Friend or foe: Hybrid proline-rich proteins determine how plants respond to beneficial and pathogenic microbes

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Abstract

Plant plastids generate signals, including some derived from lipids, that need to be mobilized to effect signaling. We used informatics to discover potential plastid membrane proteins involved in microbial responses in Arabidopsis (Arabidopsis thaliana). Among these are proteins co-regulated with the systemic immunity component AZELAIC ACID INDUCED 1, a hybrid proline-rich protein (HyPRP), and HyPRP superfamily members. HyPRPs have a transmembrane domain, a proline-rich region (PRR), and a lipid transfer protein domain. The precise subcellular location(s) and function(s) are unknown for most HyPRP family members. As predicted by informatics, a subset of HyPRPs has a pool of proteins that target plastid outer envelope membranes via a mechanism that requires the PRR. Additionally, two HyPRPs may be associated with thylakoid membranes. Most of the plastid- and nonplastid-localized family members also have pools that localize to the endoplasmic reticulum, plasma membrane, or plasmodesmata. HyPRPs with plastid pools regulate, positively or negatively, systemic immunity against the pathogen Pseudomonas syringae. HyPRPs also regulate the interaction with the plant growth-promoting rhizobacteria Pseudomonas simiae WCS417 in the roots to influence colonization, root system architecture, and/or biomass. Thus, HyPRPs have broad and distinct roles in immunity, development, and growth responses to microbes and reside at sites that may facilitate signal molecule transport.

Introduction

Plants have evolved strategies to provide immunity against a broad range of pathogens. The recognition of pathogen-derived molecules at the surface or inside the cell leads to the establishment of local or systemic immunity (Zipfel, 2008; Fu and Dong, 2013; Albert et al., 2020). During systemic immunity, the host plant responds to a primary infection to provide a faster response against a broad range of secondary infections by a phenomenon called priming (Jung et al., 2009; Jaskiewicz et al., 2011; Pieterse et al., 2014).
The recognition of pathogen-derived molecules in the leaves induces systemic acquired resistance (SAR), a broad-spectrum program that suppresses diverse pathogens at sites distal to a primary infection (Fu and Dong, 2013). Colonization of roots with beneficial bacteria can lead to induced-systemic resistance (ISR) in the aboveground part of the plant (Pieterse et al., 2014). These systemic immune programs require long-distance transport of mobile signal(s) from the infected tissue (Pieterse et al., 2014; Cecchini et al., 2019b; Carella, 2020; Kachroo and Kachroo, 2020). Many candidate signal molecules, including azelaic acid (AZA), have been identified as mobile signals for SAR. These signals may act together to confer SAR (Jung et al., 2009; Kachroo and Kachroo, 2020). Although there are differences in the mechanism of induction, some of the signaling components for SAR and ISR overlap (Pieterse et al., 1996, 1998; Beckers and Conrath, 2007; Beckers et al., 2009; Cecchini et al., 2015, 2019b). Beneficial rhizobacteria not only induce ISR, but also stimulate the growth and productivity of plants and impact the root system architecture (Berendsen et al., 2012; Efthimiadou et al., 2020). A crosstalk between the signaling pathways of rhizosphere-induced growth promotion and ISR remains unknown. However, some degree of shared signaling seems plausible (Bulgarelli et al., 2013; Haney et al., 2015).

Plastids coordinate many cellular responses to infections, generating signals that activate defenses (Bobik and Burch-Smith, 2015). Several key defense molecules including hormones and second messengers are synthesized in plastids (Mittler et al., 2004; Dempsey et al., 2011; Bobik and Burch-Smith, 2015). More importantly, signals needed for SAR, such as salicylic acid (SA), AZA, and piperocelic acid are produced at least in part within plastids (Dempsey et al., 2011; Zoeller et al., 2012; Hartmann et al., 2017; Rekhter et al., 2019). These molecules (or others regulated by them) should be mobilized from their site(s) of synthesis for long-distance transport. The SA precursor isochorismate synthesized in the chloroplasts is thought to be exported by ENHANCED DISEASE SUSCEPTIBILITY (Serrano et al., 2013; Rekhter et al., 2019). Similarly, pools of AZA INDUCED 1 (AZI1) and its putative paralog EARLY ARABIDOPSIS ALUMINUM INDUCED 1 (EARLI1) are localized in the plastid envelopes and help in the mobilization of plastid-produced AZA (Zoeller et al., 2012; Cecchini et al., 2015). These proteins also localize to the endoplasmic reticulum (ER), and plasma membrane (PM), including plasmodesmata (PD) (Cecchini et al., 2015). Pathogen infection or treatment with the microbe (pathogen)-associated molecular pattern (MAMP/PAMP) flg22, an epitope of bacterial flagellin, induces AZI1 and EARLI1 to become enriched at chloroplasts (Cecchini et al., 2015, 2021). The plastids together with other membranous organelles like nucleus, ER and PM (including PD), facilitate the efficient relay of inter-organelar signaling to achieve whole-cell immunity (Toulmay and Prinz, 2011; Helle et al., 2013; Caplan et al., 2015; Lee, 2015). AZI1 and EARLI1 are essential for SAR, MAMP/PAMP-triggered SAR, ISR, and AZA-induced systemic disease resistance (Cecchini et al., 2015, 2019b). Additional members of the AZI1 family have (or are predicted to have) similar subcellular distributions as AZI1 (e.g. AZI3 and AZI4; Cecchini et al., 2015), but their functions and those of other family members (AZI5–AZI7) have not been investigated.

AZI1 is a signal-anchored protein that has an N-terminal bipartite signal composed of a signal peptide (SP)-like hydrophobic domain followed by a charged protein region (CPR, consisting of at least three Lys and/or Arg residues, within eight amino acids at the C-terminal side of transmembrane domain [TMD]) and a proline-rich region (PRR) for the plastid targeting (Lee et al., 2011; Cecchini et al., 2015, 2021). The SP-like region acts as a TMD anchoring AZI1 to the membranes, whereas the PRR guides its plastid association (Cecchini et al., 2021). The superfamily of proteins to which AZI1 belongs are called hybrid proline-rich proteins (HyPRPs); 28 members were previously identified (Dvóráková et al., 2007). Invariant features include the SP-like region and a C-terminal 8-cysteine motif (8CM)/lipid transfer protein-like (LTP) domain. Most members of the family also have a PRR between these regions that varies in length. Although AZI1 uses a signal-anchored mechanism for plastid targeting, it does not conform to targeting features of classical signal anchor proteins whose N-termini have low hydrophobicity scores on the Wimley–White hydrophobicity scale (Lee et al., 2011).

In this work, we used bioinformatic analyses to identify proteins that, like AZI1, have a pool of protein that localizes to plastid membranes, but which are not classical signal anchored proteins. Included in the screen are proteins whose transcripts are co-regulated with AZI1 and members of the HyPRP superfamily, respectively. Herein, we show the utility of our bioinformatics approach for finding proteins with a pool that localizes to plastid membranes and show that some of them, in the AZI1 family and broader HyPRP superfamily, have different positive or negative roles in systemic immunity, development, and/or growth responses to microbes in roots and shoots.

Results
In silico analysis of subcellular targeting of the Arabidopsis proteome
Well-known targeting algorithms (TargetP, ChloroP, and SignalP; Nielsen et al., 1997; Emanuelsson et al., 1999, 2000) predict AZI1 to be a secreted protein. However, a pool of AZI1 and that of putative paralogs (having 92.71%–96.28% sequence identity) localize to plastid envelopes using an N-terminal bipartite signal (SP-like/TMD + PRR; Cecchini et al., 2015). AZI1 has similar properties to apicomplexan proteins that target apicoplasts (nonphotosynthetic plastids) using N-terminal bipartite signal sequences (Figure 1A; Cecchini et al., 2021). The PATS algorithm, trained using apicomplexa plastid proteins, can successfully predict plastid localization of AZI1 and putative paralogs by searching for this bipartite signal (Zuegge et al., 2001; Cecchini et al., 2015, 2021). We
used PATS to predict N-terminal bipartite signal sequence and plastid localization of the total Arabidopsis proteome. Overall, the PATS-generated prediction output (positive and negative selections) showed that approximately 7%–8% proteins coded by each chromosome may localize to plastids by using this bipartite targeting signal (Figure 1B). The Arabidopsis proteins predicted to target to the plastids by PATS are listed in Supplemental Table S1. Interestingly, the
gene ontology (GO) functional characterization (biological processes) of these proteins revealed that among others, the GO terms related to cell communication, response to biotic stimulus, defense response, protein phosphorylation, cell wall organization and cell–cell signaling were significantly overrepresented (Supplemental Figure S1A).

To test our predictions, we selected six PATS-positive proteins whose transcripts showed similar expression profiles and clustered together with AZI1 as assessed by the GENVESTIGATOR Hierarchical clustering tool (biological context: Perturbations; Hruz et al., 2008). As a secondary screen, we chose proteins that did not conform to a key characteristic of classical signal-anchored plastid outer envelope membrane (OEM) proteins. Specifically, their SP-like N-terminal regions lacked low hydrophobicity scores (<0.4) on the Wimley–White hydrophobicity scale (Lee et al., 2011). Instead, the scores (calculated with the MPEx algorithm (http://blando.biomol.uci.edu/mpex; Jayasinghe et al., 2001; Snider et al., 2009) were characteristic of ER-targeted proteins (≥0.4): AT5g54170: 0.58, AT1G22890/STMP2: 0.63, AT2G45570.1/CYP76C2: 0.90, AT1G65500/STMP6: 0.63, AT1G33720/CYP76C6: 0.81, AT3G04210.1/TN13: 0.50. We then assessed protein localization by confocal microscopy of C-terminal GFP fusions expressed under dexamethasone (Dex) control in Nicotiana benthamiana (Figure 1C, Left panel; Supplemental Figure S2B). We named the remaining HyPRPs as HyPRPs family were previously named (AZI1, EARLI1, AZI3, AZI4, and occasionally chloroplasts with more continuous and punctate GFP signals around many chloroplasts and PM including foci similar to PD (ELP, AZL2, CWLP, and AZL14). Although we assigned AZL7 to group 1, it may belong in group 3, as we observed weak chloroplast-associated signals for AZL7-GFP. Initially, we also cautiously assigned DRN1 to group three, because although there were some chloroplast signals in the micrographs (Figure 2A and Supplemental Figure S2B), in many cases GFP was detected in nuclei (Supplemental Figure S2C). This raised the possibility that DRN1-GFP was proteolyzed. Indeed, GFP was cleaved from a large pool of the protein (Supplemental Figure S2C). Two previous mass spectrometry experiments assigned DRN1 to plastid membranes (Peltier et al., 2004; Tomiziolli et al., 2014), providing additional confidence that the protein is plastid-associated. Generally, the chloroplast-targeted proteins showed a ring-like pattern of fluorescent signal around them. In one case (AZL3), punctate GFP signals around many chloroplasts and occasionally chloroplasts with more continuous and stronger GFP signals in addition to PM signals were observed. While AZL6 showed similar localization to AZL3, the chloroplast-associated punctate signals were less prominent. Finally, DHYPRP1 showed ER, PM/PD, and incomplete chloroplast rings (Figure 2A and Supplemental Figure S2B). The patterns showing ER, PM/PD, and chloroplast localization of HyPRPs were largely similar to the family member AZI1 and putative paralogs (Cecchini et al., 2015, 2021). Evaluation by immunoblotting of chloroplast fractions from N. benthamiana leaves transiently expressing many HyPRP-GFP fusion proteins largely corroborated the
We were unsuccessful in using immunoblotting to detect AZL3-GFP after fractionation due to interference from the rubisco large subunit. Instead, we used a proteomics approach and found AZL3 to be differentially enriched in chloroplast membranes of a constitutively defense active Arabidopsis line (ACD6-1HA; Lu et al., 2005) relative to wild-type (WT; Figure 2C). Prior proteomics studies detected AZL3 in the thylakoid and envelope fractions, respectively (Kleffmann et al., 2004; Tomizioli et al., 2014).

We next analyzed the localization patterns of Dex-inducible GFP fusions of the HyPRPs AZL3, AZL13, AZL14, and ELP in stable Arabidopsis transgenic lines. These lines were chosen because the fusion proteins showed chloroplast localization in N. benthamiana and mutants were available (Figure 2). Each transgene was expressed in the respective mutant background. A portion of the GFP signal of these four fusion proteins colocalized with chloroplast autofluorescence primarily in epidermal cells (Figure 3). Interestingly, while the GFP signals for AZL13,
AZL14, and ELP each showed a ring-like pattern surrounding the chloroplasts, AZL3 had a more diffuse signal. This was further confirmed by analyzing the fluorescence intensity profiles of the merged images (Figure 3, right most panel) and is consistent with its import and association with thylakoid membranes (Tomizioli et al., 2014). Inspection of the features of the PRR for AZL3 compared with other HyPRPs that show the ring-like pattern revealed that the AZL3 PRR lacks a KP motif present in others and also possesses a unique repeated sequence (Supplemental Table S2). These and/or other differences in the PRR composition (e.g. abundance of prolines relative to charged amino acid residues) may account for differences in the suborganellar plastid localization patterns. Altogether, our data show that for several HyPRPs, a pool of these proteins localizes to chloroplasts, as predicted.

Several chloroplast-localized HyPRPs are associated with OEMs
Except for AZL1 and AZL9, N-terminal regions of all HyPRPs have hydrophobicity scores > 0.4 on the Wimley–White scale (Supplemental Table S2). Thus, they do not have features of classical signal-anchored OEM plastid proteins (Lee et al., 2011). Moreover, AZL1 and AZL9 (TMD hydrophobicity < 0.4; plastidial score) did not target to the chloroplasts as indicated by confocal microscopy (Figure 2A). This suggests that HyPRPs follow a noncanonical mechanism of N-terminal targeting to chloroplast membranes, similar to the family member AZI1 (Cecchini et al., 2021).

To study the above possibility, we analyzed the chloroplast membrane association of HyPRPs that showed ring-like localization around chloroplasts. We isolated chloroplasts from N. benthamiana leaves transiently expressing...
GFP-tagged AZL2, AZL13, AZL14, and ELP. The chloroplasts were then partitioned into pellet (membrane) and soluble fractions. Interestingly, all these GFP-tagged HyPRPs were found to be associated with pellets (chloroplast membranes) as evaluated by immunoblotting (Figure 4). Thermolysin, a metalloprotease, is widely used to probe the surface features of chloroplasts. It can digest the portions of OEM proteins that are facing cytosol largely without affecting inner envelope or internal proteins (Cline et al., 1984). We observed that treating intact chloroplasts from plants transiently producing HyPRP-GFP fusion proteins with thermolysin resulted in HyPRP-GFP proteolysis, consistent with localization of AZL2, AZL13, AZL14, and ELP to the OEMs. In contrast, the intermembrane space-located POTRA domains of OEM protein Toc75 and stromal HSP70 were fully protected from thermolysin digestion (Figure 4B; Chen et al., 2016; Seguel et al., 2018). These experiments indicate that several chloroplast-targeted HyPRPs reside in OEMs and that their C-terminal regions topologically face the cytosol.

The PRR is required for the chloroplast targeting of HyPRPs

Multiple sequence alignment of Arabidopsis HyPRPs revealed conserved N-terminal SP-like hydrophobic and C-terminal 8CM/LTP domains, and highly variable PRRs, variable both in terms of the number of Pro residues and the length of each PRR (Supplemental Figure S3; Supplemental Table S2). AZI1 uses a noncanonical signal-anchored targeting mechanism that depends on the PRR for a pool of AZI1 to target chloroplasts (Cecchini et al., 2021). To determine whether this is true for other members, we deleted the PRRs of AZL2, AZL13, AZL14, and ELP (Figure 5A), and fused these deletion constructs with GFP at C-terminal ends of each sequence. We observed GFP cleavage for AZL14ΔPRR (AZL14Δ24–44), which was circumvented by deleting a portion of its LTP together with the PRR (AZL14Δ24–101). The deletion of PRRs prevented all these HyPRP-GFP fusion proteins from targeting to chloroplasts as the GFP signal, due to these deletions, was not found to be associated with chloroplasts (Figure 5, B and C). This is consistent with a role for the PRRs in promoting the association of some Arabidopsis HyPRPs with chloroplasts.

In AZI1 and the putative paralogs EARLI1 and AZI3, the C-terminal 8CM/LTP domains did not contribute to chloroplast targeting (Cecchini et al., 2015, 2021). To check if this domain is similarly dispensable for chloroplast targeting of a more distantly related HyPRP, we generated ELPAΔLTP-GFP. This truncated version of ELP associated with chloroplasts (Figure 5D). These data show that the PRRs play key roles in chloroplast targeting and are consistent with the N-terminal region of HyPRPs being sufficient to confer this targeting.

Plastid localization corresponds with SAR promotion

We wondered whether the plastid association of HyPRPs is important for systemic immunity. This was previously suggested, because mpk3 and mpk6 mutants, in which plastid targeting of AZI1 and EARLI1 is greatly reduced, have the same systemic immunity defects as azi1 and earli1 mutants (Beckers et al., 2009; Cecchini et al., 2019a, 2019b, 2021). Examining the role of additional AZI1-family members in systemic immunity can contribute to addressing the importance of plastid association, because there are members that do (AZI3) or do not (AZI5 and AZI6) target plastids due to the different lengths of their PRRs (Cecchini et al., 2015). AZI4, with its relatively long PRR, is also predicted to be plastid-localized (Cecchini et al., 2015; Figure 1D). Supplemental Figure S4 shows the high sequence identity in the AZI1-family proteins and the different PRR lengths.

We generated CRISPR/Cas9 single mutants in azi3 and azi4 and double mutants of azi5azi6 (AZI5 and AZI6 are identical in sequence; Supplemental Figure S4, B–D). To evaluate SAR, we mock-treated or immunized lower leaves with strain Pseudomonas cannabina pv. alisalensis carrying AvrRpt2 (avirulent strain PmaDG6) and subsequently challenged upper leaves with virulent strain PmaDG3. Whereas azi1 (the negative control), azi3, and azi4 did not show significant SAR relative to WT (by comparing immunized versus mock samples in each genotype), the azi5azi6 mutants still showed SAR (Figure 6A). We used the response gain of SAR, derived from a mathematical model, to quantitate the level of gain in immune response due to primary immunization (Jiang et al., 2021). Compared to WT, only the azi1, azi3, and azi4 mutants showed significantly reduced response gains (Figure 6B). Loss of SAR in azi3 and azi4 was not due to a lack of response to the SAR-inducing strain, as the mutants had normal basal resistance to PmaDG6 (Supplemental Figure S5A).

These findings support a model in which chloroplast targeting is important for SAR promotion in this family of proteins.

Expression of HyPRPs is regulated in response to microbes

The HyPRPs AZI1, EARLI1, AZI3, AZI4, and DRN1 are required for defense against pathogens and their transcripts are modulated during infection (Li et al., 2014; Cecchini et al., 2015; Dhar et al., 2020; Table 1). Mining of the literature and public data indicated that many other HyPRP transcripts are differentially regulated by pathogens or pathogen-derived molecules (e.g. the PAMP flg22), suggesting that they may play a role during plant defense responses (Table 1). Moreover, the expression of many HyPRPs was also responsive to inoculation of roots with the beneficial microbe P. simiae WCS417 (Stringlis et al., 2018). Analysis of publicly available gene expression data also revealed that a number of HyPRPs are co-regulated to different degrees, suggesting they may be involved in the same processes (Figure 7A). The HyPRPs with the highest co-regulation values were encoded by genes with strong chromosomal linkage (Supplemental Figure S1B).
We analyzed the transcript levels of several HyPRPs in WT after infection with a pathogen capable of inducing strong local and systemic defenses, PmaDG6. Most HyPRP transcripts assayed were downregulated by 18 h postinfection (Figure 7B). Because AZL3 showed increased protein levels in the plastid membrane fraction of the constitutively defense-active line ACD6-1HA (Figure 2C), we also assayed its transcript levels in these plants. Interestingly, the AZL3 transcript level was reduced in the ACD6-1HA line relative to WT (Figure 7C). Thus, AZL3 may be subject to posttranscriptional regulation.

Together, previously published and our data show that the HyPRPs are differentially regulated during biotic interactions (or simulated infection conditions provided by the ACD6-1HA line) and thus may play a role, positive or negative, during interactions with microbes.

Several non-AZI1-family HyPRPs regulate SAR in Arabidopsis

It seemed possible that non-AZI1-family HyPRPs with chloroplast-associated pools might be needed for SAR. To test this, we evaluated local foliar pathogen resistance and SAR using azl3, azl13, azl14, and elp T-DNA mutants. Since no azl2 mutant was available, we used anti-sense line AZL2-AS (Jülke and Ludwig-Müller, 2015) for SAR experiments.

The azl3 and azl13 and the AZL2-AS plants were SAR-deficient, whereas azl14 still showed robust SAR, similar to WT (Figure 8, A and C). SAR response gain values of the different genotypes supported this conclusion (Figure 8, B and D). Loss of SAR in azl3 and azl13 was not due to an inability to respond to SAR-inducing strain PmaDG6, since growth of PmaDG6 in these mutants and WT was similar (Supplemental Figure S5B). Additionally, basal resistance to
PmaDG3 in the mutants was similar to WT (Supplemental Figure S5C). Interestingly, elp showed significantly higher resistance levels to PmaDG3 in systemic leaves during SAR and an increased response gain compared with the WT (Figure 8, A and B). We confirmed this unusual “super-SAR” phenotype in elp-2, another T-DNA allele (Supplemental Figure S6A).

We analyzed the induction of SAR marker PR1 in the uninfected systemic leaves of WT, azl3, azl13, azl14, and elp after local immunization with PmaDG6 or mock treatment. Consistent with the observed bacterial growth (Figure 8A), there was increased accumulation of PR1 in systemic leaves of elp compared to WT, 72 h post local PmaDG6 infection (Figure 8, E and F). Moreover,
azl3 and azl13 showed significantly lower PR1 than WT in systemic leaves at both 48 and 72 hours post infection (hpi) (Figure 8, E and F).

We performed complementation experiments using the transgenic Arabidopsis in which GFP fusions were studied in Figure 3. The expression of HyPRPs was induced by spraying whole plants with Dex plus 0.04% v/v Tween 20, 21 h before infection. In addition to visualizing GFP (Figure 3), transgene expression was confirmed by RT-PCR (Figure 8G). Dex:AZL3#1 and Dex:AZL13#1 transgenic lines complemented the SAR-deficient phenotype and were indistinguishable from WT plants (Figure 8, H and I). Independent Dex:AZL3#2 and Dex:AZL13#2 lines were also complemented for SAR (Supplemental Figure S6, B and C). The Dex:ELP#1 line was completely SAR-deficient, indicating that the dosage of ELP (overexpression) strongly affects SAR (Figure 8, G and I). The contrasting phenotypes of the mutant (super-SAR) and Dex:ELP#1 line (SAR-deficient) strongly position this particular HyPRP as a negative regulator of SAR.

Together, the results obtained with the AZI1- and non-AZI1-family HyPRPs strongly support a close relationship between plastid-located HyPRPs (having longer PRRs) and the regulation of SAR.

**HyPRPs are required for ISR**

ISR is a defense mechanism in which beneficial microbes in the rhizosphere prime the whole plant for enhanced defense against secondary infections (Pieterse et al., 2014). Many HyPRPs are differentially expressed in roots, particularly during the interaction of roots with *P. simiae* WCS417 (Table 1; Stringlis et al., 2018). Moreover, AZI1 and EARLI1 are required for ISR (Cecchini et al., 2015). Therefore, we tested the role of several other HyPRPs with plastid-associated pools in ISR. As shown in Figure 9, azl3, azl13, and azl14 mutants were unable to mount ISR. In contrast, only WT and elp showed significant (and similar) ISR (Figure 9, A and B). These data indicate that several of the HyPRPs characterized herein have positive roles in promoting ISR.

**HyPRPs affect colonization, developmental and/or growth-promoting effects of root-associated bacteria**

Strain *P. simiae* WCS417, used to stimulate ISR, also promotes plant growth and lateral root formation in Arabidopsis (Pieterse et al., 2014; Haney et al., 2015). Unlike WT, upon treatment of roots with *P. simiae* WCS417, azl13 and azl14 showed no significant increase in biomass or lateral root numbers. The azl3 mutant also failed to show a biomass increase, but did display increased lateral root numbers (even higher than WT, Figure 10). As was seen with the SAR assay, elp was hyper-responsive, showing both biomass and lateral root numbers that exceeded that seen in WT upon *P. simiae* WCS417 inoculation.

We noticed that azl14 and to a lesser extent azl3 and azl13 had shorter primary root lengths than WT in the absence of bacteria (Figure 10D). However, azl3 and azl13 showed similar increased primary root growth as WT in response to WCS417. In contrast, elp failed to show increased primary root growth and azl14 showed reduced growth in response to *P. simiae* WCS417.

Differences in *P. simiae*-induced phenotypes may be related to altered levels of root colonization (Haney et al., 2015). Therefore, we analyzed the association of bacteria
with roots in WT and mutant plants after growing the different genotypes hydroponically in 48-well plates on a Teflon mesh (Figure 10E). Seedlings were initially grown with 2% w/v sucrose to avoid differences in root development between genotypes and thus allowing subsequent comparisons of association of bacteria with roots in media without sucrose (see “Materials and methods”). Interestingly, although there was no significant difference in the bacterial numbers (thought to be stimulated by root exudation) in the well media in which plants were growing, azl3, azl13 and azl14 roots supported less P. simiae colonization compared to WT or elp mutant plants (Figure 10F).

Altogether, these data suggest that HyPRPs have varied positive and negative role(s) in the roots including supporting beneficial microbe colonization and impacting root architecture and growth promotion.
Discussion

In this work, we greatly expanded the ability to correctly predict bipartite signal-anchored plastid membrane proteins and showed that a subset that belong to the HyPRP family have important roles in microbial-induced responses. Most HyPRP family proteins have SP-like/HD domains at their N termini followed by varying lengths of unstructured PRRs. We previously proposed that the N-terminal bipartite signal formed by SP-like/HD (TMD) + PRR define the subcellular targeting of AZI1 and putative paralogs to plastids (Cecchini et al., 2015, 2021). Here, we predicted with ~80% success the subcellular targeting of many members of the HyPRP family in Arabidopsis. Predictions that were not correct largely associated with HyPRPs harboring shorter PRRs. Importantly, the PATS algorithm also identified plausible bipartite signals in a large number of Arabidopsis proteins. PATS correctly predicted ≥ 33% of the non-HyPRP proteins that we tested. The non-HyPRP plastid protein STMP6 is among a group of proteins recently proposed to be secreted (Yu et al., 2020). Many proteins in this group, regulated in response to pathogens, are PATS-positive and are thus also good candidates for having a pool that localizes to plastids. We showed that four HyPRPs (AZL2, AZL13, AZL14, and ELP) are OEM plastid proteins. Two HyPRPs (AZL3 and DRN1) were previously suggested to be thylakoid and/or envelope proteins, as shown by their presence in proteomics.
Figure 8 SAR in Arabidopsis HyPRP mutants. A and C, Growth of virulent bacteria *Pma*DG3 in systemic leaves of WT (Col-0) and indicated mutants three days after infection. The plants were previously immunized in local leaves with avirulent *Pma*DG6 or mock treated, 2 days prior to the secondary infection. The mean c.f.u. of two-three independent experiments combined are plotted (*n* = 7–8 for each experiment). B, Response gain of SAR due to *Pma*DG6 immunization in (A). D, Response gain of SAR due to *Pma*DG6 immunization in (C). E, PR1 induction at the indicated time points in the uninfected systemic leaves of plants that were locally mock treated or immunized with *Pma*DG6. F, Densitometric quantification of PR1 levels in immunized samples shown in (E). The PR1 levels are relative to the nonspecific band in CB stained membrane. The mean from two replicates is shown. Quantification was done by ImageJ (Fiji). G, RT–PCR of the indicated HyPRP transcripts in Dex-inducible complementation lines. Leaves were collected 21 h after treatment with 30 μM Dex (+) or no treatment (−). Expression of *EF1α* was used as control. H, Growth of virulent bacteria *Pma*DG3 in systemic leaves of WT (Col-0) and Dex-inducible complementation lines, three days after infection. The plants were mock treated or immunized as in (A and C). About 30 μM Dex plus 0.04% v/v Tween 20 was sprayed on local and systemic leaves (continued)
datasets (Kleffmann et al., 2004; Peltier et al., 2004; Tomiziol et al., 2014). While thylakoid samples could be contaminated with plastid envelope proteins, our microscopy of AZL3 in Arabidopsis (and occasionally by transient expression in N. benthamiana) is consistent with a thylakoid location. This suggests that bipartite signals, depending on their composition, may direct the targeting of proteins to plastid envelopes or the internal plastid thylakoid membrane.

Figure 11 summarizes the localization patterns of HyPRPs, and by analogy to AZ11/EARL11, proposes that they are needed for mobilizing signals. The multiple roles of HyPRPs in microbial responses that were found previously (Cecchini et al., 2015, 2019b; Dhar et al., 2020) and herein are shown in Figure 11B. ELP is unique among the HyPRPs, as it impacts responses to microbes as a negative regulator of both SAR and growth/development responses to the root-colonizing microbe WCS417. The elp mutant shows increased response gain and PR1 accumulation during SAR, but does not affect basal resistance, suggesting increased signaling output to distal tissue or signal amplification in systemic tissues. This mutant also has normal root colonization by P. simiae WCS417, but has increased plant growth and lateral root numbers relative to WT, further supporting enhanced signaling. However, ISR is not affected in the elp mutant, indicating distinct requirements for the developmental responses and ISR. This is consistent with certain ISR-defective mutants showing robust growth and developmental responses when co-cultivated with WCS417 (Zamioudis et al., 2013).

Some mutants in this study (azl3 and azl13) are affected in both SAR and ISR, similar to the azl1 and earl11 mutants (Jung et al., 2009; Cecchini et al., 2015). For the SAR phenotypes, the defects are linked to long-distance signaling, since the growth of the immunizing pathogen is normal. However, the azl3, azl13 and azl14 mutants show possible local signaling defects demonstrated by lower colonization of roots with WCS417 relative to that seen in WT. It is possible that other root-related defects in these mutants arise from low root colonization. However, azl3 still shows lateral root induction, indicating that there is enough colonization to cause signaling for this phenotypic output. Lateral root induction in azl3 may occur due to the presence of other HyPRPs. This would implicate other HyPRPs in regulating both colonization as well as the downstream signaling for lateral root induction.

It seems possible that different phenotypic outputs (growth promotion, ISR, and lateral root number increases) require different thresholds of bacterial colonization of the roots. Alternatively, all outputs may require the same threshold, but certain HyPRPs may be differentially needed for multiple steps: colonization and downstream signaling to cause growth, ISR and/or lateral root production. A genome-wide association mapping study (Wintermans et al., 2016) identified different markers that were independently associated with shoot growth promotion, lateral root formation, and primary root length changes in response to WCS417. This finding supports the idea that there are separate pathways (or branches of a pathway) that independently regulate the different phenotypic outputs. Interestingly, two HyPRPs (DEG27 and AZL2) are within 5–10 KB of a marker associated with regulating the lateral root changes.

Despite having positive roles in regulating interactions with/responses to microbes, transcript levels of many HyPRPs are downregulated by microbe-related signals. This could reflect negative feedback to modulate signaling. However, in plants with constitutive defenses (ACD6-1HA) the transcript level of AZL3 is low, while the plastid membrane-associated protein level is high relative to WT. Some HyPRPs may become stabilized by posttranslational modification, since they have kinase motifs (SP/TP; Supplemental Table S2). AZ11’s PRR is a kinase substrate in vitro (Pitzschke et al., 2014). It is also possible (and not a mutually exclusive idea) that HyPRPs can become stabilized via interaction with another protein(s) under specific conditions, like infection.

In aerial tissue, HyPRP-GFP fusion proteins primarily accumulate in epidermal cells. Epidermal plastids have emerged as key players in defense against microbes (Caplan et al., 2015; Cecchini et al., 2015; Beltrán et al., 2018; Seguel et al., 2018; Jiang et al., 2021; Vlot, 2021). AGD2-LIKE DEFENSE RESPONSE PROTEIN1 expressed specifically in leaf epidermal cells and targeted to plastids is sufficient to confer local disease resistance and SAR (Jiang et al., 2021). In leaves, additional important defense proteins, including HyPRPs, are mainly detected in epidermal plastids (Yamasaki et al., 2013; Cecchini et al., 2015; Seguel et al., 2018). In this work, HyPRP-GFP fusions expressed in Arabidopsis were mainly in the epidermal plastids and rescued the SAR phenotypes. HyPRPs produced in epidermal leaf cells may impact the accumulation and/or mobilization of plastid-derived signals to limit bacterial growth and also to induce systemic resistance. In contrast, in roots, HyPRPs may impact plastid-derived signals needed for interactions between beneficial bacteria and root cells.

HyPRPs have similar SP-like and 8CM/LTP domains and they locate to several types of membranes. This could permit the formation of membrane contact sites and complexes with other HyPRPs/LTPs in different places. Membrane contact sites between plastids, ER and PM,

Figure 8 (Continued)

21 h before local and distal infection, respectively. The mean c.f.u. of two independent experiments combined are plotted (n = 7–8 for each experiment). I. Response gain of SAR due to PmaDG6 immunization in (H). In the graphs showing "response gain," error bars indicate variation among biological replicates (calculated from SEM); in all other graphs error bars show SEM. Different letters above the bars indicate statistically significant difference. ANOVA, SNK test or Student’s t test, P < 0.05.
comprising of LTPs, are the sites of exchange of small molecule signals (Andersson et al., 2007; Toulmay and Prinz, 2011; Li et al., 2020). HyPRPs are likely enriched in these sites for the mobilization/transport of immune and/or developmental and growth signals. Supporting this, AZI1 and EARLI1 can interact and mobilize the SAR signal AZA or AZA-lipid conjugates formed in plant cells, possibly through their 8CM/LTP domains, which also can trigger AZI1-/EARLI1-dependent root architecture changes (Cecchini et al., 2015, 2019b). AZI1/EARLI1 physically interact with a non-HyPRP LTP superfamily member (i.e. DIR1) in membrane contact sites to modulate systemic responses (Maldonado et al., 2002; Yu et al., 2013; Cecchini et al., 2015). Therefore, it is also possible that other positive regulatory HyPRPs similarly form active homo or hetero-oligomeric LTP complexes at the key sites/structures for transmission of developmental, growth, and/or defense signals to regulate systemic defense responses. The presence of so many HyPRPs may allow the movement of signals at different rates (to reach specific thresholds) or enable movement of various signals that require different membrane-paths to move. For example, to reach the vascular system and/or to move signals between aboveground tissue and roots, different contact sites may be required compared to signals that stimulate lateral root formation. Thus, different signals (or the amount of signal moved) could affect and explain different specific phenotypes.

Taken together, our findings—enabled by bioinformatics, biochemical, disease, and physiological assays—identify a mode of plastid localization of several HyPRP family members and underscore the importance of HyPRPs that target plastids in defense and microbe-mediated growth and development.

Materials and methods

Plants, bacteria, and plasmids

All the Arabidopsis (Arabidopsis thaliana) plants used were Columbia-0 (Col-0) ecotype. The plants used for infection were grown in soil (Berger, BM1:BM2; 50:50 mix) or Jiffy-7 peat pellets (for ISR, Hummert International, # 14-23700) at 12-h day/night cycle as previously described (Cecchini et al., 2015). The following Arabidopsis accessions were obtained from ABRC: At1g12090 (elp, SALK_147582C; elp-2, SAIL_532_C09), At2g10940 (azl3, SALK_083118C), At5g46890 (azl13, SALK_065132), and At5g46900 (azl14, SALK_096750C). At1g62510 AZL2-AS line was described previously (Jülke and Ludwig-Müller, 2015). The transgenic line ACD6-1HA (Lu et al., 2005) used for proteomic analysis was grown at 16-h day/8-h night cycle. pt-gk (cTP-GFP) transgenic line from ABRC (CS16266) was used as a chloroplast marker. Transgenic Arabidopsis were generated by floral dip method using Agrobacterium tumefaciens C58C1 (for HyPRP complementation lines) and GV3101 (for CRISPR/Cas9 editing) suspensions (Clough and Bent, 1998). Transformants were selected by spraying 10 day-old T1 plants grown in soil (and subsequently every three days for a total of four treatments) with BASTA (120 mg/L) solution containing Silwet-77 (500 µL/L). Nicotiana benthamiana used for localization studies were grown in soil at 24°C under 16-h light/8-h night regime. For A. tumefaciens-mediated transient assays, 4–5-week-old N. benthamiana plants were used.

Bacterial strains used for infections or colonization were virulent P. cannabina pv. alisalensis (formerly Pseudomonas syringae pv. maculicola strain ES4326) carrying an empty vector (PmaDG3), the avirulent isogenic strain carrying avrRpt2 (PmaDG6) and Pseudomonas simiae WCS417 (formerly Pseudomonas fluorescens WCS417r).
Figure 10 Growth promotion and root-colonization of HyPRP mutants by P. simiae WCS417. A, Representative images of WT (Col-0) and indicated mutants grown on agar plates without sugar and inoculated with mock or P. simiae WCS417 (WCS417). About 15-day-old seedlings, 10 days post infection (dpi) with WCS417 or mock are shown. B, Fresh weight, (C) number of lateral roots and (D) primary root length in plants inoculated with mock or WCS417 as in (A), n = 15–20 plants. Combined data from three independent experiments is plotted. E, Representative images of WT (Col-0) plants grown hydroponically in 48-well plates before (12-day-old) and 7 dpi (19-day-old) with WCS417. F, Number of c.f.u. of WCS417 in the wells or colonizing the roots of WT and mutants. "No plant" represents the number of c.f.u. of WCS417 in the well-media without any plant present (n = 20–24 plants). Error bars are SEM in (D) and (F), and in all graphs different letters above bars indicate statistical differences by ANOVA, SNK test. P < 0.05.
The full-length coding sequences or the truncated versions of HyPRPs were amplified by PCR and cloned by Gateway procedure into Dex-inducible plant expression vectors pBAV150 (C-terminal GFP tag) or pBAV154 (C-terminal HA tag) (Cecchini et al., 2015). Full-length coding sequences of HyPRPs in pBAV150 vector were used to transform Arabidopsis to generate complementation lines. To generate ΔPRR variants, proper fragments were generated with overlapping primers and linked by PCR. ΔLTP variants were generated using nonoverlapping primers in PCR. All the primers and constructs used in this study are listed in (Supplemental Table S3).

**Generation of CRISPR/Cas9 alleles**

CRISPR/Cas9 gene-editing technology was used to generate AZI3, AZI4, and AZI5/AZI6 Arabidopsis mutants (Ma and Liu, 2016). Two target adaptors for each gene of interest were designed to increase the editing efficiency (Supplemental Table S3). Next, sgRNA intermediate plasmids containing the Arabidopsis U6 small nuclear RNA promoter, the binding scaffold for the Cas9 nuclease, and the two target adaptors were constructed (Addgene # 66201 and 66198). The sgRNA cassettes were finally assembled into a CRISPR/Cas9 plasmid containing a Cas9 gene expression cassette driven by the maize ubiquitin promoter and Basta herbicide resistance (Addgene # 66188).

To determine the genotype of T3 plants, DNA was extracted from three leaf discs collected from an individual T3 plant using a cetyltrimethyl ammonium bromide-based method (Clarke, 2009). The target region was PCR-amplified using the appropriate locus-specific primers, confirmed via agarose gel electrophoresis, cleaned using a QiAquick PCR Purification/Clean-up kit (Qiagen, Hilden, Germany; #28104; Supplemental Table S3) and Sanger-sequenced using the specific sequencing primers (Supplemental Table S3). To determine allelic heterogeneity, sequencing results were analyzed using DsDecode (Liu et al., 2015; Ma et al., 2015a, 2015b). The individual alleles revealed by DsDecode were then re-aligned to the reference sequences using SnapGene (Insightful Science, snapgene.com) and expasy.org/translate to determine the amino acid sequence of each allele. Three biallelic mutants of AZI3, one biallelic mutant of AZI4, and two biallelic mutants of AZI5/6 were identified in this screen and selected for further study (Supplemental Figure S4). T4 seeds from one individual plant per sequenced T3 line that was sequenced were used for experiments.

**In silico analysis of subcellular targeting**

For bipartite plastid targeting signal prediction, we analyzed the full-length sequences of Arabidopsis proteome (ATpepTAIR10) by using the PATS algorithm (Zuegge et al., 2001) or an iterative method (iTargetP/iChloroP). In the iterative method, N-terminal SP-like regions as determined by TargetP (http://www.cbs.dtu.dk/services/TargetP-1.1/) or SignalP (http://www.cbs.dtu.dk/services/SignalP/), varying between 22 and 27 amino acids (except for AZI7 with 30 amino acids as SP-like) were removed in silico and the remaining sequences were analyzed by TargetP/ChloroP to determine the presence of chloroplast transit peptide following the SP-like region.

The GO analysis was done as described (Bonnot et al., 2019). The list of representative enriched GO terms was obtained using Panther (https://www.arabidopsis.org/tools/go_term_enrichment.jsp) and REVIGO (http://revigo.irb.hr) tools.

**HyPRP chromosome map**

Maps of HyPRP genes were drawn with the TAIR chromosome map tool (https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp) using the HyPRP locus names. The loci were then displayed on individual chromosomes.

**ClustalW**

ClustalW alignments of Arabidopsis HyPRPs were generated by MegAlign, DNASTAR, version 7.1.0. Amino acids in SP-like/HD and 8CM/LTP domains were colored according to Taylor scheme (Taylor, 1997) using JalView version 1.0.

**Confocal imaging**

About 9–12-day-old Arabidopsis seedlings or 4-week-old N. benthamiana leaves expressing the different HyPRP versions fused at C-terminal with GFP were visualized by confocal microscopy. Zeiss LSM710 and LSM800 laser scanning confocal microscopes were used to visualize GFP fluorescence (Argon laser/excitation: 488 nm; emission collection: 505–530 nm) and chlorophyll autofluorescence (He-Ne laser/excitation: 633 nm; emission collection: 650–750 nm or 645–700 nm). Images were captured using LD C-Apochromat 40x/1.1 W Korr (LSM710) and EC Plan-Neofluar 40x/1.3 Oil (LSM800) objectives, pinhole at 1AU for each channel, photomultipliers master gain between 500 and 700. The images are optical sections captured at 1,024 × 1,024 pixels scanning resolution in maximum speed mode. Fluorescence of GFP and chlorophyll was acquired in sequential acquisition mode. Plant tissues were mounted in perfluorodecalin (Strem Chemicals, Inc., Newburyport, MA, USA; #09-5960) for optical enhancement. Images were processed by ImageJ (Fiji) and Adobe photoshop software. For each N. benthamiana imaging experiment, four independent samples were assayed per construct. For each Arabidopsis imaging experiment, two or more seedlings were imaged per line.

**Fractionation**

Nicotiana benthamiana leaves, transiently transformed with A. tumefaciens harboring different constructs, were used to isolate chloroplasts as described in (Cecchini et al., 2015) with minor modifications. Briefly, leaves (0.5–1 g) were homogenized in Xpl buffer (0.33 M sorbitol, 50 mM HEPES pH 7.5, 2 mM EDTA, 1 mM MgCl₂, 0.25% w/v BSA, 0.1% w/v sodium ascorbate and protease inhibitors) using polytron (Kinematica, Lucerne, Switzerland) and filtered through two layers of Miracloth (Calbiochem, San Diego, CA, USA; #475855). The filtrate was centrifuged at 5,000 rpm for 5 min.
and the pellet was resuspended in 0.4 mL of Xpl buffer. The resuspended pellet was loaded on Percoll (GE Healthcare, Chicago, IL, USA; # 17089101) gradient (80% v/v and 40% v/v) and centrifuged at 13,000 rpm to isolate the intact chloroplasts. The recovered intact chloroplasts at the interphase were resuspended in 0.5 mL Xpl buffer and centrifuged at 3,000 rpm for 5 min to remove the Percoll impurities. The purity of the chloroplast fractions was checked using specific markers by immunoblotting.

To obtain enriched membrane and soluble fractions, the intact chloroplasts were partitioned into membrane (pellet) and soluble fractions by centrifugation. To do this, the plastsids were resuspended in protease free ice-cold water containing protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA; #A32965). For complete lysis, the chloroplasts were incubated on ice for 45–60 min followed by incubation at ~80°C O/N. The samples were then thawed on ice and centrifuged at 13,000 rpm. The soluble fraction was recovered, and the pellet was resuspended in 1XPBS (+ 1% w/v SDS and protease inhibitor cocktail).

**Thermolysin assay**

Proteolysis by thermolysin was performed as described in Cecchini et al. (2015). Briefly, intact chloroplast fractions were resuspended in 1× import buffer (0.33 M sorbitol, 50 mM HEPES, pH 8.0) and treated with thermolysin (to a final concentration of 0.1 mg/mL). The reaction was quenched by adding 10 mM EDTA. The chloroplasts were carefully separated on 40% v/v Percoll cushion.

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**Figure 11** Speculative model and summary of roles of HyPRPs in microbial interactions. A, Cellular localization and proposed roles of HyPRPs in signal mobilization. Green color indicates organelles and structures in cells where HyPRPs are found. Arrows show the potential mobilization of signals by HyPRPs, either within a cell or locally near the infection (dashed line) or the long-distance mobilization of signals to distal tissue (solid line). Within cell, movement may result in local signaling, for example due to a mobilized signal binding to a receptor (Cecchini et al., 2015). Although leaf epidermal cells are depicted, similar localization patterns are expected for root cells. The * indicates HyPRPs that have roles in local responses to microbes (root or leaf colonization); most also have roles in longer distance responses as well (B). Data from this and previous work (Peltier et al., 2004; Tomizioli et al., 2014; Cecchini et al., 2015, 2021) is summarized. The cell on the left shows HyPRPs that likely locate to plastid thylakoids and/or OEMs as well as ER and PM/PD. HyPRPs that fit the left pattern, AZL3* and DRN1*, have positive roles. The cell on the right shows HyPRPs that locate to plastid OEMs as well as ER and/or PM/PDs. HyPRPs that fit the right pattern have positive roles (AZI1, EARLI1, AZI3, AZI4, AZL2, AZL13, and AZL14), a mainly negative role (ELP) or have unknown roles (CWLP, DHyPRP) in microbial responses. B, Schematic of the steps at the whole plant level that are affected by one or more HyPRPs: 1, leaf colonization by pathogen (DRN1, positive role); 2, increased immunity to distal infections after an immunizing infection at site 1 (SAR: AZI1, EARLI1, AZI3, AZI4, AZL2, AZL13, and AZL14, positive roles; ELP, negative role); 3, growth of beneficial bacteria in association with roots (AZL3, AZL13, and AZL14, positive roles); 4, stimulation of primary root growth in response to beneficial bacteria colonization of the root (ELP and AZL14, positive roles); 5, stimulation of lateral roots in response to beneficial bacteria colonization of the root (AZL13 and AZL14, positive roles; ELP, negative role); 6, stimulation of whole plant growth by root colonization by beneficial bacteria (AZL3, AZL13, and AZL14, positive roles; ELP, negative role); 7, increased immunity of aerial tissue (ISR after root colonization by beneficial bacteria: AZI1, EARLI1, AZI3, AZL13, and AZL14, positive roles).
Immunoblotting

Equal amounts of total proteins were separated on 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA; #17000010). The concentration of proteins was determined by Bradford assay. The following primary antibodies were used in this study: PR1 antibody (Agrisera, Vännäs, Sweden; #AS10687; 1:2,500), GFP monoclonal antibody (Takara, Shiga, Japan; #632375; 1:2,000), Bip2 antibody (Agrisera; #AS09481; 1:4,000), Tic110 antibody (a gift from Masato Nakai; 1:3,000), chloroplast Hsp70 (a gift from Thomas Leustek; 1:12,000), Toc75 (Agrisera; #AS08351, 1:3000). Herosardish peroxidase-conjugated anti-mouse (Invitrogen, Waltham, MA, USA; #SA-100; 1:4,000), anti-guinea pig (Sigma, St. Louis, MO, USA; #A5545; 1:100,000), or anti-rabbit (Thermo Fisher Scientific; #32460; 1:5,000) secondary antibodies were used. The bands were detected by using SuperSignal pico/femto chemiluminescence kits (Thermo Fisher Scientific; #s1859022/3 and 1859674/5).

Transcript analysis

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized using was the Reverse Transcriptase SuperScript III and Oligo (dT)20 primers (Thermo Fisher Scientific) according to the manufacturer’s instructions. HyPRP expression (in WT/ACD6-1HA) was analyzed by quantitative real-time PCR (qPCR) using SYBR Premix Ex Taq (Takara, Shiga, Japan) in a Bio-Rad Real-Time PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. For Dex-AZL expression (in ACD6-1HA), relative transcript levels were checked by RT-qPCR (optimized for cycle #s). EF1α was used as a reference for normalization.

Pathogen growth bioassays

The evaluation of bacterial pathogen growth was conducted as described (Jung et al., 2009). Briefly, 26–28 day-old WT and HyPRP mutant plants were syringe-infiltrated with PmaDG3 or PmaDG6 (OD600 = 0.0001). Growth was quantified using eight equal sized leaf discs from different plants 3 days after bacterial inoculation. All graphs of colony-forming units (c.f.u.s) were plotted as log10 values.

For SAR assays (Jiang et al., 2021), local leaves of 26–28 day-old plants were syringe-infiltrated with mock (10 mM MgSO4) or PmaDG6/avrRpt2 (OD600 = 0.01). Two days after the primary infection, systemic leaves were infiltrated with PmaDG3 (OD600 = 0.0001). The local leaves were removed from the plants prior to the secondary challenge and the plants were covered with plastic domes following the secondary challenge. Three days postsecondary challenge, eight equal-sized leaf discs were collected and ground in 10 mM MgSO4. The ISR assay was performed exactly as described (Cecchini et al., 2019a) by colonizing the roots with P. simiae WCS417 and later challenging leaves with PmaDG3 OD600 of 0.0001–0.0002. The response gain of SAR or ISR was calculated as described (Jiang et al., 2021).

Plant growth promotion assays of whole plants and roots

Arabidopsis were grown vertically at −20°C with 16-h day/8-h night on small square plates containing Murashige and Skoog (MS) media (GibcoBRL, Life Technologies) without sugar as previously described (Haney et al., 2015). Briefly, ten seeds were germinated on plates and five days after plating, seedlings were thinned to five seedlings per plate and roots inoculated with 1 μL 10 mM MgSO4 (Mock) or P. simiae WCS417 (OD600 = 0.001). After 10 days, scanned (Epson, Nagano, Japan) plant images were used to count lateral roots and measure primary root lengths. Subsequently, the fresh weights were determined.

Root colonization assay

Root colonization assay was performed in 48-well plates as described (Haney et al., 2015). An aliquot of 250 μL MS media (Sigma; #M5519-10L) containing 2% w/v sucrose was added to the wells of 48-well tissue cultures plates (BD Falcon, Franklin Lakes, NJ, USA). Round Teflon mesh disks (McMaster-Carr, Elmhurst, IL, USA; #1100t41) were sterilized and imbibed seed was placed at the center of each disc. Seedlings were subsequently grown at −20°C with 16-h day/8-h dark for 10 days. The MS media was replaced with 270 μL of fresh MS media without sugar. Two days after the media change, wells were inoculated with 30 μL (final OD600 = 0.00002) of P. simiae WCS417 suspended in water. After 7 days, seedlings were removed, and roots and well media analyzed for bacterial amount (six seedlings were combined per data point). The root extracts prepared by grinding and the corresponding well media were plated by serial dilution to determine c.f.u.s.

LC–MS/MS

Approximately 15 g Arabidopsis shoots of WT and ACD6-1HA were homogenized and the chloroplasts separated on Percoll gradients (80% v/v and 40% v/v). The total plastid membrane fraction obtained after centrifugation was used for LC–MS/MS. Total plastid membrane proteins were analyzed by Q-Exactive Orbitrap mass spectrometer. Relative quantification was performed using Maxquant software. The raw data were searched against the Arabidopsis database, as well as reversed protein sequences, and common contaminants. The search results were filtered to be within 1% false discovery rate and label-free quantification using normalized protein abundances was performed to assess differential protein expression between WT and the transgenic expressing ACD6-1HA.

For total spectral analysis, MaxQuant data were analyzed for the normalized total spectra by the scaffold software and the peptides similar/unique to AZL3 and PHB3 were identified.

Plotting and statistical analysis

The graphs were plotted in R studio (version 3.6.1/4.1) using ggplot2 package (version 3.3.2) and all statistics (ANOVA or
Student’s t test) were done using agricolae package (version 1.3.3).

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in Supplemental Table S2.

**Supplemental data**

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

- **Supplemental Figure S1**. Over-represented GO terms for biological processes in PATS plastid predicted proteins and location of HyPRPs in Arabidopsis genome.
- **Supplemental Figure S2**. Targeting of Arabidopsis bipartite signal protein candidates to plastids visualized in separate channels and GFP cleavage in DRN1.
- **Supplemental Figure S3**. ClustalW alignment of Arabidopsis HyPRPs.
- **Supplemental Figure S4**. Amino acid sequences of AZI3, AZI4, AZI5/6, and mutants generated by CRISPR/Cas9-directed mutagenesis
- **Supplemental Figure S5**. Local disease resistance of HyPRP mutants against P. syringae pv. maculicola strains.
- **Supplemental Figure S6**. SAR in elp-2 allelic mutant and Dex-inducible complementation lines.
- **Supplemental Table S1**. List of all PATS positive proteins of Arabidopsis.
- **Supplemental Table S2**. CPRs, PRRs, WWH scores, and experimental and predicted plastid targeting of Arabidopsis HyPRPs.
- **Supplemental Table S3**. List of primers and constructs used in this study.

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