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Loss of chloroplast-localized protein phosphatase 2Cs in *Arabidopsis thaliana* leads to enhancement of plant immunity and resistance to *Xanthomonas campestris* pv. *campestris* infection

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SUMMARY

Protein phosphatases (PPs) counteract kinases in reversible phosphorylation events during numerous signal transduction pathways in eukaryotes. PP2Cs, one of the four major classes of the serine/threonine-specific PP family, are greatly expanded in plants. Thus, PP2Cs are thought to play a specific role in signal transduction pathways. Some rice PP2Cs classified in subgroup K are responsive to infection by the compatible *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight. In *Arabidopsis thaliana*, orthologous PP2C genes (*AtPP2C62* and *AtPP2C26*) classified to subgroup K are also responsive to *Xanthomonas campestris* pv. *campestris* (*Xcc*, causal agent of black rot) infection. To elucidate the function of these subgroup K PP2Cs, *atpp2c62*- and *atpp2c26*-deficient *A. thaliana* mutants were characterized. A double mutant plant which was inoculated with a compatible *Xcc* showed reduced lesion development, as well as the suppression of bacterial multiplication. AtPP2C62 and AtPP2C26 localized to the chloroplast. Furthermore, the photosynthesis-related protein, chaperonin-60, was indicated as the potential candidate for the dephosphorylated substrate catalysed by AtPP2C62 and AtPP2C26 using two-dimensional isoelectric focusing sodium dodecylsulfate-polyacrylamide gel electrophoresis (2D-IDF-SDS-PAGE). Taken together, *AtPP2C62* and *AtPP2C26* are suggested to be involved in both photosynthesis and suppression of the plant immune system. These results imply the occurrence of crosstalk between photosynthesis and the plant defence system to control productivity under pathogen infection.

Keywords: *Arabidopsis thaliana*, chloroplast, plant immunity, protein phosphatase 2C, type III secretion system, *Xanthomonas campestris* pv. *campestris*.

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INTRODUCTION

On perception of microbe-associated molecular patterns (MAMPs) by their specific receptors (Boller and He, 2009; Jones and Dangl, 2006), plants trigger a series of defence responses, including the generation of reactive oxygen species (ROS), induction of mitogen-activated protein kinases (MAPKs), pathogenesis-related (PR) gene expression and callose deposition at the cell wall (Li *et al.*, 2016; Segonzac and Zipfel, 2011). Because of MAMP-triggered immunity (MTI), most bacteria are unable to cause disease (Boller and Felix, 2009). However, pathogens have acquired MTI-interfering effector proteins to render certain plant species susceptible. In turn, plants have evolved disease resistance proteins that recognize the presence of individual effectors, resulting in effector-triggered immunity (ETI). This conforms to the 'gene-for-gene' interaction model (Chisholm *et al.*, 2006; Grant *et al.*, 2006; Jones and Dangl, 2006).

Protein phosphatases (PPs), by reversing the action of protein kinases, provide modulations of protein phosphoregulation. Members of the PP2Cs, widely distributed in eukaryotes, have been implicated as modulators of signal transduction, and are known to be activated by diverse environmental stresses or developmental signalling cascades (Schweighofer *et al.*, 2004). A comprehensive computational analysis identified 80 and 78 PP2C genes in *Arabidopsis thaliana* and *Oryza sativa*, respectively, which makes the PP2C gene family one of the largest identified in plants (Xue *et al.*, 2008). Phylogenetic analysis divided PP2Cs in Arabidopsis and rice into 13 and 11 subgroups, respectively, with characteristic features in gene structures and protein motifs. Several Arabidopsis (Umezawa *et al.*, 2010) and rice (Singh *et al.*, 2015) PP2Cs classified into subgroup A have been described as negative regulators of abscisic acid-mediated signalling and responses. Other PP2C genes, including *AP2C1* (subgroup B) (Schweighofer *et al.*, 2007), *WIN2* (subgroup F) (Lee *et al.*, 2008), *KAPP* (unclustered) (Gómez-Gómez *et al.*, 2001; Stone *et al.*, 1994) and *OsBIPP2C2* (subgroup K) (Hu *et al.*, 2009), have been revealed to possess stress-related functions. *OsBIPP2C1* has been reported to be a rice gene up-regulated by treatment with benzothiadiazole, a well-known inducer of systemic acquired resistance (Hu *et al.*, 2006). Furthermore, transgenic tobacco plants overexpressing the

OsBIPP2C1 gene show enhanced disease resistance against *Tobacco mosaic virus* and *Phytophthora parasitica*. Interestingly, most of the PP2Cs belonging to subgroup K are predicted to be localized at the chloroplast. However, there has been no clear characterization of these genes in a *Xanthomonas*-infected plant.

The primary reactions of photosynthesis occur in the thylakoid membranes of chloroplasts. These membranes harbour pigment–protein complexes called photosystem I (PSI) and photosystem II (PSII) which convert light energy into chemical energy [adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)]. The chloroplast is thought to contain around 3000 different proteins in Arabidopsis (Richly and Leister, 2004), and currently more than 40 proteins, such as kinases, phosphatases (including PP2C), proteases, transporters and chaperones, are known to associate with PS and have been shown to assist the PS repair cycle (Järvi *et al.*, 2015). Moreover, PSII is crucial for plant immunity through the production of ROS, which not only damage the pathogen and components of the photosynthetic electron transfer chain, but also act as important retrograde signalling molecules (Rodríguez-Herva *et al.*, 2012; de Torres Zabala *et al.*, 2015). Chloroplasts and mitochondria, in addition to being involved in biosynthetic pathways, energy production, redox homeostasis and retrograde signalling, also play key roles in plant immune responses (Maxwell *et al.*, 2002; Stael *et al.*, 2015). For instance, the chloroplast constitutes a platform for the synthesis of important defence hormones, such as salicylic acid (SA) and jasmonic acid (JA), and both chloroplasts and mitochondria are essential providers of redox resources to fight pathogen attack. Yang *et al.* (2011) reported that *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) infection induced a changed distribution of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activase from the soluble stroma to the thylakoid membrane of chloroplasts. MAMP perception triggers the rapid, large-scale suppression of nuclear-encoded chloroplast-targeted genes (NECGs). Virulent *Pseudomonas syringae* effectors can reprogram NECG expression in *Arabidopsis*, target the chloroplast and inhibit photosynthetic CO_2 assimilation through the disruption of PSII (de Torres Zabala *et al.*, 2015). As noted above, although the exact role of photosynthetic components in the sensing and signalling of pathogen infection has only just emerged, a wealth of information has accumulated during the past few years on the consequences of fluctuating light on the activity of the photosynthetic machinery (Allahverdiyeva *et al.*, 2015; Grieco *et al.*, 2012). Several *P. syringae* type III effectors (T3Es), including HopI1, HopN1, HopK1 and AvrRps4, target the chloroplast and suppress immune responses (Jelenska *et al.*, 2007; Li *et al.*, 2014; Rodríguez-Herva *et al.*, 2012).

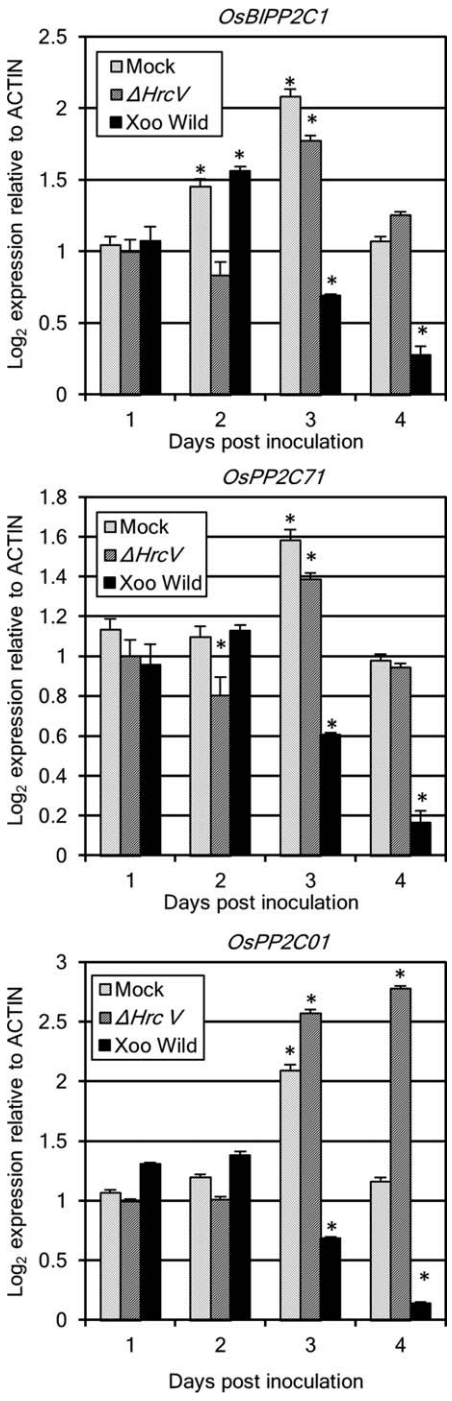


Fig. 1 The expression of three rice clade K protein phosphatase 2Cs (PP2Cs) is down-regulated by compatible *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) inoculation. Wild-type or *hrcV* (type III mutant) *Xoo* T7174R was inoculated to IR-64 by leaf clipping, and collected at the indicated time point. Gene expression was obtained by real-time reverse transcription-polymerase chain reaction (RT-PCR). Significant differences from 1-day mock were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and are indicated with an asterisk ($*P < 0.01$). Results are representative of three experiments.

Xanthomonas is one of the most agriculturally important pathogens and causes plant diseases on various plant species. Bacterial leaf blight, which is caused by *Xoo*, is one of the most devastating diseases in major rice production areas in tropical Asia (Niño-Liu *et al.*, 2006; Rajarajeswari and Muralidharan, 2006). *Xanthomonas campestris* pv. *campestris* (*Xcc*) is the causal agent of black rot, one of the most important diseases on cruciferous vegetables. The pathogen infects a wide range of brassica plants, including broccoli, mustard, cabbage, cauliflower, radish, turnip and the model plant *A. thaliana* (Meyer *et al.*, 2005). Together with other bacterial pathogens, such as *Pseudomonas*, *Xoo* and *Xcc* pathogenicity are greatly attenuated when their type III secretion system (TTSS) is defective, indicating that T3Es are the major virulence factors (Dow and Daniels, 1994; Sun *et al.*, 2011). *Xanthomonas* T3Es are translocated into the plant cytoplasm via the TTSS and are called Xanthomonas outer proteins (Xops). Many Xop proteins have been identified (Furutani *et al.*, 2009) and characterized. The functions of XopR, XopN, XopZ, XopD, XopY, XopP, XopQ, AvrAC and AvrBs2 have been reported to be essential for bacterial virulence (Akimoto-Tomiyama *et al.*, 2012; Cheong *et al.*, 2013; Gupta *et al.*, 2015; Ishikawa *et al.*, 2014; Li *et al.*, 2015; Song and Yang, 2010; Wang *et al.*, 2015).

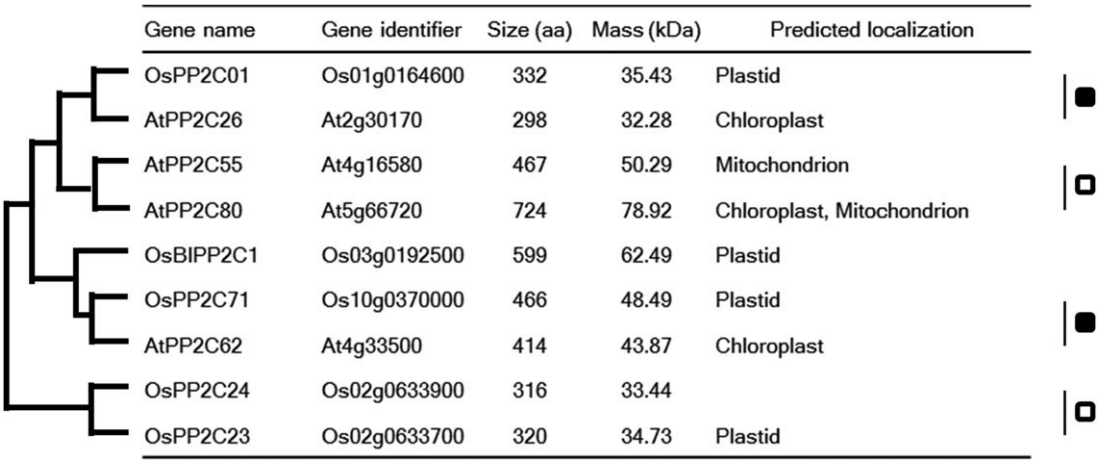
In this study, to elucidate the function of chloroplast-localized PP2Cs in plant immunity, we first used microarray analysis to identify novel PP2C genes of subgroup K in rice, which are down-regulated in a TTSS-dependent manner in response to inoculation with *Xoo* (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63047, GSE63047). We further found the orthologous genes in *Arabidopsis*: *AtPP2C62* and *AtPP2C26*. Through the analysis of the loss-of-function mutants, our results indicated that these two gene products are involved in the resistance to *Xcc* via action on the metabolic activities of the chloroplast.

RESULTS

Rice subgroup K PP2Cs are responsive to a compatible infection with *Xoo* in a TTSS-dependent manner

According to the microarray analysis, three putative PP2C genes (*OsBIPP2C1*, Os03g0192500; *OsPP2C71*, Os10g370000; and *OsPP2C01*, Os01g0164600) were responsive to a compatible *Xoo* inoculation (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63047). To confirm the results, we analysed the expression of the three rice PP2C genes in IR-64, which was inoculated with wild-type or TTSS mutant (*hrcV* deficient) of *Xoo* T7174R, by real-time polymerase chain reaction (PCR) using specific primers. At 3 and 4 days post-inoculation (dpi), the expression of each PP2C was suppressed following inoculation with wild-type *Xoo* T7174R, but not suppressed following mock or TTSS mutant inoculation (Fig. 1), consistent with the microarray results. Gene expression induced by mock treatment at 3 dpi was a response to wounding in the inoculation process, as reported previously (Hu *et al.*, 2006). The expression of *OsPP2C01* induced by the TTSS mutant of *Xoo* increased significantly at 4 dpi, even though the expression level of mock treatment returned to the same level as at 1 or 2 dpi (Fig. 1). This indicates that *OsPP2C01* is also responsive to MAMPs of the TTSS mutant as well as wounding.

These rice PP2C genes (*OsBIPP2C1*, *OsPP2C71* and *OsPP2C01*) belong to subgroup K and encode for plastid proteins (Xue *et al.*, 2008). The phylogenetic tree of subgroup K PP2Cs of rice and Arabidopsis is shown in Fig. 2 based on a previous report (Xue *et al.*, 2008). The rice genes (*OsPP2C71* and *OsPP2C01*) were predicted to be orthologous to *AtPP2C62* and *AtPP2C26* of Arabidopsis, respectively. Two other PP2Cs of subgroup K, *AtPP2C55* and



Gene name	Gene identifier	Size (aa)	Mass (kDa)	Predicted localization
OsPP2C01	Os01g0164600	332	35.43	Plastid
AtPP2C26	At2g30170	298	32.28	Chloroplast
AtPP2C55	At4g16580	467	50.29	Mitochondrion
AtPP2C80	At5g66720	724	78.92	Chloroplast, Mitochondrion
OsBIPP2C1	Os03g0192500	599	62.49	Plastid
OsPP2C71	Os10g0370000	466	48.49	Plastid
AtPP2C62	At4g33500	414	43.87	Chloroplast
OsPP2C24	Os02g0633900	316	33.44	
OsPP2C23	Os02g0633700	320	34.73	Plastid

Fig. 2 Rice and Arabidopsis subgroup K protein phosphatase 2Cs (PP2Cs). The phylogenetic relationships are indicated by the tree (shown on the left). The predicted localizations were determined for rice (http://rice.plantbiology.msu.edu/index.shtml) and Arabidopsis (http://www.arabidopsis.org/index.jsp). Putative orthologous and paralogous pairs are marked with black and white squares, respectively. aa, amino acid.

AtPP2C80, were not subjected to further analysis because they were predicted to localize to mitochondria (Fig. 2).

Arabidopsis subgroup K PP2Cs are responsive to a compatible infection with *Xcc* in a TTSS-dependent manner

Next, we examined the expression pattern of the two Arabidopsis subgroup K PP2Cs in response to *Xcc*. Fully expanded *A. thaliana* ecotype Col-0 leaves were inoculated with a compatible strain of *Xcc* MAFF106712 (Akimoto-Tomiyama *et al.*, 2014), and the expression of *AtPP2C62* and *AtPP2C26* was analysed by real-time polymerase chain reaction (PCR) (Fig. 3). The *hrcC* deletion mutant of *Xcc* MAFF106712 was used as the TTSS loss-of-function mutant. *AtPP2C62* gene expression was up-regulated on inoculation with the TTSS mutant bacterium, but not on wild-type inoculation. The expression of *AtPP2C26* was down-regulated at 1 day after mutant and wild-type inoculation, which was not observed for *AtPP2C62* expression. Although the induction of *AtPP2C26* gene expression was observed following both mock and mutant inoculation, expression was down-regulated by wild-type inoculation at 3 dpi. At 5 dpi, *AtPP2C26* gene expression was no different between mock and mutant inoculation, but was down-regulated by wild-type inoculation. These results indicate that the two Arabidopsis PP2Cs belonging to subgroup K are responsive to wounding and MAMPs. The gene *AtPP2C26* is responsive to wounding and MAMPs, but the gene *AtPP2C62* is responsive to MAMPs only.

AtPP2C62 and *AtPP2C26* redundantly affect lesion development

To characterize *AtPP2C62* and *AtPP2C26*, we obtained homozygous T-DNA insertion lines of these genes (Col-0 background; SALK_014358C and SALK_127920C, respectively, as shown in Fig. 4A) from the Arabidopsis Biological Resource Center. A double mutant (*atpp2c62/atpp2c62*) was created by crossing the single mutants, and its homozygous genotype was confirmed by PCR (data not shown). The expression of both *AtPP2C62* and *AtPP2C26* induced by the TTSS mutant of *Xcc* was abolished in the double mutant (Fig. S1, see Supporting Information).

Neither the single mutants nor the double mutant showed morphological phenotypes during the 5 weeks following germination in our culture conditions (data not shown). We inoculated *Xcc* MAFF106712 on fully expanded leaves of 5-week-old Col-0 and the two *pp2c*(s) knock-out mutants, and observed lesion development in the inoculated leaves (Fig. 4B,C). The size of the lesions was categorized according to the disease index (Fig. 4C, right panels). At 3 dpi, visible lesions in the inoculated leaves emerged (Fig. 4B) and, at 7 dpi, the lesions on some inoculated leaves had spread to the whole leaf (Fig. 4C). In Col-0 and the two single mutants, nearly 20% of total leaves had whole-leaf lesions

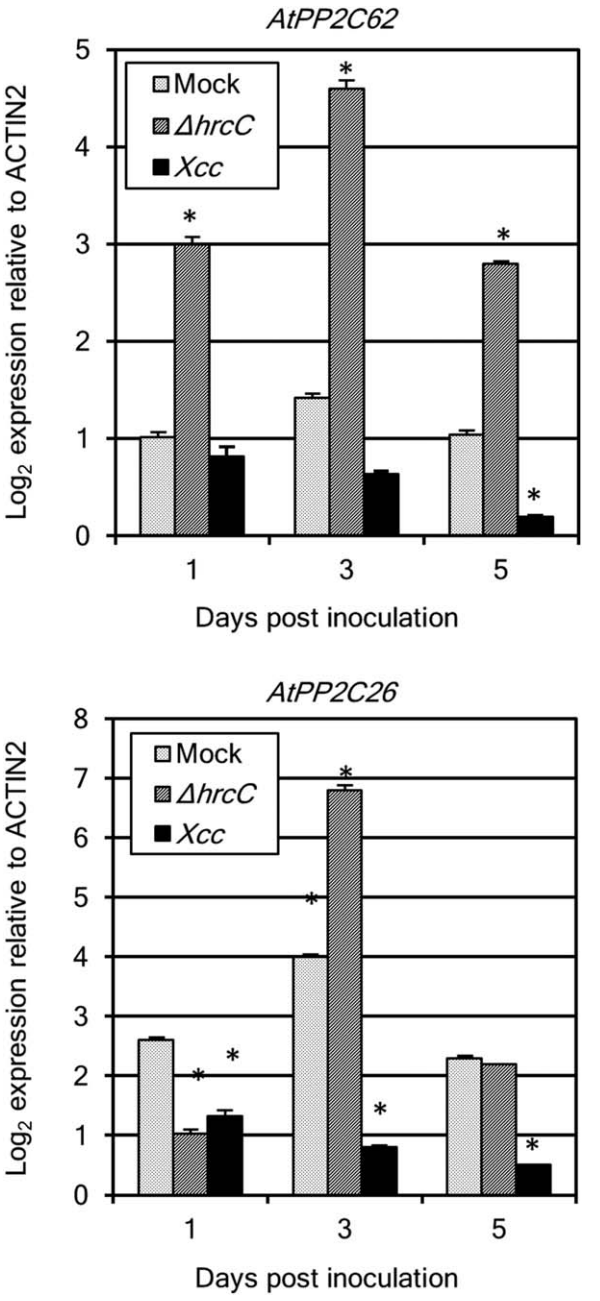


Fig. 3 The expression of two Arabidopsis subgroup K protein phosphatase 2Cs (PP2Cs) is up-regulated by the *hrcC* deletion mutant of *Xanthomonas campestris* pv. *campestris* (*Xcc*) MAFF106712 and down-regulated by wild-type *Xcc*. Five fully expanded leaves of each plant (Arabidopsis eco-type, Col-0) were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xcc* wild-type and *hrcC* mutant bacteria [10^9 colony-forming units (CFU)/mL]. Plant samples were harvested at 1, 3 and 5 days post-inoculation (dpi) with the *Xcc* wild-type, *hrcC* mutant (*hrcC*) or vehicle alone. Expression of the PP2C genes in the plants was obtained by real-time reverse transcription-polymerase chain reaction (RT-PCR). Significant differences from 1-day mock were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and are indicated with an asterisk (*$P < 0.01$). The results are representative of three experiments.

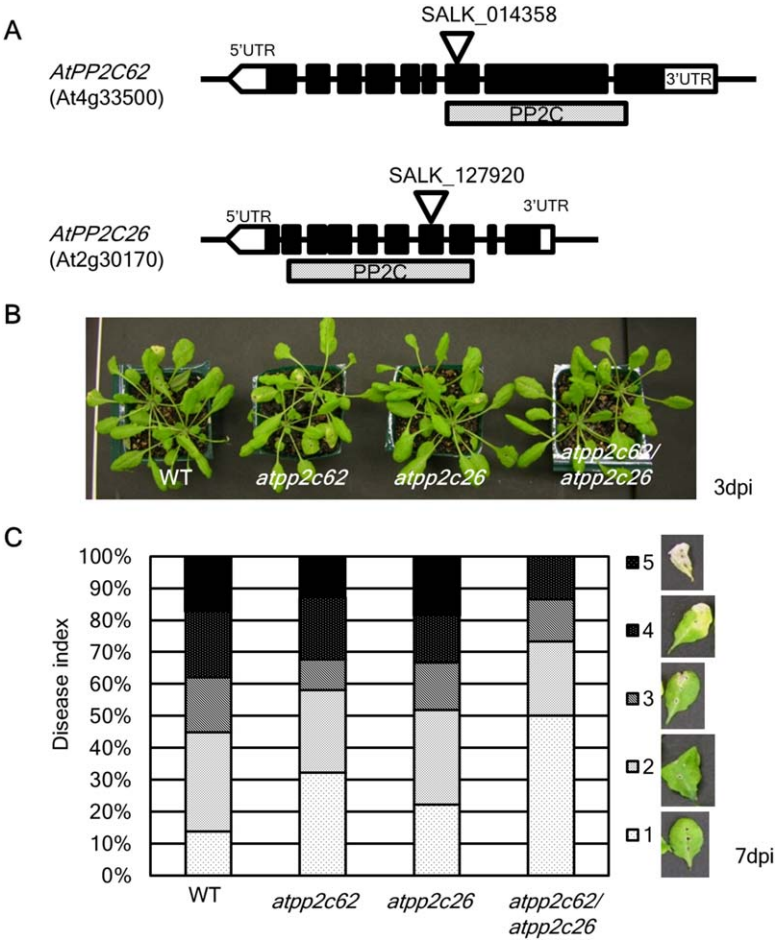


Fig. 4 The lesions caused by compatible *Xanthomonas campestris* pv. *campestris* (*Xcc*) MAFF106712 inoculation are suppressed in the *atpp2c62/atpp2c26* mutant. (A) Genomic structure of the two Arabidopsis *PP2C* genes analysed in this study. The black boxes show the exons and the dotted box shows the protein phosphatase 2C (PP2C) motif. The positions of the T-DNA insertions are indicated by triangles. UTR, untranslated region. (B) Photograph of wild-type (WT, Col-0) and *pp2c*s mutant plants inoculated by *Xcc* MAFF106712 at 3 days post-inoculation (dpi). (C) Percentage of inoculated leaves showing lesion development, categorized as disease index 1–5, at 7 dpi. Disease index examples are shown on the right.

(disease index 5), whereas none of the inoculated leaves of the double mutants showed the phenotype. Furthermore, half of the inoculated leaves showed no symptoms (disease index 1) in the double mutant. Notably, lesion development caused by compatible *Xcc* inoculation was suppressed in the double *pp2c* mutant. These results strongly suggest that *AtPP2C62* and *AtPP2C26* work cooperatively in the suppression of lesion formation.

Bacterial growth is suppressed in the double mutant (*atpp2c62/atpp2c26*)

The changes in the visible symptoms shown in Fig. 4 were further quantified by measurement of bacterial growth in the host plant (Fig. 5). Although no significant difference was observed amongst the four lines of *Arabidopsis* at 3 dpi, bacterial growth in the double and *atpp2c26* single mutant was reduced significantly ($P < 0.01$) compared with that of the wild-type at 7 dpi. The bacterial growth in the *atpp2c62* single mutant was reduced significantly ($P < 0.5$). Therefore, we conclude that the suppression of lesion development in the double mutant shown in Fig. 4C is accompanied by the inhibition of bacterial growth.

AtPP2C62, as well as AtPP2C26, is localized to the chloroplast

One of the PP2Cs analysed in this study, AtPP2C26, has been reported as a chloroplast protein (Samol *et al.*, 2012). To determine the localization of AtPP2C62, we generated citrine-tagged full-length AtPP2C62 and AtPP2C26 fusions expressed under the control of the oestrogen-inducible promoter, and transiently expressed the constructs in *Nicotiana benthamiana* (Akimoto-Tomiyama *et al.*, 2012). Citrine fluorescence was observed by confocal laser scanning microscopy. One day after treatment with oestradiol, citrine fluorescence was observed at the chloroplast in AtPP2C62::citrine-expressing (Fig. 6A) and AtPP2C26::citrine-expressing (Fig. 6B) plants. In the absence of oestradiol, citrine fluorescence was not observed (data not shown). Notably, the fluorescence was observed only in chloroplasts of the inoculated part of the leaf, and not in every chloroplast.

Screening of the *AtPP2C62* and *AtPP2C26* substrate candidates

As described above, loss of the two chloroplast-localized PP2Cs resulted in enhanced plant immunity. Thus, we hypothesized that

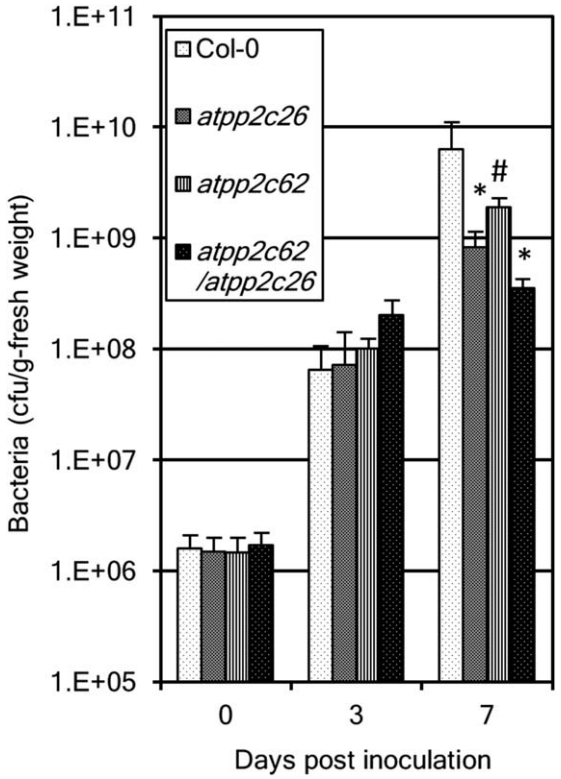


Fig. 5 Bacterial multiplication in the double mutant (*atpp2c62/atpp2c26*) is suppressed. Bacterial multiplication was measured in Col-0 (wild-type), the single mutant of *atpp2c62* or *atpp2c26* and double mutant. Five fully expanded leaves of each plant were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xanthomonas campestris* pv. *campestris* (*Xcc*) MAFF106712 bacteria [10^9 colony-forming units (CFU)/mL]. Significant differences from Col-0 at each time point were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and are indicated with an asterisk ($*P < 0.01$) or a hash ($\#P < 0.5$). The results are representative of at least three experiments per condition.

phosphorylation of the target protein(s) of the PP2Cs positively affects the plant defence system. To screen candidates, we conducted protein analysis by combining two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein was extracted from the leaves inoculated with compatible *Xcc* MAFF106712 bacteria at 1, 2 and 3 dpi, and then separated by two-dimensional electrophoresis. We selectively stained the phosphorylated proteins in polyacrylamide gels by Pro-Q diamond (Thermo Fisher Scientific, Waltham, MA, USA) (Fig. 7A,B,D,E). As shown in Fig. 7A,D, many spots of phosphoprotein were detected in both Col-0 and the double mutant. After comparison of the spot size and location for each gel, four spots which clearly changed on the basis of plant background were selected for further protein identification. By reference to the non-selective Coomassie brilliant blue (CBB)- or silver-stained spot (Fig. 7C,F), the changed spots were cut out and subjected to amino acid sequence analysis. The annotation of the four spots is shown in Table 1. Peptides derived from spot 1 of the mutant were identified as glycine decarboxylase P-protein 1, whereas the peptides from spot 1 of Col-0 were unknown. Spots 2 and 3 were physically close to each other and peptides from both spots 2 and 3 were annotated as chaperonin-60α. The peptides from spot 4 were annotated to the large subunit of Rubisco. The spot size of spot 2 in the double mutant (Fig. 7B) was much larger than that in Col-0 (Fig. 7A), suggesting abundant phosphorylated chaperonin-60α. This is consistent with the predicted role of PP2C in the dephosphorylation of the target(s). However, the sizes of spots 1 and 4 in the double mutant were smaller than those in Col-0 (Fig. 7A,B,D,F). This indicates that these are not direct target(s) of the PP2Cs.

DISCUSSION

The two chloroplast-localized PP2Cs are wounding and/or MAMP responsive, and their induction is abrogated by successful pathogen infection. This strongly suggests that the induction of PP2Cs is required for plant defence. Indeed, in previous work, the overexpression of *OsBIPP2C1* in tobacco plants led to enhanced disease resistance against *Tobacco mosaic virus* and *Phytophthora parasitica* (Hu *et al.*, 2006). However, surprisingly, the bacterial growth in the PP2C knock-out plants was significantly reduced in this study, indicating that the loss of PP2Cs enhances Arabidopsis immunity. This implies that the constant dephosphorylated status of the potential substrate of these PP2Cs acts in a physiological or physical negative manner with regard to defence. Although no visible phenotype change was observed in the experiment, the *atpp2c26* single mutant has been reported to show a concomitant reduction in the number of layers in the grana stacks (Samol *et al.*, 2012), and further analysis of the thylakoid membrane protein composition of this mutant characterized its smaller PSII antenna, whereas cytochrome (Cyt) b_6f complexes and PSI were similar to those of the wild-type (Puthiyaveetil *et al.*, 2014). Thus, the attenuation of the PSII antenna may lead to the enhancement of the basal defence system. From a physiological viewpoint, we observed *PR1* gene expression in the double mutant. The *PR1* gene is induced by both MTI (Silipo *et al.*, 2005) and ETI (Rong *et al.*, 2010) triggered by *Xanthomonas* or *Pseudomonas* infection (Hamdoun *et al.*, 2013), and leads to the enhancement of plant immunity. The induction of *PR1* gene expression in the double mutant was observed for both *Xcc* wild-type and the TTSS-deficient mutant (Fig. S2, see Supporting Information). The induction of *PR1* was concomitant with the decrease in bacterial growth. Notably, the expression level of *PR1* by mock treatment was not impaired by the loss of PP2Cs, indicating that they are not responsible for *PR1* induction by wounding. It has been shown that photosynthesis is involved in the activation of SA biosynthesis, *PR1* gene expression and the hypersensitive response (HR) against

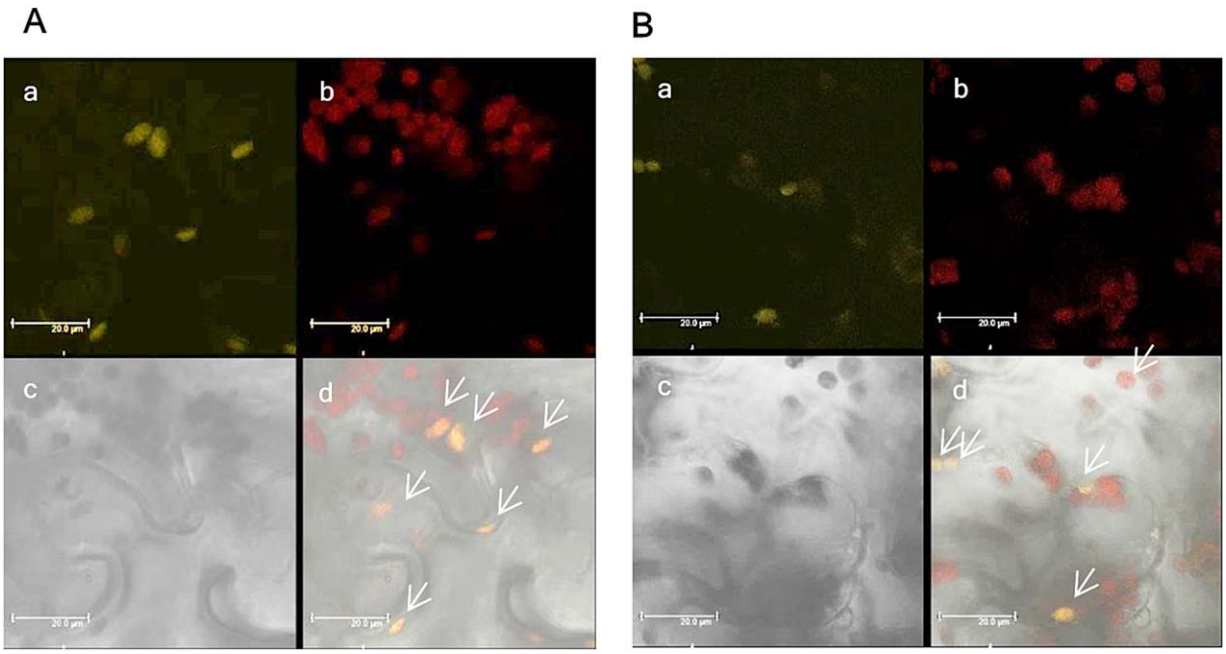


Fig. 6 AtPP2C62::citrine localizes to the chloroplast. *Agrobacterium tumefaciens* EHA105, which mediates T-DNA-based transfer of AtPP2C62::citrine, was co-infused into *Nicotiana benthamiana* leaves at an optical density at 600 nm of 1.0 with HC-Pro in pMD1 at an optical density at 600 nm of 0.1. Samples were observed by confocal microscopy 1 day after oestradiol treatment. (A) AtPP2C62::citrine: a, citrine fluorescence is shown in yellow; b, chlorophyll autofluorescence is shown in red; c, bright field image; d, merged view. (B) AtPP2C26::citrine: a, citrine fluorescence is shown in yellow; b, chlorophyll autofluorescence is shown in red; c, bright field image; d, merged view. Arrows indicate the chloroplasts expressing protein phosphatase 2Cs (PP2Cs). Scale bars, 20.0 μm. The fluorescence was stable at least 3 days after oestradiol treatment. For microscopic observation: 514 nm excitation and 520–540 nm emission for citrine fluorescence; 514 nm excitation and 650–700 nm emission for chlorophyll autofluorescence.

infection by pathogens (Jelenska *et al.*, 2007; Kangasjärvi *et al.*, 2012; Kim *et al.*, 2012). We found that PP2Cs are not involved in HR development triggered by *AvrRpm1* under continuous light conditions (Fig. S3, see Supporting Information). In addition, the bacterial growth of the TTSS mutant was not impaired in the double mutant plant (Fig. S4, see Supporting Information), suggesting that the PP2Cs are responsible for T3E-mediated virulence. It could be hypothesized that the PP2Cs are virulence targets of an *Xcc* effector and, in its absence, the pathogen no longer has such an advantage. As light is required for *PR1* induction and HR development (Zeier *et al.*, 2004), a detailed analysis of thylakoid protein composition and examination of *PR1* induction and HR development under various light intensities in the double mutant may elucidate this phenomenon. Through an analysis of protein phosphorylation by bacterial infection in *pp2cs* knock-out plants, three photorespiratory cycle-related proteins were identified as potentially differentially phosphorylated proteins in the *pp2c* double mutant during *Xcc* MAFF106712 infection: chaperonin-60α2 (Cpn60α2), glycine decarboxylase P-protein 1 and the large subunit of Rubisco (Fig. 7, Table 1). Amongst them, Cpn60α2 is a probable potential substrate candidate for the PP2Cs. Cpn60 plays a critical role in Rubisco assembly (Cannon *et al.*, 1986; Milos and Roy, 1984) and, moreover, the reduction of *AtCpn60* results in impaired Arabidopsis plastid division and reduction of chlorophylls (Suzuki *et al.*, 2009). *OsCpn60α1* has been shown to be essential for the folding of the Rubisco large subunit in rice (Kim *et al.*, 2013). In addition, AtCpn60α2 has been identified as a phosphoprotein (Reiland *et al.*, 2009), but its internal regulation and the effect of its phosphorylated status are still obscure. As there is no evidence that the maintenance of the phosphorylated status of Cpn60α2 alters or affects the photorespiratory activity or interaction between the photorespiratory activity and plant immunity, further elucidation is needed.

Following pathogen infection, the expression of the chloroplast-localized PP2Cs in subgroup K decreased dramatically in a TTSS-dependent manner for both rice–*Xoo* wild-type and Arabidopsis–*Xcc* wild-type interactions at the late stage (3–5 dpi, Figs 1 and 3). Furthermore, *AtPP2C62* and *AtPP2C26* expression is induced by the TTSS mutant within 1 or 3 dpi, respectively. This suggests that the PP2Cs are potential targets of T3Es. In support of this idea, previous microarray analysis with a different bacterial

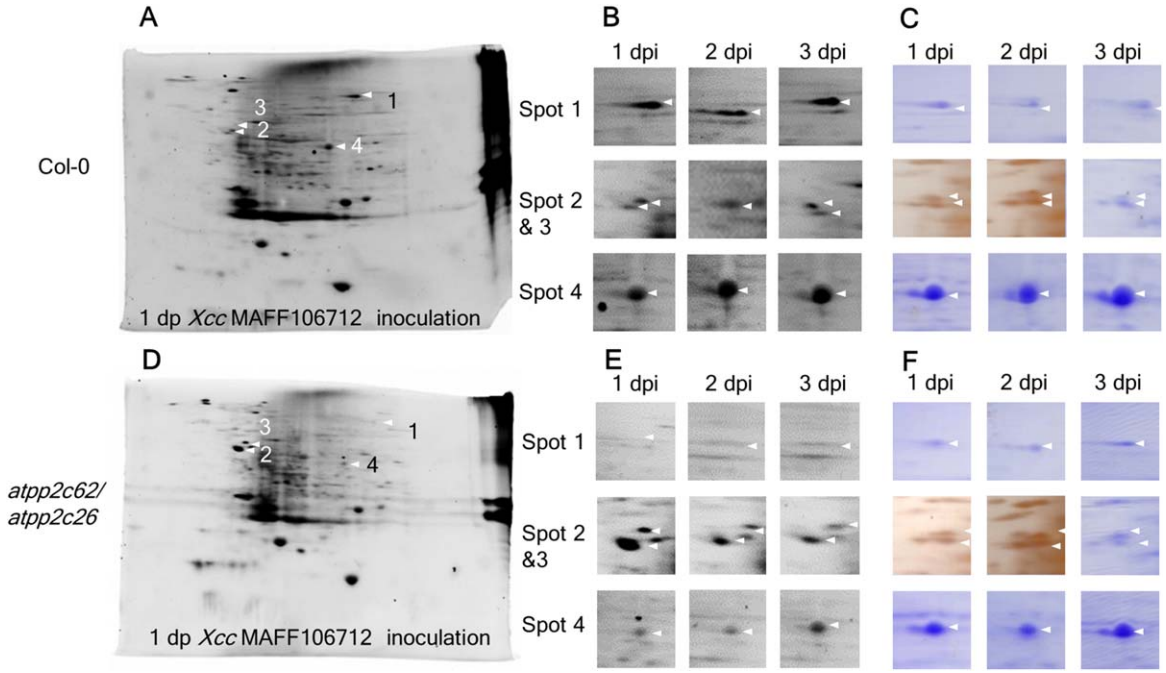


Fig. 7 Loss of the protein phosphatase 2Cs (PP2Cs) impairs the protein phosphorylation triggered by compatible infection. Five fully expanded leaves of Col-0 and the double mutant were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xanthomonas campestris* pv. *campestris* (*Xcc*) MAFF106712 bacteria [10^9 colony-forming units (CFU)/mL]. Protein was extracted from an inoculated leaf and separated by two-dimensional isoelectric focusing sodium dodecylsulfate-polyacrylamide gel electrophoresis (2D-IDF-SDS-PAGE). Protein was stained by Pro-Q diamond and the spots were cut and then identified by liquid chromatography-mass spectrometry (LC-MS). Arrows indicate the spot changes in Col-0 (A–C) compared with the double mutant (D–F). (A, D) Whole-gel images stained by Pro-Q diamond-separated proteins extracted from Col-0 (A) and the double mutant (D) at 1 day post-inoculation (dpi) of *Xcc* MAFF106712. (B, E) High-magnification image of (A, D); the spot number is given on the left and the time point is shown at the top. (C, F) Images of the protein stained by Coomassie brilliant blue (CBB) or silver.

pathogen, *Arabidopsis–Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, revealed that both *AtPP2C62* and *AtPP2C26* were significantly down-regulated within 12 hpi in a TTSS-dependent manner (Truman *et al.*, 2006). Furthermore, many chloroplast proteins essential for plant defence have been suggested to be targets for pathogen effectors (de Torres Zabala *et al.*, 2015). In the case of the *Arabidopsis–Xcc* TTSS mutant interaction, *AtPP2C62* is induced within 1 dpi. This strongly indicates that *AtPP2C62* is responsive to MAMPs. Indeed, both *AtPP2C62* and *AtPP2C26* were induced by flg22 for 30 min in a light-dependent manner (Sano *et al.*, 2014). Thus, T3Es which impair the MAMP receptor itself or downstream signal transduction may indirectly control PP2C expression. Based on the analysis of the whole-genome information of *Xoo* (KACC10331, MAFF311018, PXO99A) and *Xcc* (ATCC33913, 8004, B100) strains, they share at least 13 T3E proteins, including AvrBs2, XopF, XopG, XopK, XopL, XopN, XopP, XopQ, XopR, XopX, XopZ, XopA and HpaA. Although the genome sequence of *Xcc* MAFF106712 is not available so far, it harbours at least XopN, XopK, XopQ, XopZ and XopR (Akimoto-Tomiyama *et al.*, 2014). Single mutations of XopN, XopQ, XopZ (Cheong *et al.*, 2013; Gupta *et al.*, 2015; Song and Yang, 2010) and XopR (Akimoto-Tomiyama *et al.*, 2012) have already been reported to affect virulence. Further analysis is needed to determine the T3E(s) involving PP2C expression and those T3Es with the potential to attenuate photosynthesis.

In this study, we suggest that two chloroplast PP2Cs are responsible for plant immunity. One of these, AtPP2C26, has been reported previously as a PSII core phosphatase (PBCP) required for the efficient dephosphorylation of PSII (the first protein complex in the light-dependent reactions of oxygenic photosynthesis) proteins involved in light acclimation (Samol *et al.*, 2012). By contrast, AtPP2C62 has not been shown to be required for the dephosphorylation of PSII (Samol *et al.*, 2012). We screened potentially differentially phosphorylated proteins in the *pp2c* double mutant, such as glycine decarboxylase P-protein 1, chaperonin-60α and the large subunit of Rubisco, which were all related to the photorespiratory cycle of light-independent reactions, but not light-dependent reactions. It is suggested that the functions of *AtPP2C62* and *AtPP2C26* in plant immunity relate to the light-independent reactions (photorespiratory cycle) of photosynthesis. In support of this idea, Järvi *et al.* (2016) have indicated the interconnection between light acclimation and plant immunity,

Table 1 List of the identified proteins.

Spot name	GI	Possible function	Score	No. of peptides	Peptides
#1 Col-0	gi|21592723	Unknown	12	1	K.QVASVIPVTRK.L
#1 *atpp2c62/atpp2c26*	gi|14596025	Glycine dehydrogenase	41	1	K.CSDAHAIADAASK.S
#2 Col-0	gi|15226314	Chaperonin-60α	73	3	K.VGAATETELEDR.K K.HGLLSVTSGANPVSLK.R K.DSTTLIADAASKDELQAR.I
#2 *atpp2c62/atpp2c26*	gi|15226314	Chaperonin-60α	152	5	K.VGAATETELEDR.K K.ITAIKDIIPILEK.T K.HGLLSVTSGANPVSLK.R K.TNDSAGDGTTTASILAR.E K.DSTTLIADAASKDELQAR.I
#3 Col-0	gi|15226314	Chaperonin-60α	145	3	K.VGAATETELEDR.K K.TNDSAGDGTTTASILAR.E K.DSTTLIADAASKDELQAR.I
#3 *atpp2c62/atpp2c26*	gi|15227717	UDP-glycosyltransferase 87A1	0	1	R.VGMGIER.K
#4 Col-0	gi|7525041	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	99	10	K.LGLSAK.N K.ALAALR.L R.GGLDFTK.D R.AVYECLR.G + propionamide (C) R.VALEACVQAR.N + propionamide (C) K.LTYYTPEYETK.D R.LEDLRIPPAYTK.T R.LSGGDHIHAGTVVGK.L R.LSGGDHIHAGTVVGK.L R.LSGGDHIHAGTVVGK.L K.TFQGPPHGIQVER.D K.EITFNFPTIDKLDGQE.-
#4 *atpp2c62/atpp2c26*	gi|7525041	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	64	5	K.ASVGFK.A R.AVYECLR.G + propionamide (C) K.DTDILAAFR.V R.LSGGDHIHAGTVVGK.L K.TFQGPPHGIQVER.D

The mass spectrometry data were obtained by one-time analysis using the spots excised from the five or six gels.

where the appropriate repair cycle of PSII plays a key role in the process. Moreover, a rice leaf colour mutant, which is defective in chlorophyll synthesis and photosynthesis, conferred resistance to *Xoo* (Chen *et al.*, 2016), and the white sector of an Arabidopsis variegation mutant promoted susceptibility to the pathogen *P. syringae* (Pogorelko *et al.*, 2016). These results strongly indicate that the chloroplast is relevant to immunity. Further experiments are needed to elucidate the function of PP2Cs in plant defence, especially the point of tradeoff of carbon.

In previous work, the *pbcp* mutant, which is exactly the same genotype as the *atpp2c26* mutant used in this study, showed retarded growth under normal light conditions (illumination for 9 h with 220 μmol quanta/m^2/s at 23 °C; dark temperature, 21 °C) in two of three experiments (Puthiyaveetil *et al.*, 2014). With our experimental conditions (illumination for 8 h with 300 μmol quanta/m^2/s at 23 °C; dark temperature, 21 °C), in contrast, biomass loss of the *atpp2c26* mutant was not observed. Considering the inconsistent results with previous studies, we conclude that there is a low correlation between AtPP2C26 (or Pbcp) and biomass gain. The variability may be a result of the difference in culture conditions, especially light conditions.

Interestingly, the PP2Cs focused on here belong to the 'PP2C7s', which are almost universally distributed in Eukaryotes (Kerk *et al.*, 2015). The 'PP2C7s' are distributed in most crops, vegetables and fruits, which are potential hosts for *Xanthomonas*. Therefore, to establish a disease-tolerant variety, breeding for PP2Cs may be effective. Interestingly, the 'PP2C7s' have been suggested to originate from Bacteria group II PP2Cs. In addition, *Xanthomonas* contains group II PP2Cs. How could this horizontal gene transfer have resulted in the acquisition of effector-triggered susceptibility? Furthermore, how is the host protein selected as the target by pathogen effectors? Evolutionary analysis may help to provide an answer to these questions.

EXPERIMENTAL PROCEDURES

Bacterial strains and media

Xoo strain T7174R and *Xcc* strain MAFF106712 (Akimoto-Tomiyama *et al.*, 2014) were grown at 28 °C in nutrient broth–yeast extract (NBY) medium. *Agrobacterium tumefaciens* was grown in Luria broth at 28 °C. For solid medium, agar was added at a final concentration of 1.5% (w/v). All media were supplemented with antibiotics at the following concentrations: for *Xcc* and *Xoo*, 50 μg/mL rifampin, 50 μg/mL kanamycin and 40 μg/mL spectinomycin; for *Agrobacterium*, 50 μg/mL ampicillin, 25 μg/mL kanamycin and 40 μg/mL spectinomycin.

Plant material, growth conditions and infection tests

Rice cv. IR-64 (susceptible to *Xoo* MAFF311018) was grown in a growth chamber set at 28 °C with a 14-h photoperiod and 24 °C with a 10-h dark period. The fully expanded upper leaves of 5-week-old rice were inoculated by the clipping method (Kauffman *et al.*, 1973). *Arabidopsis* plants were grown on soil in pots, as described previously (Akimoto-Tomiyama *et al.*, 2012). Homozygous Arabidopsis T-DNA insertion lines SALK_014358C and SALK_127920C (Col-0 background) were obtained from the Arabidopsis Biological Resource Center. A double mutant (*atpp2c62/atpp2c26*) was created by crossing the single mutants, and its homozygous genotype was confirmed by PCR (data not shown). *Xcc* pathogenicity was assayed on *A. thaliana* by piercing inoculation of a bacterial suspension at 10^9 colony-forming units (CFU)/mL, as described previously (Akimoto-Tomiyama *et al.*, 2012). Disease development was scored at 7 dpi using a disease index ranging from 1 (no symptoms) to 5 (full leaf necrosis). For assays of bacterial growth in leaves, four leaves were weighed, ground in 1 mL of 10 mM $MgCl_2$ and mixed with serial dilutions of bacteria on appropriate media to calculate bacterial numbers.

Transient expression of AtPP2C62::citrine protein in *N. benthamiana*

The open reading frame of AtPP2C62 was cloned into the pENTR/d-TOPO vector (Thermo Fisher Scientific), and AtPP2C62::citrine and AtPP2C26::citrine in pMDC7 (Akimoto-Tomiyama *et al.*, 2012) were generated by LR clonase II (Thermo Fisher Scientific) according to the manufacturer's instructions. Transient expression in *N. benthamiana* was conducted as reported previously (Akimoto-Tomiyama *et al.*, 2012). In brief, *A. tumefaciens* EHA105 transformed with either AtPP2C62::citrine or AtPP2C26::citrine in pMDC7 or HC-Pro of *Potato virus Y* (a silencing suppressor protein) in pMD1 (Kubota *et al.*, 2003) was cultivated overnight at 28 °C in the presence of appropriate antibiotics. Bacterial cells were collected by centrifugation and resuspended in induction buffer (10 mM $MgCl_2$ and 10 mM MES, pH 5.6, supplemented with 0.5 mM acetosyringone) and incubated for 2 h with gentle mixing. Bacteria were diluted with induction buffer to a final optical density at 600 nm of 1.0 for AtPP2C62::citrine and AtPP2C26::citrine and 0.1 for Hc-Pro, and then co-infused into the leaves of 4–6-week-old *N. benthamiana* using a needleless syringe. Two days after infiltration, leaves were sprayed with a combination of 30 μM 17-oestradiol (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% Silwet L-77 to induce protein expression. After 1 day, the expression and accumulation of AtPP2C62::citrine and AtPP2C26::citrine protein in plant cells were observed using a TCS SP5 confocal microscope (Leica Microsystems, Solms, Germany).

Quantitative real-time PCR

Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). First-strand cDNA was synthesized from 500 ng of total RNA using a PrimeScript RT reagent Kit (Takara Bio Inc., Otsu, Japan) with an oligo (dT) primer and a random 6-mer primer, according to the manufacturer's instructions. For quantitative real-time PCR, 20 ng of cDNA were combined with SYBR premix Ex Taq (Takara Bio Inc.). PCRs were performed in triplicate with an MX 3000P (Agilent Technologies, Santa Clara, CA, USA), with actin RNA as an internal control. Primers for the genes were as follows: *OsBIPP2C1*, 5′-TGCCTGGATGTCCGTGTTTTG-3′ and 5′-TCAGCGCCATCCTCAGAGCACA-3′; *OsPP2C71*, 5′-GTCTCGAAATC TTTACAAGCCGATCT-3′ and 5′-CGGCTGCAGATCTACCAACTT-3′; *OsPP2C 01*, 5′-TTCGCGCCGCCAAGTTGGAA-3′ and 5′-AGCTGAGGATC ATGATTGACCTCCT-3′; *AtPP2C62*, 5′-TCGCAGCAACTGATGGGCTC-3′ and 5′-GCTGCGTCAGCAAATGGCGT-3′; *AtPP2C26*, 5′- GGTTGGGCTGAAC AAGATGT-3′ and 5′-CCCACTTCCTCAAGCATAGC-3′; *actin*, 5′-AGTGGT CGTACAACCGGTATTGT-3′ and 5′-GAGGAAGAGCATTCCCCTCGTA-3′; *actin* (*Os03g50890*), 5′-CTCCCCCATGCTATCCTTCG-3′ and 5′-TGAAT GAGTAACCACGCTCCG-3′; *PR1*, 5′- TCATGGCTAAGTTTGCTTCC-3′ and 5′-AATACACACGATTTAGCACC-3′.

Protein analysis

Three inoculated leaves were dissociated in lysis buffer (10 mg of tissue/300 μL of buffer) containing 7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate, 2% Nonidet P-40, 5% 2-mercaptoethanol, 0.2% ampholine (pH 3.5–10) and ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor. The dissociated tissue was subjected to repeated freeze–thaw cycles in liquid nitrogen and then stored at −80 °C until use for electrophoresis. The two-dimensional polyacrylamide gel electrophoresis (PAGE) gels were stained with CBB or Pro-Q Diamond Phosphoprotein Gel Stain (Thermo Fisher Scientific) following the manufacturer's instructions. MS and data analysis were performed largely as described previously (Kajiwara *et al.*, 2009). Spots were excised from five or six gels, and amaZon SL (Bruker Daltonics, Bremen, Germany) was used for MS analysis. The MS data were analysed with Mascot software (Perkins *et al.*, 1999) using the amino acid sequence data in the National Center for Biotechnology Information data bank (www.ncbi.nlm.nih.gov/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 The double mutant (*atpp2c62/atpp2c26*) was a null mutant. Five fully expanded leaves of Col-0 and the double mutant were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xanthomonas campestris* pv. *campestris* (*Xcc*) MAFF106712 *hrcC* mutant bacteria [10^9 colony-forming units (CFU)/mL]. Expression of the *AtPP2C62* and *AtPP2C26* genes in the plants harvested at 1 day post-inoculation (dpi) was obtained by real-time reverse transcription-polymerase chain reaction (RT-PCR). $*P < 0.001$ represents the significance vs. the expression level in Col-0 by Student's *t*-test. Results are representative of three experiments.

Fig. S2 Pathogenesis-related 1 (*PR1*) expression was up-regulated by either *Xcc* MAFF106712 or the *hrcC* deletion mutant after inoculation of the plant double mutant (*atpp2c62/atpp2c26*). Five fully expanded leaves of Col-0 and the double mutant were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xanthomonas campestris* pv. *campestris* (*Xcc*) wild-type and *hrcC* mutant bacteria [10^9 colony-forming units (CFU)/mL] or vehicle alone. Expression of the *PR1* gene in the plants harvested at 1 day post-inoculation (dpi) was obtained by real-time reverse transcription-polymerase chain reaction (RT-PCR). Significant differences from Col-0 mock were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and are indicated with an asterisk ($*P < 0.05$). The results are representative of three experiments.

Fig. S3 Hypersensitive response (HR) caused by AvrRPM1 is not changed in the double mutant (*atpp2c26/atpp2c62*). The HR phenotypes of the plant lines indicated following inoculation with 5×10^7 colony-forming units (CFU/mL) of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 carrying *avrRpm1* or empty vector (EV). Representative leaves are shown at 20 h (hpi, left panel) and 3 days (dpi, right panel) post-inoculation. Beneath each leaf is the number of leaves showing HR from the total number of leaves infiltrated. Black and red marks indicate the inoculated leaves. WT, wild-type.

Fig. S4 The multiplication of type III secretion system (TTSS) mutant bacteria is not changed in the double mutant. Bacterial multiplication was measured in Col-0 (wild-type) and the double mutant. Five fully expanded leaves of each plant were inoculated by piercing three holes in the central vein with a needle dipped in a suspension of *Xanthomonas campestris* pv. *campestris* (*Xcc*) *hrcC* bacteria [10^9 colony-forming units (CFU)/mL]. Results are representative of three experiments. Significant differences from Col-0 were not identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.