PR gene PR1 measured by quantitative RT-PCR. Individual analysis of variance (ANOVA) tests detected significant PR1 induction over time in all genotypes (Figure 2B). No significant differences in PR1 expression were detected between rar1-13, rar1-13 rsp1, and rar1-13 rsp2 (Figure 2B). These data show that the increased resistance of the rar1-13 rsp mutants cannot be explained by enhanced basal or Hpa-induced SA defenses. We infected plants with a virulent strain (DC3000) of the bacterial pathogen Pseudomonas syringae pv tomato (Pst) to test whether increased resistance of rar1-13 rsp mutants extended to an unrelated hemibiotrophic pathogen. Pst DC3000 grew more in rar1-13 and eds1-2 compared with Ler wild type, as expected (Figure 2C; Muskett et al., 2002). The rar1-13 rsp1 and rar1-13 rsp2 double mutants supported similar (rsp1) or higher (rsp2) bacterial growth compared with rar1-13 (Figure 2C). These data argue against the rsp mutations causing constitutive disease resistance.

To test whether increased resistance to Hpa observed in the rar1-13 rsp mutants extends to a different host-adapted obligate biotrophic pathogen, we inoculated plants with the adapted powdery mildew fungus Golovinomyces orontii (Go). Fungal entry into Arabidopsis epidermal cells is used as an indicator of early infection competence. The host cell entry rate of Go in rar1-13 rsp1 and rar1-13 rsp2 cells was therefore compared with that in wild-type Ler and the rar1-13 single mutant. Accession Col (susceptible) and the Col mlo2-6 (MILDEW RESISTANCE LOCUS O2) mutant, which is more resistant to Go (Consonni et al., 2006), were included as additional controls in the experiment. Fungal host entry rates were similar in Col and Ler and not altered by the rar1-13, rsp1, or rsp2 mutations, but significantly reduced in mlo2-6 (Figure 2D). Development of macroscopic disease symptoms was evaluated between 4 and 7 d after infection. There was enhanced sporulation on rar1-13 rsp2 plants compared with Ler and rar1-13 (see Supplemental Figure 2 online). Symptom formation on rar1-13 rsp1 was accompanied by leaf yellowing but Go sporulation itself was not altered. As expected, sporulation on mlo2-6 was reduced compared with Col (see Supplemental Figure 2 online). Since resistance of the rsp mutants does not extend to another host-adapted biotrophic pathogen, we concluded that the rsp1 and rsp2 defects perturb the Arabidopsis–Hpa interaction in quite a specific manner. This prompted us to clone and characterize the RSP1 and RSP2 genes.

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**Figure 1.** rsp1 and rsp2 Confer Strong Resistance against Different Hpa Isolates.

(A) Disease symptom formation on rsp mutant leaves. Three-week-old plants were infected with Hpa isolate Noco2, and first true leaves were stained with TB at 7 d after inoculation and examined under a light microscope. h, hyphae; TN, trailing necrosis. Bar = 0.5 mm.

(B) Conidiospore formation 6 d after infection of 3-week-old plants with Hpa isolate Cala2. Accession Col was included as a control expressing RPP2 resistance to Cala2. Standard deviations of three biological replicates are shown. Significantly different classes are indicated by lowercase letters (one-way ANOVA, Tukey’s post-hoc test, P < 0.05 each). FW, fresh weight.

**rsps1 and rsp2 Are Not Constitutive Resistance Mutants**

The rar1-13 rsp1 and rar1-13 rsp2 mutants are smaller than wild-type Ler or rar1-13 when grown under normal conditions in soil (see Supplemental Figure 1B online). This feature, together with their reduced susceptibility to a virulent Hpa isolate, suggested high susceptibility. By contrast, pathogen sporulation was strongly reduced on rar1-13 rsp1 and rar1-13 rsp2 mutant plants that grouped statistically with the fully resistant Columbia-0 (Col-0) accession (Figure 1B). We concluded that the mutations in rsp1 and rsp2 cause reduced susceptibility to Hpa infection independently of RPP5.
rsp2 is a Loss-of-Function Allele of DIHYDRODIPICOLINATE SYNTHASE2

When backcrossed rar1-13 rps2/RS2 plants were selfed, resistance to Hpa Noco2 segregated in a 1:3 ratio (296:108; χ² = 1.07) in the progeny indicative of a monogenic recessive trait. rar1-13 rps2 was crossed to the Col rar1-28 null mutant to generate segregating F2 families for map-based cloning. Although RPP5 also segregates in the progeny (Parker et al., 1997), we observed a clear 1:3 ratio of resistant to susceptible plants in the mapping population consistent with the enhanced resistance of rps2 being largely independent of RPP5 (Figure 1B). Linkage between rps2 resistance and the erecta morphology was found and rps2 was mapped in ~600 phenotyped F2 plants to an 86-kb interval on the lower arm of chromosome 2. This interval contains 26 annotated genes. DNA sequencing revealed a G-to-A exchange in the coding region of DIHYDRODIPICOLINATE SYNTHASE2 (DHDPS2, At2g45440), resulting in a predicted exchange of Gly-243 to Glu in DHDPS2 (Figures 3A and 3B).

DHDPS enzymes catalyze the formation of dihydrodipicolinate from L-aspartate-4-semialdehyde (ASA) as the committing step for biosynthesis of Lys in the biosynthesis of the Asp-derived amino acids Lys, Met, Thr, and Ile, also called the Lys superpathway (Jander and Joshi, 2010; see also scheme in Figure 5A). All reactions of this pathway from Asp phosphorylation to the amino acid end products Lys, Thr, Met, and Ile take place in the chloroplast (Ravanel et al., 2004). The mutated Gly residue in rps2 is highly conserved among DHDPS proteins (Figure 3B) and has a high bias in the DHDPS motif (http://pfam.sanger.ac.uk/family?acc=DHDPS#tabview=tab3). The recessive nature of rps2 suggests it is a reduced or loss-of-function (LOF) allele of DHDPS2. To test this, we searched for additional Arabidopsis dhdps2 mutations. From several candidate T-DNA insertions in DHDPS2 (http://signal.salk.edu/cgi-bin/tdnaexpress), one insertion in the 5' untranslated region of DHDPS2 was confirmed (Figure 3A). Homozygous plants of this line exhibited strong resistance compared with a control line containing the wild-type DHDPS2 locus isolated from the same population (Figure 3C).
We therefore concluded that *rsp2* is a LOF allele of *DHDPS2* and the mutant was renamed *dhdps2-2*.

The *Arabidopsis* genome contains two genes encoding DHAPS enzymes (http://www.Arabidopsis.org). To test whether *DHDPS1* also contributes to *Hpa* resistance, a T-DNA insertion mutant in *DHDPS1* (*At3g60880*) was isolated (see Supplemental Figure 3 online). Insertion of the T-DNA in an exon strongly reduced *DHDPS1* transcript abundance measured by RT-PCR, suggesting that this line is a null dhdps1 mutant. In contrast with *dhdps2-2* (*rsp2*), homozygous *dhdps1-1* mutant plants did not exhibit reduced susceptibility to *Hpa* Noco2 (see Supplemental Figure 3 online).

**rsp1** Carries a Mutation in ASPARTATE KINASE2

The *rsp1* phenotype was initially scored as a recessive monogenic trait and crossed to Col rar1-28 for mapping. However, scoring of mapping populations infected with *Hpa* Noco2 revealed that resistance was not strictly recessive and, depending on the infection conditions could appear dominant, suggesting dosage effects. *rsp1* was placed on the upper arm of chromosome 5 and a high-confidence interval of 360 kb containing 143 annotated loci was defined using a mapping population of 500 phenotyped F2 plants. From this interval, a locus encoding ASPARTATE KINASE2 (*AK2*, *At5g14060*) was sequenced because *rsp1* and *dhdps2-2* share common developmental defects (see Supplemental Figures 1 and 4 online), and we therefore suspected the mutations may affect the same pathway (see Figure 5A). A G-to-A exchange resulting in a predicted change of Val-430 to Met in *AK2* was detected (Figures 4A and 4B). Asp kinases catalyze the conversion of Asp to L-aspartyl-4-phosphate as the first step of the Lys superpathway. The *Arabidopsis* genome contains three genes encoding monofunctional Asp kinases and two genes encoding bifunctional aspartate kinase/homoserine dehydrogenase (HSDH) enzymes (Figure 5A). Little is known about the in vivo roles of the different isoenzymes, but kinetic parameters in vitro are well described (Curien et al., 2005, 2007). The monofunctional *AK2* and *AK3* enzymes are allosterically inhibited by Lys, while AK1 is synergistically inhibited by Lys and S-adenosylmethionine. The recently reported crystal structure of the *Arabidopsis* AK1 dimer revealed binding of both effectors to its ACT1 domain (Mas-Droux et al., 2006). We built a structural model for the AK2 dimer using the AK1 crystal structure as a template (Figure 4C). The Val-430 residue mutated in *rsp1*, which is not completely conserved among AK enzymes from phylogenetically distant organisms, is located at a homodimer interface built by the two ACT1 domains, and contacts the Val-430 in the other monomer (Figure 4C). Hence, the *rsp1* mutation might affect protein function by impairing homodimerization, although the mutation V430M could be modeled without provoking major steric clashes.

We isolated a putative *AK2* LOF allele from the T-DNA collections in accession Col in which the T-DNA insertion is located in an exon upstream of the mutation detected in *rsp1* (Figure 4B; see Supplemental Figure 5 online). Homozygous Col *ak2* plants were as susceptible as wild-type Col when tested with *Hpa* Noco2 (see Supplemental Figure 5B online). Therefore, we reasoned that *rsp1* is not a LOF allele of *AK2*. This is further supported by the presence of a Met residue at the same position in an AK from *Streptococcus equi* (YP_002743803). Val-430 is also very close to the binding site of the allosteric Lys molecule in AK1, which is conserved with that of the Lys-inhibited Escherichia coli enzyme (Mas-Droux et al., 2006). We therefore reasoned that the V430M mutation might instead affect feedback inhibition of the enzyme in vivo in accordance with the nonrecessivity of *rsp1* resistance.

**Figure 3.** Molecular Cloning of the *rsp2* Mutation.

(A) Organization of the *DHDPS2* locus. Positions of the *rsp2* mutation and T-DNA insertion in GABI_180_F08 are indicated. LB, left border.

(B) Alignment of DHAPS proteins and position of the amino acid exchange in *rsp2*. (At, Q0WSN6; Os, Q7XP85; Pp, A9SGY8; Ec, C9QP6).

(C) Conidiospore formation 7 d after infection of 3-week-old plants with *Hpa* isolate Noco2. Biological replicates were pooled and treated as one sample. Error bars are derived from five technical replicates. FW, fresh weight.

[See online article for color version of this figure.]
Both \textit{rsp1} and \textit{rsp2/dhdps2-2} Strongly Perturb Amino Acid Homeostasis

Large differences in accumulation of products of the Lys superpathway have been reported for the previously characterized \textit{dhdps2-1} mutant (Craciun et al., 2000; Sarrobert et al., 2000). We investigated whether this is also the case for our newly identified \textit{rsp2/dhdps2-2} mutant and possibly \textit{rsp1}. Polar metabolite extracts from aerial tissue of soil-grown plants were therefore prepared and analyzed by gas chromatography–mass spectrometry (GC-MS). Relative peak areas for signals corresponding to Lys, Ile, Met, and Thr were determined and normalized to \textit{Ler} wild type, which was set at 100% (Figure 5B). No significant changes were detected between the wild type and \textit{rar1-13}. In \textit{rsp2/dhdps2-2}, higher accumulation of all pathway end products was observed, except Lys, which remained unchanged. Remarkably, a 60-fold increase in Thr levels was detected in \textit{rsp2/dhdps2-2} compared with the wild type. The amino acid profile of \textit{rsp1} was similar to \textit{rsp2/dhdps2-2} except that Lys levels increased three- to fourfold (Figure 5B). Altered amino acid levels, including Lys in \textit{rsp1} mutant plants, are consistent with our hypothesis that the \textit{rsp1} mutant protein has altered inhibition properties leading to deregulated flux of Asp, since the two DHDPS enzymes are normally tightly regulated by feedback inhibition through Lys (Craciun et al., 2000; Vauterin et al., 2000).

\textit{rsp1} Is a Loss-of-Inhibition Allele of \textit{AK2}

We tested whether the \textit{AK2} \textit{V}_{430}M mutation in \textit{rsp1} could indeed give rise to an enzyme refractive to Lys inhibition by expressing wild-type \textit{AK2} and mutant \textit{AK2^{rsp1}} proteins lacking the chloroplast targeting peptide in \textit{E. coli} as C-terminal fusions to glutathione S-transferase (GST). Affinity-purified GST-\textit{AK2} exhibited strong AK activity when tested in the presence of saturating concentrations of Asp and ATP (80 and 20 mM, respectively), whereas reactions containing a control protein purified under the same conditions did not show activity. The observed activity was fully dependent on the presence of Asp in the reaction mixture (data not shown). Hence, the GST-tagged protein is active and could be used for inhibition assays. GST-\textit{AK2} and GST-\textit{AK2^{rsp1}} activities were compared under physiological conditions (2 mM ATP and 1 mM Asp) for inhibition by Lys (Figure 6A, top graph). A decrease in activity of the wild-type enzyme was observed with low concentrations of 10 to 25 \textit{mM} Lys, as previously described (Curien et al., 2007). By contrast, even high Lys concentrations (up to 250 \textit{mM}) did not lead to appreciable inhibition of \textit{AK2^{rsp1}} in our assays. We confirmed this result under conditions of higher substrate availability (5 mM Asp and 10 mM ATP). Although the Lys concentration range for efficient inhibition of GST-\textit{AK2} shifted, differential inhibition properties of GST-\textit{AK2^{rsp1}} remained (Figure 6A, bottom graph). A decrease in activity of the wild-type enzyme was observed with low concentrations of 10 to 25 \textit{mM} Lys, as previously described (Curien et al., 2007). By contrast, even high Lys concentrations (up to 250 \textit{mM}) did not lead to appreciable inhibition of \textit{AK2^{rsp1}} in our assays. We confirmed this result under conditions of higher substrate availability (5 mM Asp and 10 mM ATP). Although the Lys concentration range for efficient inhibition of GST-\textit{AK2} shifted, differential inhibition properties of GST-\textit{AK2^{rsp1}} remained (Figure 6A, bottom graph). Thus, a considerably higher activity of the \textit{AK2^{rsp1}} enzyme would be expected at physiological levels of Lys sufficient to inhibit the wild-type enzyme. We also tested whether the \textit{AK2^{rsp1}} mutation causes accumulation of amino acids generated by the Lys superpathway in \textit{Arabidopsis} aerial tissues by transforming Col with constructs of \textit{AK2} or \textit{AK2^{rsp1}} driven by the constitutive cauliflower mosaic virus 3SS promoter. In T1 plants (selected for the cotransformed BASTA resistance marker and confirmed by PCR), 4/30 3SS-\textit{AK2^{rsp1}} transformants exhibited abnormal growth reminiscent of \textit{rsp1},
Accumulation of the Asp-derived amino acids Lys, Met, Thr, and Ile in the phenotype. TCA, tricarboxylic acid.

Figure 5. The Lys Superpathway and Accumulation of Its End Products in rsp Mutants.

(A) Scheme showing the biosynthesis of Asp-derived amino acids in Arabidopsis. Bifunctional AK-HSDH enzymes are shown in brackets. Allosteric inhibition mechanisms mentioned in the text are indicated with negative impacting lines. Mutants identified in this or previous studies are shown in black (not tested), red (resistant to Hpa), or green (no resistance to Hpa). AK2rsp1 hyphal outgrowth and no host cell death (Figure 7A). In susceptible Ler wild type, 75% of primary infection sites produced hyphal extension from these sites (Figure 7A). In susceptible Ler wild type, 75% of primary infection sites produced hyphal extension from these sites (Figure 7A). In susceptible Ler wild type, 75% of primary infection sites produced hyphal extension from these sites (Figure 7A).

(B) Accumulation of the Asp-derived amino acids Lys, Met, Thr, and Ile in rsp mutant and control plants. Polar metabolites were extracted from aerial tissue of 4-week-old soil-grown plants and analyzed by GC-MS. Values were normalized to Ler = 100%, and standard deviations from three independent samples are shown. Significantly different classes are indicated by lower-case letters (one-way ANOVA, Tukey’s post-hoc test, P < 0.05 each). Trends were confirmed in an independent experiment.

whereas all 35S:AK2 transformants (>30) grew normally (see Supplemental Figure 6 online). Thr accumulation in aerial tissues of individual 35S:AK2 and 35S:AK2rp1 transformants was measured by HPLC (Figure 6B). Analysis was restricted to the T1 generation because most of the abnormal 35S:AK2rp1 transformants failed to produce seed. Two biological replicates of Col tissue produced highly similar Thr values (37.9 ± 1.2 pmol/mg fresh weight), indicating that major differences could be detected by single measurements. Also, the effect of BASTA pretreatment was evaluated with a control transgenic line not expressing AK2. Thr content measured for this line without treatment was highly similar to Col wild type and decreased by ~25% after BASTA treatment. The Thr content of 35S:AK2 transformants ranged between 13 and 32 pmol/mg (Figure 6B). Similar values were obtained from the phenotypically normal 35S:AK2rp1 transformants. By contrast, massive accumulation of Thr was detected in the rps1-like transformants, rising to ~150-fold higher Thr content than the wild type. When 35S:AK2 and 35S:AK2rp1 T1 plants were inoculated with Hpa Noco2, pathogen sporulation was observed on all plants except rps1-like 35S:AK2rp1 transformants included in the experiment, consistent with loss of susceptibility caused by AK2rp1. The increased accumulation of Lys, Thr, Ile, and Met in rps1 (Figure 5), the reduced Lys sensitivity of recombinant AK2rp1 protein (Figure 6A), and the high Thr content of 35S:AK2rp1 transformants phenotypically resembling the original rps1 mutant lead us to conclude that rps1 is a loss-of-inhibition allele of AK2. rps1 was therefore renamed AK2rp1 to differentiate it from LOF alleles.

AK2rp1 and rps2/dhdps2-2 Impede Hpa at an Early Stage of Infection

Having identified the molecular lesions underlying the rps mutant phenotypes, we isolated single AK2rp1 and rps2/dhdps2-2 mutants to characterize their impact on Hpa infection in a RAR1 background with a fully functioning innate immune system. When infected with virulent Hpa Cala2, the rps single mutants exhibited similar levels of resistance as the corresponding rps rar1-13 double mutant combinations (see Supplemental Figure 7 online), confirming that the rps phenotype manifests independently of the RAR1 status. We examined early stages of Hpa Cala2 infection (24 h after inoculation) by staining oomycete structures on the leaf surface with the optical brightener calcofluor. At this time point, germinating and fully germinated spores were visible on Ler wild-type leaves, although the germination rate was low (~1%). Spore germination on rps1 leaves was indistinguishable from the wild type. By contrast, the germination rate appeared lower on rps2, and we rarely located a germinating spore. This result suggested that Hpa colonization is impeded at a very early time point in rps2. We then examined infection sites in the first pair of true leaves of wild-type and rps mutant plants at 48 h after inoculation with Cala2 by TB staining. In the genetically resistant accession Col (due to RPP2 recognition; Sinapidou et al., 2004), each infection site was associated with host cell death and there was no hyphal extension from these sites (Figure 7A). In susceptible Ler wild type, 75% of primary infection sites produced hyphal outgrowth and no host cell death (Figure 7A). In AK2rp1 and rps2/dhdps2-2 mutant leaves, the proportion of infection
sites producing hyphal outgrowth reduced to 16 and 5%, respectively, but at no site was this associated with host cell death. Therefore, the reduced susceptibility of \( \text{AK2}^{\text{rsp1}} \) and \( \text{rsp2/} \text{dhdp} \text{s2-2} \) mutants to \( \text{Hpa} \) infection is not due to activation of a classical resistance response. We found that \( \text{Hpa} \) could grow and form conidiophores at a low level on cotyledons of \( \text{AK2}^{\text{rsp1}} \) and \( \text{dhdp} \text{s2-2/} \text{dhdp} \text{s2-2} \) and occasionally on true leaves of \( \text{AK2}^{\text{rsp2}} \) plants (Figure 7B). These phenotypes are in line with a metabolic imbalance or nutrient deficiency in the host-limiting early \( \text{Hpa} \) colonization of tissues.

The reduced growth of \( \text{rsp} \) mutant plants could be partially complemented on synthetic media by addition of Suc but not other osmolytes or signaling sugars to the media (see Supplemental Figures 8A and 8B online). Since this suggested that the \( \text{rsp} \) mutants might be limited for carbohydrate, we tested whether Suc could also restore susceptibility of \( \text{rsp} \) mutant plants to \( \text{Hpa} \). Plants were initially grown on synthetic media containing Suc and were then transferred to soil at different time points before infection with \( \text{Hpa} \) and infections monitored by TB staining. Transfer of plants prior to infection increased disease susceptibility of wild-type and \( \text{rsp} \) mutant plants to a similar extent (see Supplemental Figure 8C online). Nutrient or carbohydrate deficiency is therefore unlikely to underlie \( \text{rsp} \) resistance to \( \text{Hpa} \).

Testing of Candidate Metabolites for Resistance Induction in \( \text{rsp} \) Mutants

We reasoned that imbalances within the Lys superpathway might lead to increased production of secondary metabolites with resistance properties. For example, camalexin and indolic glucosinolates derived from Trp are important for resistance to adapted and nonadapted fungi (Bednarek et al., 2009, and references therein) and the hemibiotrophic oomycete pathogen *Phytophthora brassicae* (Schlaeppi et al., 2010). We measured levels of indole-derived secondary metabolites in wild-type and \( \text{rsp} \) mutant leaves. There were no differences in accumulation of the phytoalexin camalexin, but two- to threefold increased 1-methoxyindol-3-ylmethylglucosinolate levels were detected in \( \text{rsp1} \) and \( \text{rupt} \) mutant extracts (see Supplemental Figure 10 online). We therefore tested the importance of indolic secondary metabolites in \( \text{Hpa} \) resistance by infecting \( \text{Hpa} \) mutant supported lower sporulation of the phytoalexin camalexin, but two- to threefold increased 1-methoxyindol-3-ylmethylglucosinolate levels were detected in \( \text{rsp1} \) and \( \text{rupt} \) mutant extracts (see Supplemental Figure 10 online). We therefore tested the importance of indolic secondary metabolites in \( \text{Hpa} \) resistance by infecting \( \text{Hpa} \) mutant supported lower sporulation of virulent \( \text{Hpa} \) isolate Noco2 (Figure 8A), suggesting that indolic compounds do not contribute to *Arabidopsis* resistance to \( \text{Hpa} \).

Both \( \text{rsp1} \) and \( \text{rupt} \) accumulate high levels of Met, which is the precursor of 5'-adenosylmethionine, polyamines, ethylene, and aliphatic glucosinolates (Figure 5A). To test whether high Met content, either directly or through increased production of downstream metabolites, might contribute to resistance in the \( \text{rup} \) mutants, we measured \( \text{Hpa} \) sporulation on \( \text{mto1-1} \) mutant plants carrying a mutation in a gene encoding cystathionine \( \gamma \)-synthase (Figure 5A), which leads to high Met accumulation (Inaba et al., 1994; Chiba et al., 1999). The \( \text{mto1-1} \) mutant plants supported similar levels of \( \text{Hpa} \) sporulation as the wild type (Figure 8B). Therefore, increased Met and its downstream metabolites do not explain the resistance in \( \text{rsp1} \) and \( \text{rup} \).

\( \text{rup1} \) and \( \text{rup2} \) also accumulate high levels of Thr and Ile. *Arabidopsis* mutants with increased levels of Thr and/or Ile in aerial tissue have been reported (Kim and Leustek, 2000; Garcia and Mourad, 2004), but these were unobtainable. Therefore, we...
spray applied amino acid solutions onto hypersusceptible *L. eridania* plants to mimic the mutant metabolic state prior to infection with *H. pannorum* Cala2. HS was included in these experiments as induction of resistance to *Hpa* by this metabolite was recently reported (van Damme et al., 2009). Pretreatment of plants with Ile did not alter *Hpa* sporulation (Figure 8C). By contrast, sporulation was significantly reduced after HS and, to a stronger extent, Thr pretreatment. We detected only minor HS increases in *rsp* mutant tissues (see Supplemental Figure 11 online), suggesting that Thr accumulation is more likely to be causal for reduced *Hpa* growth on *rsp* mutant plants. Notably, suppression of *Hpa* growth by Thr application occurred in a dose-dependent manner (see Supplemental Figure 12 online).

Although our experiments indicate that indolic glucosinolates themselves are unlikely to contribute to *Hpa* resistance (Figure 8A), we examined whether jasmonate (*JA*)-regulated defenses...
might underlie the \( \text{rsp} \) mutant phenotypes since JA application is known to induce indolic glucosinolate metabolites (Mikkelsen et al., 2003). The expression of JA marker genes was not significantly different between wild-type and \( \text{rsp} \) mutant tissues, and Thr application induced resistance to \( \text{Hpa} \) to the same extent in plants defective for JA biosynthesis or signaling (see Supplemental Figure 13 online). Therefore, JA-mediated defenses are not responsible for Thr-induced suppression of \( \text{Hpa} \) infection.

**Thr Treatment Recapitulates \( \text{rsp} \) Mutant Resistance Phenotypes**

Suppression of \( \text{Hpa} \) growth by Thr application might result from general toxicity of this metabolite and thus be unrelated to the \( \text{rsp} \) mutant phenotypes. To exclude this possibility, the same set of plants pretreated with Thr was infected with \( \text{Hpa} \) or Go. Col plants were used for this assay because Go sporulation was most uniform on this accession. Leaves from plants infected with virulent \( \text{Hpa} \) isolate Noco2 were stained with TB and examined under the microscope at 7 days after inoculation. \( \text{Hpa} \) growth was observed in all mock-treated leaves but not in Thr pretreated leaves (Figure 9A, left panel). By contrast, similar levels of Go sporulation were observed on mock- and Thr-treated plants (Figure 9A, right panel). Selective suppression of \( \text{Hpa} \) growth observed with the \( \text{rsp} \) mutants can therefore be reproduced by Thr application, excluding broad toxicity of Thr in the chemical treatment experiments.

We then tested whether Thr indeed accumulates in plant tissues after application. Since spray application would not allow us to discriminate between absorbed and surface-deposited Thr, we grew plants on synthetic media containing different concentrations of Thr and measured Thr accumulation in aerial tissues that were not in direct contact with the metabolite. We found that the \( \text{Hpa} \) infection-suppressing action of Thr could be reproduced under these conditions. Ler \( \text{eds} \)-1-2 plants grown on Murashige and Skoog medium in the presence of 0 to 1.5 mM Thr were infected with \( \text{Hpa} \) Cala2 and symptom formation scored at 6 days after inoculation. The numbers of conidiophores produced diminished with increasing concentrations of Thr added to the medium (Figure 9B). Thr accumulation was measured by HPLC. Whereas aerial tissues of control plants contained \( \approx 0.3 \text{ nmol/mg (fresh weight)} \) Thr, this increased to \( \approx 3 \text{ nmol/mg in tissues grown on 1 mM Thr (Figure 9C, left graph). We concluded that Thr accumulation in aerial tissue upon spray treatment is therefore highly likely. As inclusion of 1 mM Thr to the medium strongly suppressed \( \text{Hpa} \) infection in the plate assay, we analyzed the extent to which the Thr amounts measured under these conditions relate to \( \text{rsp} \) mutant Thr contents. Because \( \text{rsp} \) mutant plants cannot be cultivated on synthetic media lacking Suc (see Supplemental Figure 8 online) leaf samples of soil-grown \( \text{rsp} \) mutant and control plants were included in the HPLC analysis (Figure 9C, right graph). Thr amounts were similar in the in vitro- and soil-grown control plants, ranging from 0.33 to 0.36 nmol/mg, respectively (Figure 9C). Notably, Thr accumulation in aerial tissues of plants grown on media containing 1 mM Thr was comparable to levels found in \( \text{rsp} \) mutant tissues (ranging from 3.1 to 3.4 nmol/mg; Figure 9C). Altogether, the results suggest that Thr application closely mirrors the \( \text{rsp} \) mutant phenotypes, both with regard to Thr accumulation and suppression of \( \text{Hpa} \) infection.

**Effects of Thr on Plant and \( \text{Hpa} \) Growth Correlate with Deployment of the Diaminopimelate Pathway**

Thr toxicity for \( \text{Arabidopsis} \) grown in vitro has been described (Sarróbert et al., 2000), and expression of an \( \text{E. coli} \) Thr synthase in \( \text{Arabidopsis} \) produced Thr overaccumulating plants with wrinkled and thickened rosette leaves and infertility (Lee et al., 2005), phenotypes broadly resembling the \( \text{rsp} \)1 and \( \text{rsp} \)2 mutants and \( \text{AK2}^{\text{Hpa}^{-1}} \) transgenic plants (see Supplemental Figures 1, 2, and 6 online). Also, lethality of \( \text{tha} \)2-1 mutant plants defective in \( \text{THRE-ONINE ALDOLASE2} \), one of two \( \text{Arabidopsis} \) enzymes converting Thr to Gly, can be rescued by expression of \( \text{omr} \)1-5, a feedback-insensitive Thr deaminase (Figure 5A; Garcia and Mourad, 2004; Joshi et al., 2006), suggesting that \( \text{tha} \)2-1 lethality is due to Thr toxicity (Joshi et al., 2006). Thr overaccumulation therefore appears to be detrimental to \( \text{Arabidopsis} \). The \( \text{Hpa} \) infection phenotypes of \( \text{rsp} \) mutants (Figure 1) and chemical treatment results (Figure 9) point to a negative effect of Thr on \( \text{Hpa} \) but not Go infection. We reasoned that the target of Thr interference or biosynthetic pathway negatively affected by high Thr accumulation might be conserved among plants and oomycetes but not in the fungal ascomycete Go. The presence of genes for biosynthesis of the amino acids Lys, Thr, and Met was therefore compared in a targeted manner between the three different phyla. Thr and Met are derived from Asp in all organisms and their biosynthesis appears to be broadly similar. Ascomycetes use the \( \alpha \)-aminoadipate (AAA) pathway with \( \alpha \)-ketoglutarate serving as a precursor for biosynthesis of Lys, which thus belongs to the Glu family of amino acids (reviewed in Xu et al., 2006). By contrast, Lys is produced through the diaminopimelate (DAP) pathway in plants and oomycetes and belongs to the Asp family (Figure 5A; Randall et al., 2005; Hudson et al., 2006).

In order to test for deployment of the DAP pathway by Thr, homologs of DHDPIS, dihydrodipicolinate reductase (DHDPPR), and diaminopimelate decarboxylase (LysA), which are common to all types of DAP pathway, were searched for in the newly available \( \text{Hpa} \) genome (see Supplemental Table 1 online; Baxter et al., 2010). Sequences with high similarity to \( \text{E. coli} \) DHDPSS and LysA were identified for \( \text{Hpa} \) DAPPR and LysA were detected. For DHDPPR, a sequence with moderate similarity to \( \text{E. coli} \) DapB was found. Notably, the Pfam DHDPPR N-terminal (PF01113) and C-terminal (PF05173) domains were identified in the predicted \( \text{Hpa} \) DHDPPR protein. The presence of all three marker genes suggests that Lys biosynthesis occurs via the DAP pathway in \( \text{Hpa} \). No sequences supporting the presence of these enzymes were obtained when searching the Go and related \( \text{Blumeria graminis} \) \( \text{Bg} \) genome assemblies (Spanu et al., 2010). By contrast, there was strong evidence for the presence of the AAA pathway in Go and \( \text{Bg} \) (see Supplemental Table 1 online). Sequences similar to genes of the AAA pathway were also detected in \( \text{Hpa} \). Reciprocal sequence comparisons generally revealed higher similarity to proteins of different function, as previously described for \( \text{Phytophthora infestans} \) (Randall et al., 2005), arguing against co-option of both DAP and AAA pathways for Lys biosynthesis in oomycetes. We concluded that detrimental effects of Thr on \( \text{Arabidopsis} \) growth and \( \text{Hpa} \) infection likely
reflect operation of a complex and highly regulated DAP pathway in both systems.

DISCUSSION

Obligate biotrophy implies strong interdependence between host metabolism and nutrient uptake by the pathogen, but the processes allowing establishment and maintenance of a compatible interaction are poorly understood. Here, we show that the primary amino acid metabolic status of a plant can profoundly affect its suitability as an infection substrate for the adapted obligate biotrophic oomycete pathogen, Hpa.

The rsp2 LOF mutant carries a lesion in DHDPS2 (Figure 3), one of two Arabidopsis DHDPS enzymes catalyzing the conversion of ASA to L-2,3-dihydricolinate as the committing step in Lys biosynthesis (Figure 5A). Although expression of DHDPS2 in Arabidopsis was initially reported to be restricted to the root tip (Sarrobert et al., 2000) and mainly vascular tissue in aerial parts (Craciun et al., 2000), analysis of public microarray data (http://www.genevestigator.com) suggests similar levels of DHDPS2 expression in roots and rosette leaves, both of which are colonized by Hpa (Coates and Beynon, 2010). DHDPS1 has a similar expression pattern but with lower overall signal intensity according to microarray data. Since dhdps1 mutant plants did not exhibit altered growth or pathogen resistance (see Supplemental Figure 3 online), DHDPS2 probably accounts for the main DHDPS activity in Arabidopsis. We did not attempt to generate double mutants, as these would be expected to be lethal because no alternative route for Lys biosynthesis is known. Both DHDP and HSDH, which catalyzes the committing step toward the biosynthesis of Met, Thr, and Ile, use ASA as a common substrate. Due to flux partitioning at the DHDPS/HSDH node, loss of the major DHDPS isoform leads to increased accumulation of products of the Met, Thr, and Ile branch (Figure 5). As Lys is a key regulator of Asp kinases (Curien et al., 2007), flux into the entire pathway is likely increased through compensatory control to reestablish Lys accumulation (for a kinetic model of the pathway, see Curien et al., 2009).

The rsp1 mutation isolated in our study perturbs the Lys superpathway in a different manner to rsp2/dhdps2-2. We show that rsp1 is a loss-of-inhibition allele of AK2, which renders the mutant AK2<sup>rsp1</sup> protein refractive to allosteric inhibition by Lys under physiological conditions (Figures 4 and 6). Allosteric transitions deduced from the structures of E. coli AKIII suggest that subtle interdomain movements at the dimer interface are involved in the R- to T-state transition (Kotaka et al., 2006). The exchange of Val-430 located in the dimer interface to Met in AK2<sup>rsp1</sup> might impede state transition or directly interfere with inhibitor binding. This molecular characterization of a feedback-insensitive AK variant from Arabidopsis adds to knowledge...
gained by prior isolation of presumed aspartate kinase mutants with altered regulatory properties (Heremans and Jacobs, 1997). Consistent with a failure in feedback inhibition, expression of AK2<sup>ras1</sup> from its endogenous locus increases flux into the Lys superpathway leading to accumulation of all pathway end products (Figure 5B).

AK2 and DHDS2 were not obviously linked to plant immunity. This is probably because mutations in these genes do not lead to broad spectrum resistance (Figure 2) but appear to specifically impede colonization by Hpa. Basal innate immunity to adapted Hpa isolates is normally mediated by SA-dependent processes and numerous mutants with constitutively induced SA defenses display enhanced resistance to Hpa (Lu et al., 2003; Zhang et al., 2003). In contrast with these mutants, neither rspl nor rsps2 has characteristics of primed or constitutive SA pathway activation (Figure 2). Importantly, in an immune-competent wild-type background, rspl and rsps2 suppressed colonization by virulent Hpa isolate Cala2 at an early stage of infection, although the sporadic microcolonies formed in rspl did eventually grow and produce some spores (Figure 7). None of the successful or unsuccessful infection sites were associated with host cell death in the rsps2 mutants. Therefore, the reduced Hpa growth on rspl and rsps2 leaves is not through activation of a classical immune response but rather due to a loss of susceptibility. We conclude that perturbations in host metabolism render tissues unsuitable as an Hpa infection substrate.

We performed a number of experiments to elucidate how the host metabolic status might interfere with Hpa infection. While provision of Suc partially restored rspl mutant plant growth in vitro, scarcity of carbohydrates is unlikely to underlie the reduced susceptibility of rspl and rsps2 plants because Hpa growth was not appreciably restored by Suc (see Supplemental Figure 8 online). Suppression of Hpa growth through increased accumulation of indolic glucosinolates or Met-derived compounds is also not supported by our data (Figure 8; see Supplemental Figure 10 online). In chemical application experiments, we identified Thr as a potent inhibitor of Hpa growth (Figure 8C). Indeed, the consequences of Thr application compare well with rspl mutant phenotypes both with regard to selective suppression of Hpa growth and to absolute Thr concentrations (Figure 9). It is possible that a toxic metabolite derived from or induced by Thr accumulates upon increased Thr abundance. Since neither rspl mutant metabolic state nor Thr application interfered with growth of the adapted biotrophic ascomycete Go (Figure 9A; see Supplemental Figure 2 online), such a metabolite would have to act specifically on Arabidopsis and Hpa, but not Go. Alternatively, Thr itself could interfere with host and pathogen biosynthetic pathways. Negative effects of Thr by feedback inhibition of the Lys superpathway have been proposed, although failure to rescue lethality of tha2-1 mutant plants accumulating excess Thr by amino acid supplementation suggests otherwise (Joshi et al., 2006). Similarly, we could not restore Hpa growth on rspl mutant plants by providing an amino acid solution upon infection. Surprisingly, however, we also could not rescue the poor growth of the rsps2/ dhdsps2-2 mutant by Lys supplementation of synthetic media lacking Suc. This suggests an unexpected degree of compartmentalization or additional regulatory mechanisms operating within the Lys superpathway. The precise molecular processes underlying Thr interference remain to be elucidated. We think it likely that Thr overaccumulation interferes with both host and oomycete metabolic processes due to the relatively close phylogeny of the two organisms and the deployment of the DAP pathway in contrast with the more distant ascomycete Go (see Supplemental Table 1 online; Burki et al., 2007).

Two additional loci, AGD2 and DMR1, encoding enzymes of the Lys superpathway have previously been described as having effects on plant immune responses. AGD2 was shown to be a LL-diaminopimelate decarboxylase catalyzing the last step of Lys biosynthesis (Figure 5A; Hudson et al., 2006), and agd2 mutant plants exhibited increased resistance to bacteria and Hpa (Song et al., 2004). We measured the amino acid content of agd2 and detected an approximately twofold increase in Thr content (see Supplemental Figure 15 online), supporting relevance to Lys biosynthesis in vivo. While this small increase in Thr content might contribute to agd2 Hpa resistance, the mutant does not resemble rspl and rsps2 since it displays constitutive resistance (Song et al., 2004). By contrast, dmr1 mutant plants defective in homoserine kinase were resistant to Hpa without the hallmarks of constitutive resistance (van Damme et al., 2009). The similar phenotypes of three independent mutants (rsps1, rsps2, and dmr1) affected in enzymes of the Lys superpathway point to a common mechanism leading to impairment of Hpa infection. Our data support Thr as being causal for Hpa growth suppression in rsps2 mutant tissues, whereas dmr1 mutant plants preferentially accumulate HS (van Damme et al., 2009). It is conceivable that HS taken up by Hpa is subsequently converted to Thr, which might then accumulate in Hpa tissues. In Arabidopsis, HS is rate limiting for the accumulation of downstream metabolites under normal conditions and HS supplementation leads to Hpa accumulation (Lee et al., 2005). Genes encoding enzymes of the initial and final reactions of Thr, Ile, and Met biosynthesis and for putative amino acid transporters/permeases are present in Hpa according to primary transcript annotations (http://vmd.vbi.vt.edu/query.php). Also, van Damme et al. (2009) showed HS-induced Hpa growth suppression to be independent from known defense pathways. These data lend support to our hypothesis that incompatibility with Hpa arises by an imbalance in amino acid homeostasis. Here, characterization of the Arabidopsis rsps1 and rsps2 mutants provides a new insight to how plant metabolic status can selectively determine interactions with pathogens. It also prompts a deeper comparative analysis of biotrophic pathogen metabolism uptake and assimilation systems that may also influence host plant selection.

METHODS

Plant Material, Growth Conditions, and Pathogenicity Assays

Wild-type Arabidopsis thaliana accessions used were Col-0 and Ler. The Ler rar1-13, rar1-15 (Musket et al., 2002), eds1-2 (Aarts et al., 1998), and Col mlo2-6 (Consonni et al., 2006), mto1-1 (Inaba et al., 1994), and cyp79B2 cyp79B3 (Zhao et al., 2002) mutants are published. Col dhdp2-1 (SALK_147470), dhdp2-2 (GABI-KAT_180F08) (Rosso et al., 2003), ak2-1 (SAIL_258_E06, Syngenta), Ler rsps2/dhdp2-2, and rsps1/AK2<sup>ras1</sup> are characterized here. Oligonucleotides used for genotyping are listed in Supplemental Table 2 online. Plants were grown in soil in controlled environment chambers under a 10-h light regime (150 to 200 μE/m²s) at
Expression Analyses

Plants were spray infected with Hpa Noco2 as described above, and leaf samples (~50 mg) from different plants were taken at the indicated time points. Total RNA was extracted from leaves using TRI reagent (Sigma-Aldrich), and RNA was reverse transcribed into cDNA using SuperScriptII reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR experiments were performed in an iQ5 Real-Time PCR detection system (Bio-Rad) using Brilliant SYBR Green QPCR Core Reagent (Invitrogen) following the manufacturer's instructions. Primers used are listed in Supplemental Table 2 online.

GC-MS Analyses

SA quantification was done as previously described (Straus et al., 2010). For amino acid analysis, metabolites were extracted from 100 mg ground leaf tissue in 1 mL CHCl3/CH3OH/water (1:2:0:3). After shaking for 10 min at 70°C, samples were centrifuged and reextracted with 500 μL CHCl3/CH3OH (2:1). Five hundred microliter of water was added to the pooled supernatants, which were then centrifuged for phase separation. The upper phase was collected and dried. To separate polar from semipolar metabolites, the dried extract was resuspended in 0.5 mL of water with 0.1% trifluoroacetic acid and loaded onto a 100 mg DCS-18 solid phase column (Agilent). Amino acids were identified by running commercial standards under the same conditions and quantified with Chemstation software from Agilent.

HPLC Analysis

Analysis of indolic glucosinolates was performed as previously described (Bednarek et al., 2009). For amino acid analyses, 100 mg leaf material was used for extractions if available, but less tissue was used for severely affected 3SS:AK2STOP transgenic plants. Free amino acids were determined using a modified protocol from Scheible et al. (1997). Plant material was extracted for 20 min at 4°C with 400 μL 80% (v/v) aqueous ethyl acetate (2.5 mM HEPES-KOH, pH 7.5) and 400 μL 50% (v/v) aqueous ethanol (2.5 mM HEPES-KOH, pH 7.5) and then 200 μL 80% (v/v) aqueous ethanol. Amino acids were measured in the collected supernatants by precolumn derivatization with orthophthaldehyde in combination with fluorescence detection (λex 330/λem 450) as described (Kreft et al., 2003). Elution was achieved on a Hyperclone C18 BDS column (Phenomenex) on a Summit HPLC system ( Dionex), applying a solvent gradient with increasing hydrophobicity (buffer A: 0.2% [v/v] THF, 8.5 mM sodium phosphate buffer [NAPI], pH 7.5; buffer B: 32.5% [v/v] methanol, 20.5% [v/v] acetonitrile, and 18.5 mM NAPI, pH 7.5; flow: 0.8 mL/min; 0 to 2 min: 100% A, 16 min: 77% A, 13% B; 23.25 min: 15% A, 85% B; 32.23 min: 50% A, 50% B; 43.30 min: 40% A, 60% B; 49 to 52 min 100% B, 53 to 60 min: 100% A).

Protein Alignments and Modeling

Protein alignments were made using T-Coffee (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee cgi/index.cgi) and graphical views generated with ESPript (http://esprit.ibcp.fr/ESPript/ESPript/). Comparative modeling of the At AK2 structure was performed with the modeller9v3 program (Sali and Blundell, 1993) using the structure of At AK1 (PDB: 2CDQ) as template. The high sequence identity between At AK1 and At AK2 (77%) ensures that errors in the model should not exceed 1 Å in Cα root mean square deviation. Mutation V785M in each chain of the dimer At AK2 could be modeled using the backrub module of the rosettav3 program (Smith and Kortermme, 2008). Figures were generated using Pymol.

Stable Transgenic Lines

The AK2 coding region was amplified from cDNA and cloned into pENTR/D-TOPO (Invitrogen), and the rps1 mutation was introduced using site-directed mutagenesis by PCR with complementary oligonucleotides. Primers are listed in Supplemental Table 2 online. Wild-type and mutant AK2 sequences were recombined into Gateway-converted pAM-PAT-MCS. Plasmids were transformed into Agrobacterium tumefaciens strain GV3101:pMP90RK for transformation of Arabidopsis plants using the floral dip method (Logemann et al., 2006).

Recombinant Protein Expression and Purification

A cDNA sequence coding for AK2 with the initiating Met introduced at position 61 to remove the chloroplast targeting peptide was amplified by PCR and cloned into pENTR/D-TOPO, yielding pE AK2_STOP. The rps1 mutation was introduced by site-directed mutagenesis giving rise to pE AK2rps1STOP. Sequences were recombined into Gateway-converted pDEST15 (Invitrogen) and confirmed plasmids were transformed into Escherichia coli Rosetta (Novagen). For protein expression, bacteria were grown at 37°C in Luria-Bertani media to an OD600 of 0.6, and expression was then induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, and bacteria were grown for additional 16 h at 15°C. Bacteria were harvested by centrifugation, taken up in buffer A (50 mM Tris, pH 7.4, 50 mM KCl, 2 mM l lysine, 2 mM DTT, 1 mM EDTA, and 10% glycerol) supplemented with Complete Protease inhibitor without EDTA (Roche), DNasel, and Lysozyme, and lysed by sonification. Lysates were cleared by centrifugation (30 min, 4°C, 30,000g), filtered through 0.22-μm PES membrane filters.
and batch incubated with 1 mL preequilibrated GST-Sepharose (GE Healthcare) for 1 h. Beads were transferred to 10-mL filter columns (Bio-Rad) and washed with buffer A, then with buffer A containing 300 mM NaCl and finally reequilibrated with buffer A. Proteins were eluted with buffer A containing 10 mM reduced glutathione, concentrated using Vivaspin devices (Sartorius), and buffer exchanged on a HiTrap column (GE Healthcare) for 1 h. Beads were transferred to 10-mL filter columns (Bio-Rad) and washed with buffer A, then with buffer A containing 300 mM FeCl₃, 0.5 M HCl, and 20% (w/v) trichloractetic acid, and absorbance was measured at 490 nm. The assays were repeated with higher substrate concentrations (5 mM Asp and 10 mM ATP), as these conditions yielded more robust data.

Biokinase Activity Assay

AK was assayed using the hydroxamate assay as previously described (Ferreira et al., 2006) with minor modifications. Fortytwo microliters of reaction buffer (25 mM Tris, pH 7.4, 1 mM DTT, 5% glycerol, 2 mM ATP, 1 mM Asp, 10 mM MgSO₄, and 500 mM hydroxylamine) was made up to 50 μL with buffer B, proteins in buffer B, and/or buffer B containing Lys. After incubation at 35°C, 1 volume of STOP solution was added (0.67 M FeCl₃, 0.5 M HCl, and 20% [w/v] trichloractate acid), and absorbance was measured at 490 nm. The assays were repeated with higher substrate concentrations (5 mM Asp and 10 mM ATP), as these conditions yielded more robust data.

Bioinformatic Analysis

The genomes of Hpa (gene models v8.3; http://vmd.vbi.vt.edu/), Bg (https://www.bluorgen.org/), and Go (local BLAST server) were queried for sequences with similarity to the sequences of AAA and DAP pathway listed in Supplemental Table 1 online using TBLASTn with default settings. Returned sequences producing significant alignments (E ≤ 1e-5) were considered possible presence of a gene, listed in Supplemental Table 1 online, and used for a reciprocal BLAST search. The first iteration of the PSI-BLAST algorithm (http://www.ncbi.nlm.nih.gov/Tools/ssa/psiblast/) was used with default settings against the UniProt Knowledgebase. From this reciprocal BLAST search, the first entry with an informative annotation was listed in Supplemental Table 1 online to support the results of the first BLAST search. Presence of a gene was concluded if an alignment with E ≤ 1e-35 was obtained in the first BLAST search. The result of the reciprocal BLAST was used as judgment for support or not the results of first BLAST searches. Presence of a gene was concluded if an alignment with E ≤ 1e-35 was obtained in the first BLAST search. The result of the reciprocal BLAST was used as judgment for returned results with 1e-35 ≤ E ≤ 1e-5.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: RAR1 (At5g51700), EDS1 (At3g48090), PR1 (At2g14610), MLO2 (At1g11310), DHDP51 (At3g60880), DHDP52 (At2g45440), AK2 (At5g14060), PDF1.2 (At5g44420), VSP2 (At5g24770), OPR3 (At2g06050), JAR1 (At2g48370), JIN1 (At1g32640), AOS (At5g42850), THA2 (At3g04520), and OMR1 (At3g10050).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phenotypes of rra1-13 rrs1 and rrs2 Double Mutants.

Supplemental Figure 2. Macroscopic Disease Symptom Formation upon Golovinomyces orontii Infection.

Supplemental Figure 3. Isolation and Characterization of a Col dhdps1-T1 Mutant.

Supplemental Figure 4. Germination Phenotype of rrs Mutants.

Supplemental Figure 5. Characterization of a Putative ak2 Loss-of-Function Mutant.

Supplemental Figure 6. Macroscopic Phenotypes of T1 Plants Overexpressing Wild-Type AK2 or Mutant AK2<sup>nn</sup>.

Supplemental Figure 7. Hpa Resistance of rrs Mutants Is Not rar1-13 Dependent.

Supplemental Figure 8. Effects of Sugar on Growth and Hpa Susceptibility of rrs Mutants.

Supplemental Figure 9. Hpa Infection Structures of Virulent Hpa Isolate Cala2 on rrs1 and rrs2 Mutant Plants.

Supplemental Figure 10. Indole Glucosinolate Content of rrs Mutant Plant Tissues.

Supplemental Figure 11. Homoserine Content of rrs Mutant Plants.

Supplemental Figure 12. Dose Dependency of Hpa Growth Suppression by Thr.

Supplemental Figure 13. Expression of JA-Regulated Genes in rrs Mutant Plants and Thr-Induced Hpa Growth Suppression on JA-Signaling Mutants.

Supplemental Figure 14. Growth of Hpa on Mock- or Threonine-Treated Col Plants.

Supplemental Figure 15. Threonine Content of agd2 Mutant Tissues.

Supplemental Table 1. Comparative Genome Analysis for Genes Coding DAP/AAA Pathway Enzymes in Hyaloperonospora arabidopsis, Golovinomyces orontii, and Blumeria graminis.

Supplemental Table 2. Oligonucleotides Used in This Study.

ACKNOWLEDGMENTS

We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants and the Nottingham Arabidopsis Stock Centre for distribution of Arabidopsis lines. We thank G. Jander and P. Schulze-Lefert for helpful discussions, S. Laguacze, D. Becker, and N. Moret for technical assistance, and Emil, Ver Loren van Themaat for help with bioinformatic analysis. We also thank L. Nussaume, J. Greenberg, G. Van den Ackerveken, R. Ros, and Y. Yoshikawa for providing mutant Arabidopsis seed. This work was funded by the Max-Planck Society, Deutsche Forschungsgemeinschaft government within Collaborative Research Centre (SFB) ‘635’ (J.E.P. and J.S.), and Deutsche Forschungsgemeinschaft Grant PA 917/3-1 (J.E.P. and S.R.).

AUTHOR CONTRIBUTIONS


Received May 25, 2011; revised June 27, 2011; accepted July 7, 2011; published July 22, 2011.

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