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The *Pseudomonas syringae* effector AvrPtoB targets abscisic acid signaling pathway to promote its virulence in *Arabidopsis*

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Abstract

Phytohormones play an essential role in plant immune responses. Many phytopathogens secret effector proteins to promote infection and plant hormone signaling pathways are considered to be the potential targets of effectors. Here we found that abscisic acid (ABA) signaling was activated rapidly upon infection with *Pseudomonas syringae* pv. *tomato* (*Pst*). *Pst* secretes the effector AvrPtoB to target ABA 8′-hydroxylase CYP707As for degradation in *Arabidopsis thaliana*. CYP707As hydroxylate ABA to an inactive form. The degradation of CYP707As resulted in ABA accumulation and compromised plant immune responses. Our study demonstrated that *Pst* could hijack the key components of *Arabidopsis* ABA signaling pathway to cause disease.

Keywords: *Pseudomonas syringae*, ABA, AvrPtoB, CYP707A

Background

Due to immovable feature, plants are constantly challenged by abiotic and biotic stresses, such as drought, high salinity and pathogens. Many phytohormones have been demonstrated to play essential roles in plant immune response. Of the investigated hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene are the most important ones in plant basal defenses (Li et al. 2019; Ding and Ding 2020). The phytohormone ABA (abscisic acid) is known to regulate plant responses to abiotic stresses, but its role in biotic stress responses remains inconclusive and controversial (Cutler et al. 2010; Cao et al. 2011; Chen et al. 2020).

In response to stress, ABA binds to its receptors PYRABACTIN RESISTANCE1(PYR1)/PYR1-LIKE (PYL) and mediates the binding to clade A protein phosphatase2Cs (PP2Cs), leading to suppression of phosphatase activities. This results in the immediate release of sucrose non-fermenting-1 (SNF1)-related protein kinases (SnRK2s), and induces stomatal closure as well as downstream gene expression via the phosphorylation of S-type anion channels and some transcription factors, such as ABI5 (a basic leucine zipper transcription factor) and HAT1 (an HD-ZIP II transcription factor) (Meyer et al. 1994; Ma et al. 2009; Umezawa et al. 2009; Brandt et al. 2012; Dai et al. 2013). Endogenous ABA levels are regulated by both biosynthesis and catabolism (Nambara and Marion-Poll 2005). The *Arabidopsis* cytochrome P450 (CYP) super-family genes *CYP707A* encode ABA 8'-hydroxylases. These enzymes catalyze the first committed step in ABA catabolic pathway, resulting in the production of 8'-hydroxy ABA. 8'-hydroxy ABA is then isomerized spontaneously to phaseic acid (PA), leading to the significant reduction in biological activity of ABA (Kushiro et al. 2004; Saito et al. 2004). In *Arabidopsis*, there are four *CYP707A* homolog genes; of which the expression of *CYP707A1* is dramatically induced by exogenous ABA application (Okamoto et al. 2006). *Arabidopsis cyp707a* single and double mutants can accumulate high levels of

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ABA in seeds, whereas *CYP707A* overexpression lines display lower ABA levels (Kushiro et al. 2004; Okamoto et al. 2006), suggesting the key roles of *CYP707A* in ABA accumulation.

ABA not only regulates stomatal closure, leaf abscission, seed germination and dormancy, but also regulates plant responses to a wide range of biotic stresses. However, the effect of ABA signaling on basal defenses depends on the type of pathogens. ABA-deficient mutants *aba1-6*, *abi1-1* and *abi2-1* exhibit enhanced susceptibility to the soil-borne bacterium *Ralstonia solanacearum*, but they are resistant to infection of the necrotrophic fungus *Plectosphaerella cucumerina* (Hernández-Blanco et al. 2007). The ABA biosynthesis mutants *aba2-12*, *aao3-2* and ABA-insensitive mutant *abi4-1* show enhanced susceptibility to oomycete pathogen *Pythium irregular*, necrotrophic pathogen *Alternaria brassicicola,* but exhibit strong resistance to necrotrophs *Botrytis cinerea* (Adie et al. 2007); while ABA biosynthesis mutant *aba3-1* is susceptible to biotrophic oomycete pathogen, *Hyaloperonospora arabidopsis* (Fan et al. 2009). By contrast, the ABA biosynthesis mutants *aba2-1* and *aba3-1* display enhanced resistance to the biotrophic powdery mildew fungus *Golovinomyces cichoracearum* (Xiao et al. 2017).

During *Pseudomonas syringae* infection, ABA plays a positive role in pre-invasive stomatal immunity by inducing stomatal closure to prevent pathogen entry; however, it plays a negative role in post-invasive immunity (Cao et al. 2011). *aba3-1*, *aba2-3* and *pyr1-2* are more resistant to *P. syringae* by syringe infiltration (García-Andrade et al. 2020). Application of exogenous ABA enhances plant susceptibility to *Pst* and *Pst* hrpA$^-$, a type III protein secretion system (T3SS)-defective mutant (de Torres-Zabala et al. 2007; Fan et al. 2009; Tan et al. 2019; García-Andrade et al. 2020). Notably, *Pst* infection has been reported to induce the accumulation of endogenous ABA, which is likely one of the reasons that this pathogen causes disease in *Arabidopsis* plants (de Torres-Zabala et al. 2007; Gao et al. 2016).

Many phytopathogens deploy effector proteins to subvert host immune response or target susceptible genes to promote infection. *Pst*, for instance, can deliver a set of effector proteins to host cells, which dramatically suppress host immune responses. Introducing *Pst* effector HopAM1 to *Arabidopsis* markedly increases water availability and colonization ability of the pathogen. HopAM1 also suppresses host basal defense and improves the sensitivity to ABA in plants (Goel et al. 2008). Likewise, the effector protein AvrPtoB has E3 ligase activity and can target host receptor-like kinases (RLKs) such as FLS2, CERK1 and LecRK-IX.2 for degradation, which subsequently suppresses the immune responses mediated by these receptors (Janjusevic et al. 2006; Göhre et al. 2008; Gimenez-Ibanez et al. 2009; Xu et al. 2020). The *Arabidopsis* genome harbors 23 EXO70 protein family members, some of which are involved in plant immunity. AvrPtoB can ubiquitinate and mediate the degradation of EXO70B1 to overcome EXO70B1-mediated resistance (Wang et al. 2019). Conditional expression of AvrPtoB in *Arabidopsis* results in a significant increase in ABA level and an enhanced susceptibility to *Pst* hrpA$^-$ (de Torres-Zabala et al. 2007).

We previously demonstrated that AvrPtoB targets LecRK-IX.2 for degradation, leading to immune suppression in host plants. AvrPtoB can also mediate the degradation of NON-EXPRESSER OF PR1 GENES1 (NPR1) to interfere with SA signaling and subvert plant innate immunity (Chen et al. 2017). However, how AvrPtoB manipulates host's ABA signaling pathway is unclear. In this study, we reveal that AvrPtoB targets ABA 8'-hydroxylase CYP707As for degradation, which subsequently facilitates ABA accumulation and promotes *Pst* infection.

Results

cyp707a mutants demonstrate compromised PTI responses

Recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) is essential for plants to distinguish self- and nonself-components (Zipfel 2014). To explore this process, we screened a stock of *Arabidopsis* mutants by treating with flg22, a peptide of bacterial flagellin that can trigger strong PTI response (Gómez-Gómez et al. 1999). Two mutants, *cyp707a1* (*cyp707a1-1,* SALK_069127) and *cyp707a2* (*cyp707a2-2,* SALK_083966) showed a reduction in both flg22-induced ROS production and the expression of PTI responsive gene *FRK1* (Fig. 1a and Additional file 1: Fig. S1a) (Asai et al. 2002). These two mutants also showed a reduced ROS burst by another immune elicitor elf18 that is derived from translation elongation factor Tu of bacterial pathogens (Fig. 1b). Pathogen-induced callose deposition has been reported to function as a chemical and physical defense mechanism for host to avoid pathogen attack (Kunze et al. 2004). We then examined the callose deposition in plant leaves treated with flg22. The result showed that flg22-induced callose deposition was significantly suppressed in both *cyp707a1* and *cyp707a2* mutants when compared to Col-0 (Fig. 1c, d). The expression of *GSL6*, a gene encoding GLUCAN SYNTHASE-LIKE (GSL) callose synthases, was also remarkably down-regulated in the two mutants (Additional file 1: Fig. S1b). MAPK activation is one of the early events that can be triggered by various PAMPs molecules. The flg22-induced MAPK activation displayed slight reduction in *cyp707a2* mutant compared with that in Col-0 (Additional file 1: Fig. S1c). The callose deposition also reduced

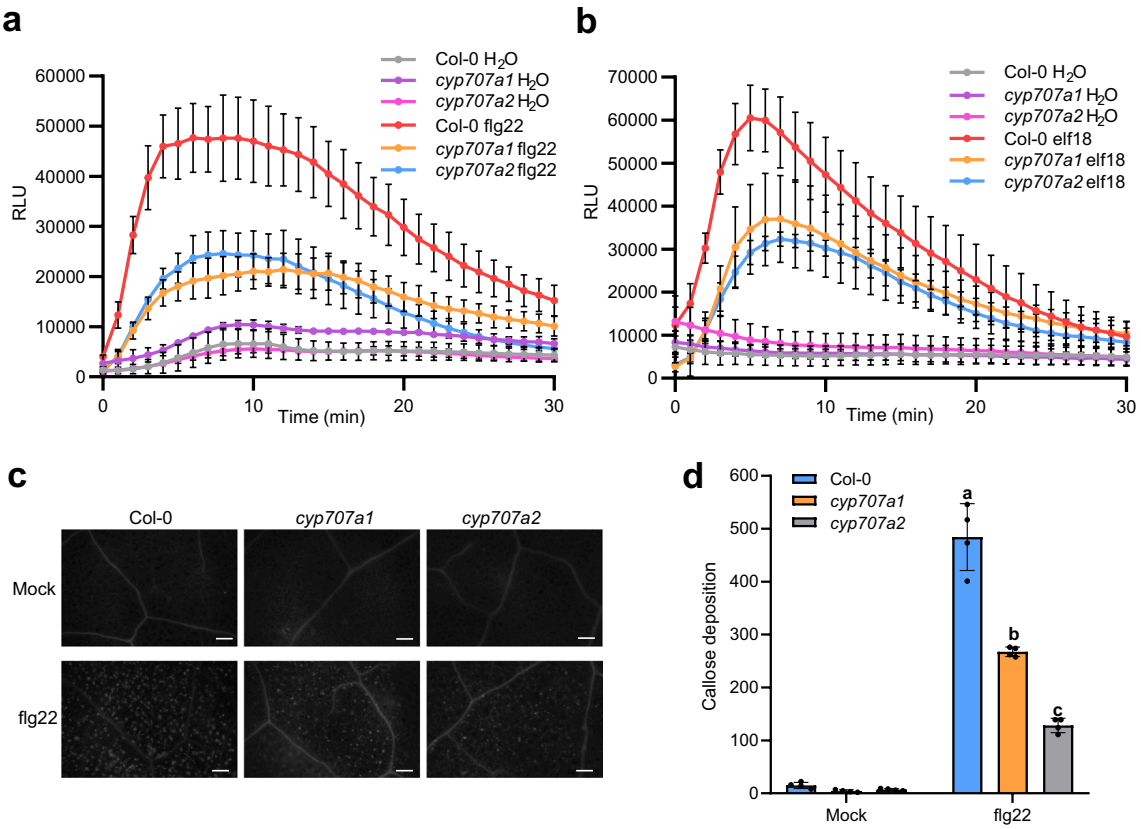

Fig. 1 The *cyp707a* mutants display reduced PTI response. **a** and **b** flg22- and elf18-induced ROS burst is reduced in *cyp707a* mutants. Leaf discs from three-week-old *Arabidopsis* plants were treated with 100 nM flg22 or 100 nM elf18, and the ROS burst was recorded. H_2O served as a control. Error bars represent means ± SD (n = 3 biological replicates). RLU, Relative light units. **c** and **d** *cyp707a* mutants display decreased callose deposition in response to flg22 treatment. Four-week-old Col-0 and *cyp707a* mutants were syringe infiltrated with Mock (10 mM $MgCl_2$) or 100 nM flg22. The calloses were stained with aniline blue (**c**) and counted under microscope (**d**) at 12 hpi (hours post-inoculation). Scale bar = 100 μm. Error bars represent means ± SD (Two-way ANOVA, $P < 0.001$, n = 4)

in *cyp707a1* and *cyp707a2* mutants after *Pst* hrcC$^-$ treatment (Additional file 1: Fig. S1d, e). Taken together, the above results indicate that CYP707A1 and CYP707A2 positively regulate plant responses to flg22 treatment.

CYP707As are required for disease resistance to *Pst*

There are four members of *CYP707A* genes in *Arabidopsis* genome, namely *CYP707A1*, *CYP707A2*, *CYP707A3* and *CYP707A4*. The transcription levels of all four *CYP707As* were induced by dehydration and exogenous ABA treatment (Saito et al. 2004). We checked the expression patterns of these four genes in Plant eFP database. The results showed that all of the genes are induced by abiotic stress, such as auxin (IAA), ABA, methyl jasmonate (MeJA), cold, osmotic, salt and drought treatment. *CYP707A1* and *CYP707A4* transcription can also be induced by heat treatment (Additional file 1: Fig. S2a, b). For biotic stress, four genes showed reduced expression after flg22, *Pst* hrcC$^-$ (a T3SS deficient mutant) and *Pst* treatment, except for *CYP707A1* and *CYP707A4* which, by contrast, were induced by *Pst* (Additional file 1: Fig. S2c). To confirm the results from the database, we used RT-qPCR to analyze the expression levels of *CYP707A1* and *CYP707A2*, and found that both genes can be slightly induced by flg22 and *Pst* compared with mock treatment at 6 hpi, but returned to the base level at 24 hpi (Fig. 2a, b). In addition, *cyp707a2* displayed enhanced susceptibility to *Pst* hrcC$^-$ and was more susceptible to *Pst* inoculation; however, *cyp707a1* was more susceptible to *Pst* treatment but not *Pst* hrcC$^-$ (Fig. 2c, d). These data reveal that CYP707A1 and CYP707A2 are positive regulators of plant disease resistance to *Pst*, and suggest that *Pst* effector(s) likely targets CYP707s to promote pathogenicity.

AvrPtoB interacts with CYP707As

As the effector(s) may interfere with CYP707A-mediated immune response, we then investigated the potential

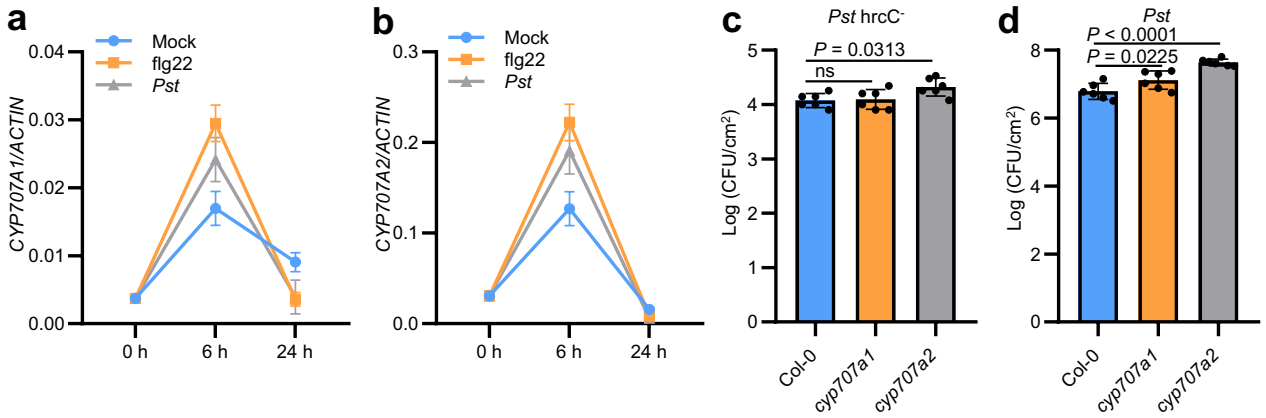

Fig. 2 *CYP707As* positively regulate disease resistance to *Pst*. **a** and **b** *CYC707As* expression after flg22 and *Pst* treatment. Four-week-old *Arabidopsis* leaves were inoculated with Mock (10 mM $MgCl_2$), 100 nM flg22 and *Pst* at a concentration of 5×10^7 CFU/mL. Samples were collected for RT-qPCR at indicated time. Error bars represent means ± SD (n = 3 biological replicates). **c** and **d** Growth of *Pst* hrcC⁻ and *Pst* in Col-0 and *cya707a* mutants. Four-week-old *Arabidopsis* leaves were inoculated with *Pst* hrcC⁻ (**c**) or *Pst* (**d**) at a concentration of 5×10^4 CFU/mL. The plants were subjected to growth curve analysis at 3 days post-inoculation (dpi). Error bars represent means ± SD (Two-way ANOVA, ns, no significance; n = 6)

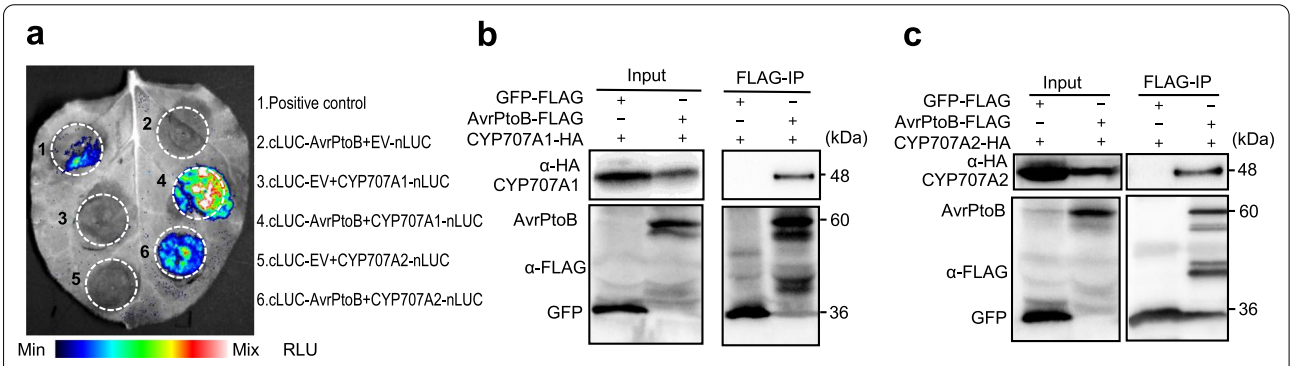

Fig. 3 AvrPtoB interacts with CYP707As. **a** AvrPtoB interacts with CYP707As in *N. benthamiana* by split luciferase assays. *N. benthamiana* leaves were co-infiltrated with *35S:cLUC-AvrPtoB* and *35S:CYP707A1-nLUC* or *35S:CYP707A2-nLUC*. Luciferase complementation imaging assays were performed 48 h later. This experiment was repeated three times with similar results. RLU, Relative light units. The combination of *35S:C2-nLUC* and *35S:cLUC-S1* was used as a positive control. **b** and **c** AvrPtoB interacts with CYP707As in vivo by co-immunoprecipitation assays. *N. benthamiana* leaves were co-infiltrated with *35S:CYP707A1-HA* or *35S:CYP707A2-HA* and *35S:AvrPtoB-FLAG* or *35S:GFP-FLAG*. Plant leaves were immunoprecipitated with anti-FLAG beads, and the proteins were immunoblotted by anti-FLAG antibody. Co-IP proteins were immunoblotted by anti-HA antibody

effector(s) in this event. *Pst* secretes ca. 30 effectors into plant cells (Xin and He 2013). In order to find the effector(s) that may target CYP707A1 or CYP707A2, we cloned all the effectors and screened them by spilt luciferase assays. The result showed that AvrPtoB interacted with both CYP707A1 and CYP707A2 (Fig. 3a). Subcellular localization assays showed that CYP707A1 and CYP707A2 were co-localized with the plasma membrane marker LTI6b-mCherry (Additional file 1: Fig. S3a, b), and exhibited the same localization pattern as AvrPtoB (Xu et al. 2020). To verify the interaction of AvrPtoB with CYP707A1 in vitro, we expressed the proteins in *Escherichia coli* and purified the recombinant proteins by affinity purification. The result showed that MBP-CYP707A1 successfully pulled down GST-AvrPtoB (Additional file 1: Fig. S4a). In addition, in anti-FLAG co-immunoprecipitation (Co-IP) assays, CYP707A1 and CYP707A2 interacted with AvrPtoB but not GFP alone in *N. benthamiana* leaves (Fig. 3b, c).

AvrPtoB targets CYP707As for degradation

The above results showed that AvrPtoB interacted with CYP707A1 and CYP707A2 in vitro and in vivo. We therefore explored the biological significance of the interactions. AvrPtoB is a 553-amino-acid protein. Its N-terminus and C-terminus contain a Pto-interacting

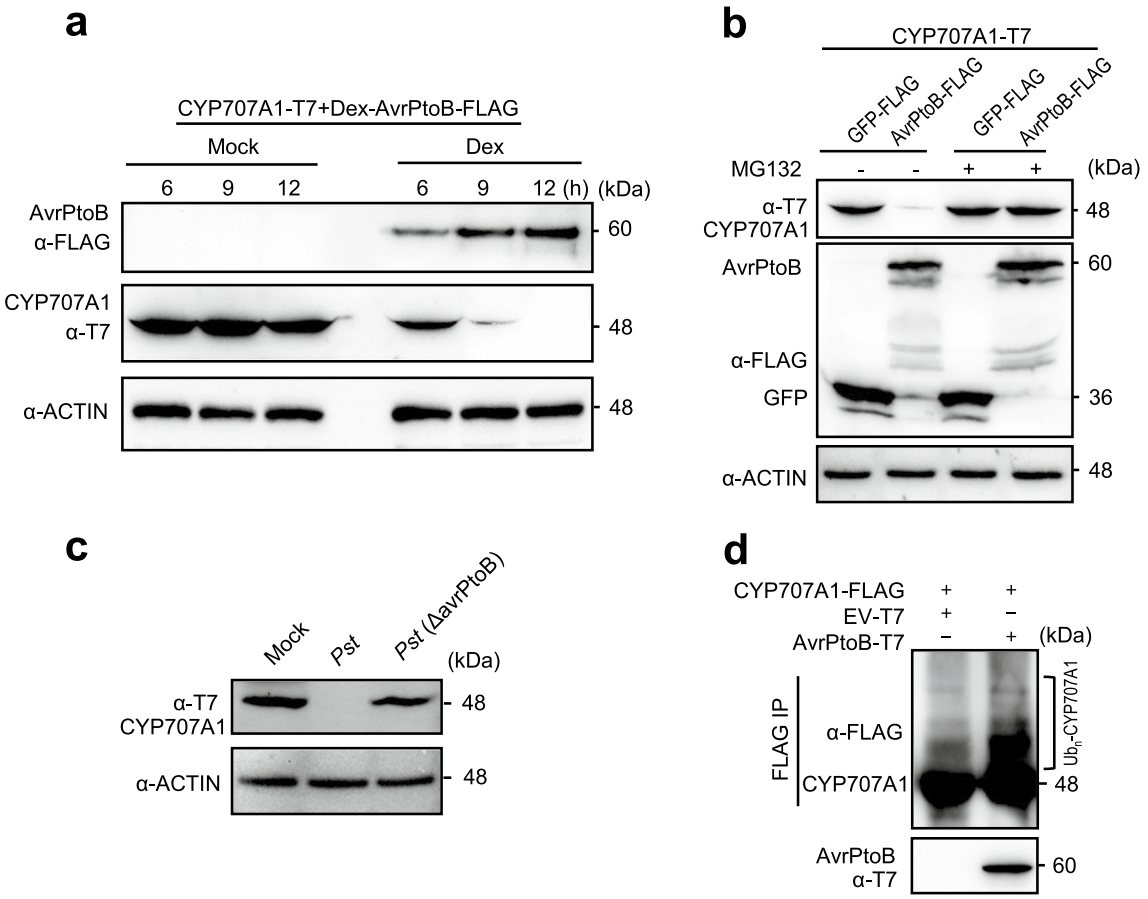

Fig. 4 AvrPtoB mediates CYP707A1 degradation by 26S proteasome. **a** AvrPtoB targets CYP707A1 for degradation. *N. benthamiana* leaves were co-infiltrated with *35S:CYP707A1-T7* and *Dex:AvrPtoB-FLAG*. Plants were infiltrated with 3 μM Dex at 48 hpi to induce the expression of AvrPtoB-FLAG. Leaf extracts were sampled for immunoblotting after mock or Dex treatment at indicated times. Mock is 10 mM $MgCl_2$. **b** The proteasome inhibitor MG132 prevents CYP707A1 from degradation. *35S:CYP707A1-T7* was transiently expressed with *35S:GFP-FLAG* or *35S:AvrPtoB-FLAG* in *N. benthamiana*. MG132 (100 μM) was used to inhibit 26S proteasome-mediated protein degradation at 36 hpi. Samples were harvested at 8 h after MG132 treatment for immunoblotting. **c** *Pst* harbored AvrPtoB degrades CYP707A1. *35S:CYP707A1-T7* stable transgenic plants were inoculated with Mock (10 mM $MgCl_2$), *Pst* or *Pst* (ΔavrPtoB) at a concentration of 2.5×10^3 CFU/mL, respectively. Infected leaves were sampled for immunoblotting at 12 hpi. **d** AvrPtoB ubiquitinates CYP707A1 in vivo. *35S:CYP707A1-FLAG* was transiently expressed with *35S:AvrPtoB-T7* or *35S:EV-T7* in *N. benthamiana*. MG132 (100 μM) was used to inhibit 26S proteasome-mediated protein degradation at 36 hpi. Samples were harvested at 8 h after MG132 treatment. Plant leaves were immunoprecipitated with anti-FLAG beads, and the proteins were immunoblotted by anti-FLAG and anti-T7 antibodies

domain (PID) and a U-box type E3 ubiquitin ligase domain, respectively (Janjusevic et al. 2006; Xiao et al. 2007). We further detected whether AvrPtoB can also mediate CYP707As degradation. In *N. benthamiana* leaves co-expressing dexamethasone (Dex)-inducible *AvrPtoB-FLAG* and *35S:CYP707A1-T7*, Dex treatment significantly reduced the CYP707A1-T7 protein levels (Fig. 4a). However, CYP707A1-T7 protein levels were completely rescued in the presence of the 26S proteasome inhibitor MG132 (Fig. 4b). To examine the degradation event during *Pst* infection, we inoculated the *35S:CYP707A1-T7* transgenic plant with *Pst* and *Pst* (ΔavrPtoB). The result revealed that *Pst* rather than *Pst* (ΔavrPtoB) infection led to the degradation of CYP707A1 (Fig. 4c).

To assess whether AvrPtoB can degrade other CYP707As and key regulators in ABA signaling, we also detected their protein levels when co-expressed with AvrPtoB in *N. benthamiana* leaves. As shown in Additional file 1: Fig. S4b, CYP707A3 protein level was reduced when co-expressed with AvrPtoB, the same as that for CYP707A1 and CYP707A2. But the PP2Cs ABI1 and ABI2, two negative regulators of ABA signaling pathway, showed no significant differences when co-expressed with AvrPtoB or GFP alone (Additional file 1: Fig. S4c). NGATHA (NGA1) is a transcriptional activator of the

key enzyme *NINECIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) in ABA biosynthesis (Sato et al. 2018). The protein level of NGATHA was not affected by AvrPtoB, neither was VirE2-INTERACTING PROTEIN1 (VIP1), a transcriptional activator of *CYP707A1* and *CYP707A3* (Additional file 1: Fig. S4d) (Tsugama et al. 2012). To investigate whether AvrPtoB can ubiquitinate CYP707As in vivo, we co-expressed CYP707As and AvrPtoB in *N. benthamiana*. By FLAG Co-IP assays, we found that CYP707A1 and CYP707A2 were highly ubiquitinated when co-expressed with AvrPtoB but not with EV (Fig. 4d and Additional file 1: Fig. S4e). In summary, these results demonstrate that CYP707As are the target of AvrPtoB and can be degraded via 26S proteasome.

AvrPtoB promotes ABA sensitivity in *Arabidopsis*

Previous studies have demonstrated that AvrPtoB transgenic seedlings are hypersensitive to SA-induced toxicity and *Dex:HopAM1* transgenic lines are severely inhibited by ABA (Goel et al. 2008; Chen et al. 2017). *Dex:AvrPtoB* transgenic plant can induce a significant increase in ABA levels after Dex treatment for 6 h (de Torres-Zabala et al. 2007). We therefore examined the responses of the *pEst:AvrPtoB* transgenic seedlings in the presence of ABA. The two AvrPtoB transgenic lines exhibited a lower cotyledon greening rate than Col-0 and *pEst: EV* (empty vector) transgenic line (Fig. 5a–c). AvrPtoB also markedly induced the expression of *NCED3* and *RAB18*, two ABA-responsive genes (Fig. 5d, e). Furthermore, we determined whether AvrPtoB can also regulate auxin and JA signaling pathways. AvrPtoB transgenic seedlings showed no significant difference in seed germination compared with Col-0 and *pEst:EV* transgenic lines after auxin (IAA), methyl jasmonate (MeJA) and extradiol treatment (Additional file 1: Fig. S5a, b), but they exhibited hypersensitivity to SA treatment (Additional file 1: Fig. S5c) (Chen et al. 2017). Nevertheless, these results indicate that AvrPtoB promotes sensitivity to ABA in *Arabidopsis*.

CYP707As are virulent targets of AvrPtoB

To gain insight into the function of CYP707As in plant immunity, we next analyzed the role of AvrPtoB in CYP707As-mediated defense during *Pst* infection. We compared bacterial replication in Col-0, *cyp707a1* and *cyp707a2* after inoculation with *Pst* or *Pst* (ΔavrPtoB). There was no significant difference between Col-0 and *cyp707a1* under *Pst* (ΔavrPtoB) treatment, while the difference between Col-0 and *cyp707a2* was reduced when compared to *Pst* treatment (Fig. 6a). Exogenous application of ABA treatment resulted in the proliferation of *Pst*. We found that when the plants were pre-treated with ABA or ABA inhibitor fluridone, *Pst* proliferated to a similar level in Col-0 and *cyp707a1*; however, the difference between Col-0 and *cyp707a2* was significantly reduced than mock treatment (Fig. 6b, c). Notably, fluridone did not inhibit the growth of *Pst* (Fig. 6d). These results further suggest that AvrPtoB targets CYP707A1 and CYP707A2 to promote infection.

Discussion

ABA is a major phytohormone that is involved in a variety of biotic and abiotic responses in plants. Although ABA has been demonstrated to have a clear role in abiotic stresses, it remains disputed for its roles in plant immunity (Adie et al. 2007; Hernández-Blanco et al. 2007; Fan et al. 2009; Cao et al. 2011; Xiao et al. 2017; Tan et al. 2019). ABA induces stomatal closure under drought stress, and this prevents plants from water loss. It is known that stomatal closure can prevent *Pst* invasion through these natural pores (Melotto et al. 2006). However, during post-invasive stage, stomatal closure facilitates the establishment of an aqueous intercellular space with high humidity, which benefits *Pst* proliferation (Xin et al. 2016).

In addition to manipulating stomata to help prevent water loss, endogenous ABA has been found to facilitate *Pst* infection. In fact, *Pst* infection could induce ABA accumulation in *Arabidopsis*, and the effector protein AvrPtoB has been suggested to dictate this process (de Torres-Zabala et al. 2007). By screening the *Arabidopsis* mutant stock, we discovered that c*yp707a1* and *cyp707a2* mutants were susceptible to *Pst* infection, and identified CYP707A proteins as the targets of AvrPtoB to induce ABA accumulation. Therefore, we resolved the mystery of *Pst*–induced ABA accumulation in *Arabidopsis* (Fig. 7). CYP707As are key enzymes in the oxidative catabolism of ABA and their roles in plant immunity are unclear (Kushiro et al. 2004; Saito et al. 2004). ABA can attenuate callose deposition which is associated with basal defense (de Torres-Zabala et al. 2007; García-Andrade et al. 2011). ABA pre-treatment can reduce flg22-induced H_2O_2 generation (Tan et al. 2019). The reduced production of flg22-induced ROS in *cyp707a* mutants may be attributed to the high level of endogenous ABA. Although only the *cyp707a2* mutant showed enhanced susceptibility to *Pst* hrcC$^-$, both *cyp707a1* and *cyp707a2* mutants were susceptible to *Pst* (Fig. 2), highlighting the CYP707As' role in plant basal defense.

By in vitro and in vivo protein–protein interaction assays, we were able to show that CYP707A1 and CYP707A2 physically interacted with AvrPtoB. AvrPtoB is a C-terminal U-box type E3 ubiquitin ligase and targets multiple immune regulators in host cells, such as FLS2, CERK1, LecRK-IX.2 and NPR1 (Göhre et al. 2008; Gimenez-Ibanez et al. 2009; Chen et al. 2017; Xu et al. 2020). Unlike the immune regulators, CYP707 family proteins

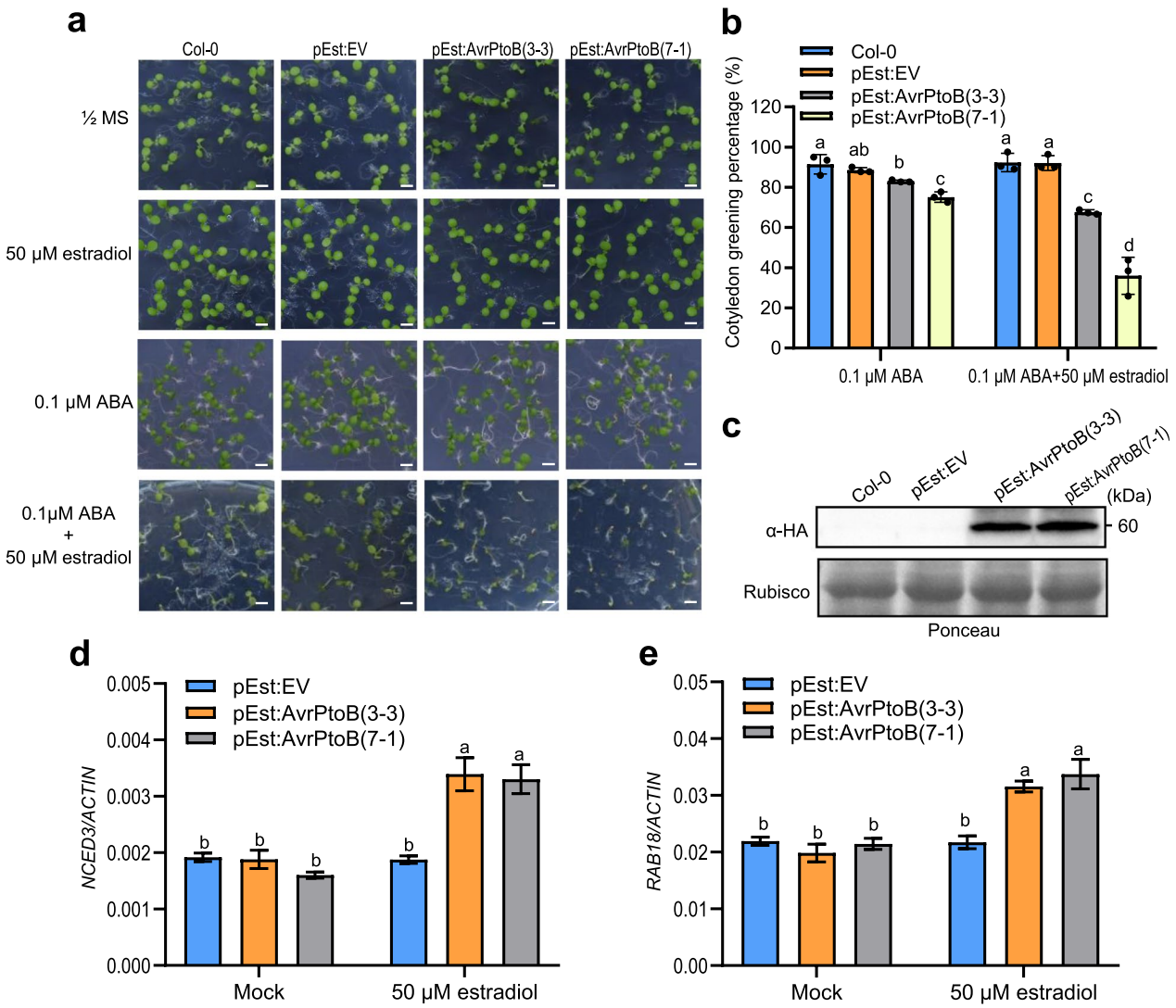

Fig. 5 AvrPtoB increases ABA sensitivity in *Arabidopsis*. **a** Ecotopic expression of AvrPtoB increases the sensitivity to ABA in plants. The *Arabidopsis* seedlings were grown on ½ MS containing 0.1 μM ABA or 50 μM estradiol or both for 7 days. Scale bar = 2.5 mm. **b** Seed germination greening ratio in (**a**). Error bars represent means ± SD (Two-way ANOVA, $P < 0.01$, n = 3). **c** AvrPtoB protein levels in *pEst:AvrPtoB* transgenic plants. The AvrPtoB proteins were shown in two independent AvrPtoB transgenic lines after 50 μM estradiol treatment (**a**). **d** and **e** AvrPtoB induces ABA regulatory gene expression. The two-week-old transgenic plants were sprayed with 50 μM estradiol. The plant leaves were sampled at 12 hpi for RT-qPCR assays. Error bars represent means ± SD (Two-way ANOVA, $P < 0.0001$, n = 3)

have not been shown to act in plant immune responses yet. CYP707A proteins contribute to ROS burst and callose deposition during pathogen infection, indicating their roles in early immune responses (Fig. 1). However, CYP707As are key enzymes that catalyze ABA to an inactive form. The *cyp707a* mutants accumulated high levels of endogenous ABA and were susceptible to *Pst*, suggesting that CYP707A could inactivate ABA to attenuate *Pst* infection. AvrPtoB can increase the expression of *NCED3* and foliar ABA levels in *Arabidopsis*, however, it is unknown how AvrPtoB manipulates plant ABA signaling pathway (de Torres-Zabala et al. 2007). In this article, we revealed that CYP707A1 and CYP707A2 are additional targets of AvrPtoB.

It has been reported that many effectors promote pathogenicity through manipulating plant hormone signaling pathway. HopAM1 is the first type III effector that was reported to aid pathogen adaptation to water availability in plant. Although the expression of HopAM1 in transgenic plants does not induce ABA production, it does enhance ABA responses and suppress basal defenses (Goel et al. 2008). HopZa1 targets the orthologues of

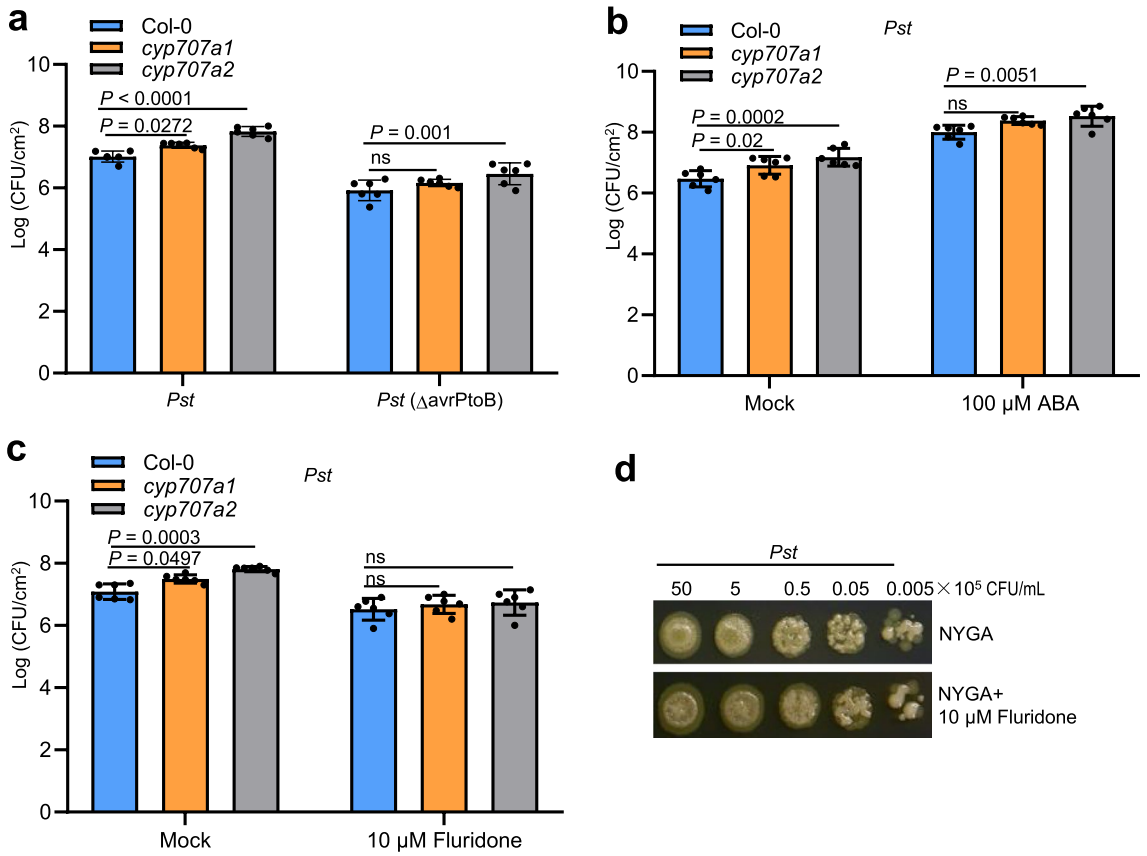

Fig. 6 CYP707As are virulent targets of AvrPtoB. **a–c** Growth of *Pst* and *Pst* (ΔavrPtoB) in Col-0 and *cya707a* mutants. Four-week-old *Arabidopsis* leaves were inoculated with *Pst* and *Pst* (ΔavrPtoB) at a concentration of 5×10^4 CFU/mL (**a**) or sprayed with Mock (H_2O) or ABA (100 μM) (**b**) or 10 μM fluridone (**c**) 12 h before pathogen treatment. The plants were subjected to growth curve analysis at 3 dpi. Error bars represent means ± SD (Two-way ANOVA, ns, no significance; n = 6). **d** 10 μM fluridone doesn't inhibit *Pst* growth on NYGA medium. The *Pst* was serially diluted on NYGA medium with or without 10 μm fluridone. The photographs were taken after incubation for 4 days

JAZ1 in both soybean (*Glycine max*) and *Arabidopsis* to promote their degradation in a COI1-dependent manner, thereby activating JA signaling to enhance *Pst* infection (Jiang et al. 2013). For the hemi-biotrophic fungus *Fusarium oxysporum*, the effector SECRETED IN XYLEM4 (FoSIX4) can contribute to disease development caused by *F. oxysporum* when expressed in *Arabidopsis*. *Arabidopsis* plants inoculated with the *six4* mutant strain show reduced expressions of JA-responsive genes, demonstrating that FoSIX4 promotes pathogen virulence via activating host JA signaling pathway (Thatcher et al. 2012). In addition to JA, ethylene is a gaseous hormone that regulates various biological processes in plants, including defense against pathogens. The *Xanthomonas euvesicatoria* (Xcv) effector protein XopD, carrying a C-terminal SUMO protease domain, is reported to target the tomato ethylene responsive transcription factor SlERF4 to suppress ethylene production, which is required for anti-Xcv immunity and symptom development (Kim et al. 2013). HopAF1 secreted by *P.syringae* inhibits host defense response by manipulating MTN (methylthioadenosine nucleosidase) activity and consequently dampens ethylene production (Washington et al. 2016). As a counter-defense strategy, oomycetes pathogen *Phytophthora sojae* secretes the RXLR effector PsAvh238 to destabilize plant Type2 1-aminocyclopropane-1-carboxylate synthases (ACSs), the key enzymes in catalyzing the rate-limiting step of ET biosynthesis, to reduce ET production and promote infection (Yang et al. 2019).

Conclusions

Taken together, we discovered an additional virulence target of the *Pst* effector AvrPtoB in *Arabidopsis*. We demonstrated that AvrPtoB induced ABA accumulation by degrading ABA 8'-Hydroxylase CYP707As to promote *Pst* infection. Because AvrPtoB targets multiple proteins in plants, it is interesting to unravel the dynamic interactions of AvrPtoB with these proteins in future studies.

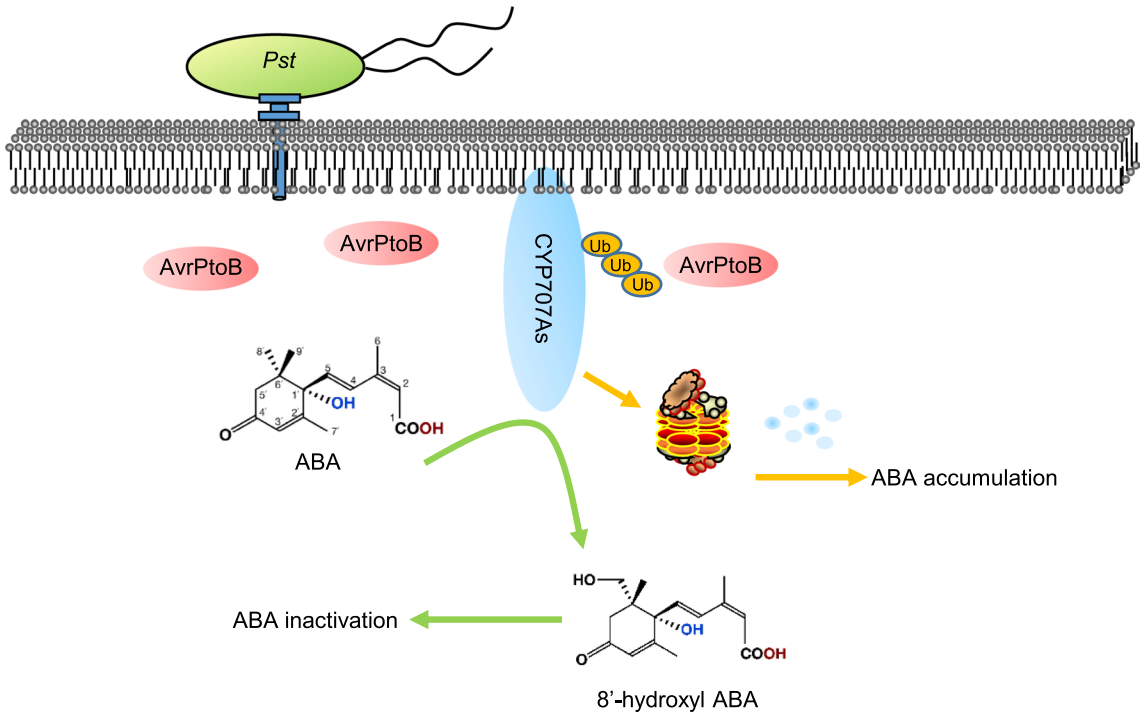

Fig. 7 The working model. ABA is catalyzed to 8'-hydroxyl ABA by CYP707As, a group of P450-type mono-oxygenases, to maintain ABA signal homeostasis in *Arabidopsis* plants. During *Pst* infection, the pathogen-delivered effector AvrPtoB degrades CYP707As via 26S proteasome. As a result, ABA is accumulated, which leads to increased susceptibility to *Pst*

In addition, how ABA enhances plant susceptibility to *Pst* is still unknown. It is worth investigating if ABA could increase interior humidity in plant cells, thereby facilitating pathogen proliferation.

Methods

Plant materials and growth conditions

A. thaliana T-DNA insertion mutants *cyp707a1-1* (SALK_069127) and *cyp707a2-2* (SALK_083966c) were used. Plants were grown at 23 °C under 10 h of light/14 h of dark for 4 weeks. *pEst:EV*, *pEst:AvrPtoB* and *35S:CYP707A1-T7* transgenic plants were generated via floral dip transformation procedure (Clough and Bent 1998). For phytohormones phenotyping assays, surface-sterilized seeds were sowed on 1/2 MS medium with or without phytohormones or estradiol. The seeds were stratified at 4 °C for 3 days in dark before being planted on media. Then the plates were moved to a growth chamber at 23 °C under short-day conditions.

ROS burst measurement

Three-week-old *Arabidopsis* seedling leaves were sampled for leaf disks and kept in 96-well plate with ddH_2O overnight. Before measurement, ddH_2O was replaced by reaction mixtures containing 17 mM luminol L-012 (Wako), 10 mg/mL horseradish peroxidase, 100 nM flg22 or 100 nM elf18. Each treatment contained at least three replications. Luminescence was measured continuously for 30 min using Infinite F200 (TECAN).

MAPK assay

Two-week-old *Arabidopsis* seedlings on the plate were sprayed with ddH_2O or 100 nM flg22. Samples were collected and frozen in liquid nitrogen at indicated time points. The total proteins were extracted with 1 × laemmli (0.0625 M Tris–HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 5% 2-mercaptoethanol, pH 6.8) buffer and separated on a 12% SDS-PAGE. Activated MAPKs were detected by immunoblotting with phospho-p44/42 MAPK antibody (Cell Signaling).

Callose staining and quantification

Leaves of four-week-old *Arabidopsis* plants were infiltrated with H_2O, 100 nM flg22 or *Pst* hrcC$^-$ at a concentration of 5×10^7 CFU/mL in 10 mM of $MgCl_2$ for 12 h or 24 h. The leaves were transferred into FAA solution (10% formaldehyde, 5% acetic acid and 50% ethanol) for 12 h, de-stained in 95% ethanol for 6 h and washed twice with ddH_2O, then stained with 0.01% aniline blue in 150 mM KH_2PO4 (pH 9.5) for 1 h at room temperature.

The callose deposits were visualized with a fluorescence microscope (OLYMPUS IX71). Callose deposits were counted by Image J software.

RT–qPCR

Total RNA was isolated from plants treated with different conditions at indicated time points by TRIzol Reagent (Invitrogen) according to the technical manual. One microgram of total RNA was subjected to synthesize the first-strand cDNA by HiScript Q RT SuperMix with a genomic DNA wipe (Vazyme, China) according to the technical manual. qPCR was performed by the Bio-Rad system using ChamQ SYBR qPCR Master Mix (Vazyme, China). *Actin2* was used as an internal control. Each sample was performed in triplicate (Additional file 2: Table S1).

Pathogen inoculation assay

Bacterial strains were grown on NYGA medium (0.5% Peptone, 0.3% yeast extract and 0.2% glycerin) at 28 °C. Four-week-old Col-0, *cyp707a1* and *cyp707a2* were infiltrated with *Pst*, *Pst* hrcC^{-} or *Pst* (ΔavrPtoB) at a concentration of 5×10^4 CFU/mL, respectively. Three days after inoculation, plants were subjected to growth curve analysis as described by Liu (Liu et al. 2011). The *Pst* (ΔavrPtoB) deletion mutant were described previously (Xu et al. 2020).

Transient expression in *Nicotiana benthamiana*

For split-luciferase complementation assay, *Agrobacterium tumefaciens* (strain EHA105) carrying the indicated nLUC and cLUC constructs was mixed and infiltrated into 4-week-old *N. benthamiana* leaves. *35S:C2-nLUC* and *35S:cLUC-S1* were used as a positive control (Zhang et al. 2011). Two days after infiltration, *N. benthamiana* leaves were rubbed with 0.5 mM D-luciferin (Gold Biotechnology) and kept in the dark for 5 min. The luciferase images were captured by Tanon-5200 (Chen et al. 2008).

For subcellular localization assay, CYP707A1 or CYP707A2 were fused to GFP at their C-terminal under the control of 35S promoter (*35S:CYP707A1-GFP* or *35S:CYP707A2-GFP*) and transiently expressed in *N. benthamiana*. The images were observed using a Leica SP8 confocal laser microscope at 48 hpi. LTI6b-mCherry was used as a marker.

For Co-IP assay, CYP707A1-HA, CYP707A2-HA, GFP-FLAG and AvrPtoB-FLAG under the control of 35S promoter were transient expressed in *N. benthamiana* by *A. tumefaciens* strain GV3101. At about 48 h, the infiltrated leaves were sampled and total proteins were extracted with extraction buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Triton, 0.2% NP-40, 6 mM 2-mercapto-Ethanol and proteinase inhibitor cocktail (Roche), pH7.5). The anti-FLAG IP was performed by incubating the proteins with 30 µL anti-FLAG (R) M2 Affinity Gel (Sigma-Aldrich, catalog # A2220) for 2 h on an end-over-end shaker at 4 °C. After washing three times with extraction buffer, the eluted proteins were separated by SDS–PAGE and revealed by immunoblot analysis using anti-FLAG and anti-HA antibody.

Recombinant protein purification

GST-AvrPtoB were purified as described previously (Xu et al. 2020). CYP707A1 were cloned into the vector pMal-C4X. The positive clones were transformed into *Escherichia coli* (BL21). Bacterial cells were grown in Luria Broth (LB) medium at 37 °C with shaking until the OD600 reaches 0.6. The MBP-CYP707A1 was induced with 0.5 mM IPTG at 16 °C overnight and purified using amylose beads according to the technical manual. The purified proteins were ultrafiltrated and diluted in PBS buffer containing 10% glycerin to 1 µg/µL and stored at − 80 °C before use.

MBP pull-down assays

MBP pull-down assays was performed as described by Liu et al. (2011) with minor modification. In brief, 3 µg of each MBP-CYP707A1 and GST-AvrPtoB were incubated in TEN100 buffer (20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1 mM EDTA and 0.2% Triton X-100) with 30 µL amylose beads on an earthquake shaker for 2 h at 4 °C. Then the beads were washed at least 4 times with NETN300 buffer (20 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA and 0.5% NP-40). The proteins were eluted by adding 50 µL 1 × laemmli buffer and boiled for 5 min at 95 °C. Eluted proteins were separated on a 12% SDS-PAGE gel and immunoblotted with anti-MBP and anti-GST antibody, respectively.

Abbreviations

ABA: Abscisic acid; Co-IP: Co-immunoprecipitation; Dex: Dexamethasone; flg22: A 22-amino-acid peptide of bacterial flagellin; GST: Glutathione S-transferase; IAA: 3-Indoleacetic acid; JA: Jasmonic acids; MAMPs: Microbe-associated molecular patterns; MBP: Maltose-binding protein; MeJA: Methyl Jasmonate; MTN: Methylthioadenosine nucleosidase; MS: Murashige and Skoog; PA: Phaseic acid; PAMPs: Pathogen-associated molecular patterns; PP2Cs: Phosphatase 2Cs; RT-qPCR: Reverse transcription quantitative PCR; ROS: Reactive oxygen species; SA: Salicylic acid; T3SS: Type III protein secretion system.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00110-8.

Additional file 1: Figure S1. The *cyp707a* mutants demonstrates defective PTI response. **Figure S2.** Expression profiles of CYP707A1, CYP707A2, CYP707A3 and CYP707A4 in response to biotic stress, abiotic stress and

hormone treatment. **Figure S3.** Subcellular localization of CYP707A1 and CYP707A2. **Figure S4.** AvrPtoB targets CYP707As for degradation. **Figure S5.** AvrPtoB does not interfere with IAA, SA and MeJA signaling pathways.

Additional file 2: Table S1. Primers used in this study.

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Authors' contributions
JL and NX conceived and designed the project. YL and NX performed the experiments and analyzed the data. JL, NX, YL, and M. R. Mahmud wrote the article. JL supervised research. All authors read and approved the final manuscript.

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Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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