INDUCED RESISTANCE FOR PLANT DEFENCE

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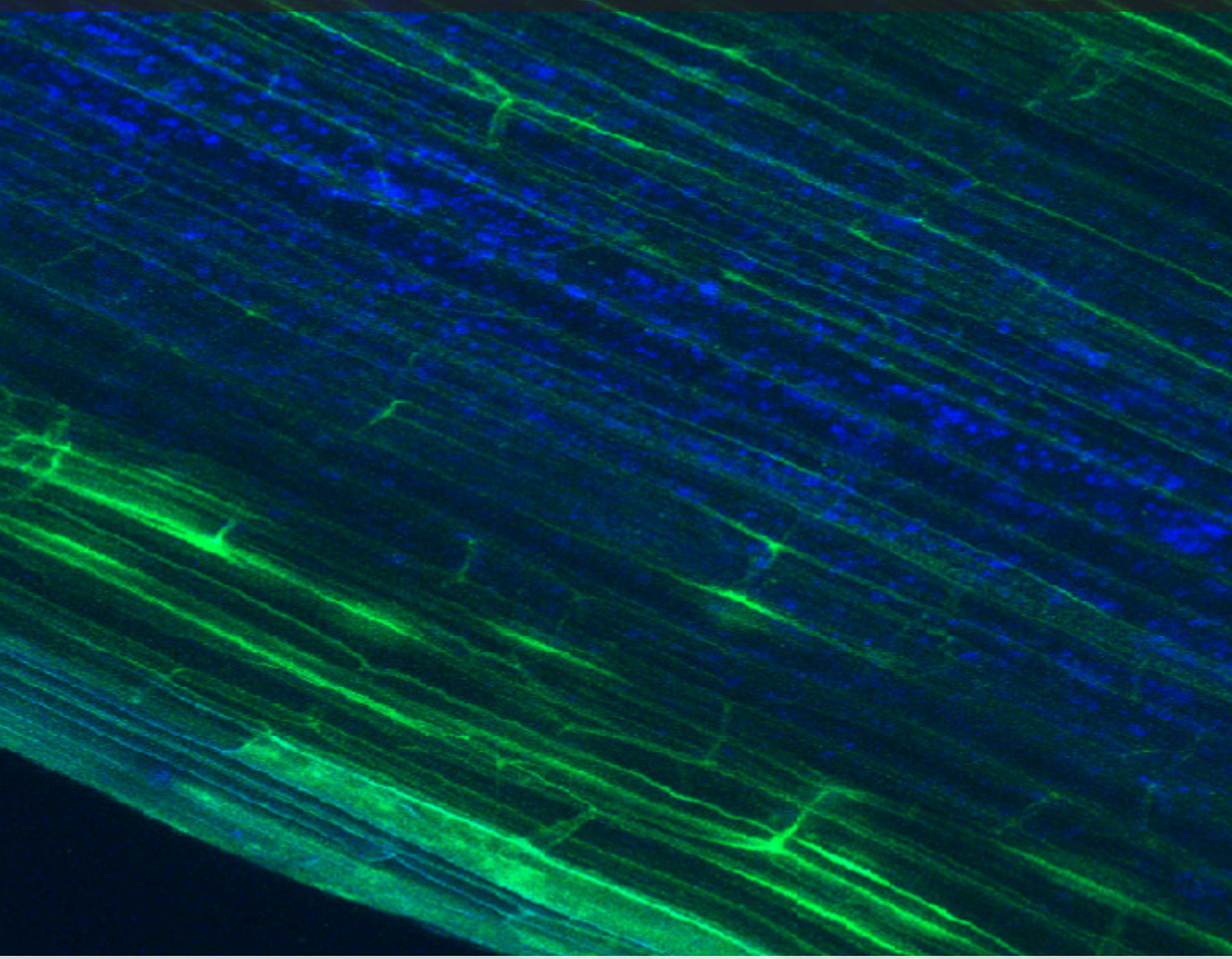

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INDUCED RESISTANCE FOR PLANT DEFENCE

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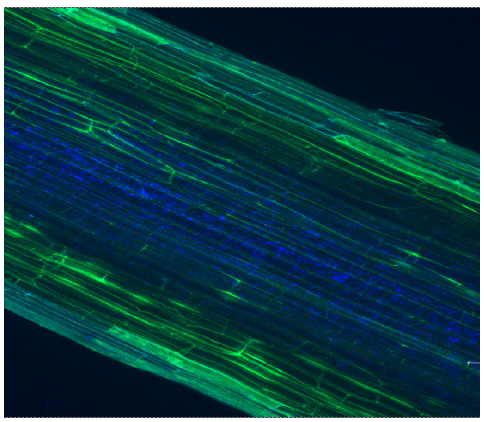

NO imaging in pea roots exposed to Cd. The image is the projection of several optical sections collected by confocal microscopy showing the autfluorescence (blue) and NO-dependent diaminofluorescein diacetate fluorescence (green). Image by María Rodríguez-Serrano and Luisa M. Sandalio (Estación Experimental del Zaidín, CSIC, Granada, Spain).

In this century the human being must face the challenges of producing enough to feed a growing population in a sustainable and environmentally friendly way. The yields are with increasing frequency affected by abiotic stresses such as salinity, drought, and high temperature or by new diseases and plagues. The Research Topic on Induced Resistance for Plant Defense focuses on the understanding the mechanisms underlying plant resistance or tolerance since these will help us to develop fruitful new agricultural strategies for a sustainable crop protection.

This topic and its potential applications provide a new sustainable approach to crop protection. This technology currently can offer promising molecules capable to provide new long lasting treatments for crop protection against biotic or abiotic stresses. The aim of this Research Topic is to review and discuss current knowledge of the mechanisms regulating plant induced resistance and how from our better understanding of these mechanisms we can find molecules capable of inducing this defence response in the plant, thereby contributing to sustainable agriculture we need for the next challenges of the XXI century.

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Induced resistance for plant defense

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Keywords: induced resistance, priming, biotic stress, abiotic stress, priming agents, crop protection, sustainable agriculture

In this century the human being must face the challenges of producing enough to feed a growing population in a sustainable and environmentally friendly way. The yields are with increasing frequency affected by abiotic stresses such as salinity, drought, and high temperature or by new diseases and plagues. The Research Topic on *Induced Resistance for Plant Defense* focuses on the understanding the mechanisms underlying plant resistance or tolerance since these will help us to develop fruitful new agricultural strategies for a sustainable crop protection. This Topic and their potential applications provide a new sustainable approach to crop protection. This technology currently can offer promising molecules capable to provide new long lasting treatments for crop protection against biotic or abiotic stresses.

One of the most studied plant defense inducers and priming agents, the β-aminobutyric acid or BABA, has been used for investigating the transgenerational epigenetic basis of priming defense and the mechanistic of long-lasting induced resistance (Luna et al., 2014a). Interestingly, these authors found that BABA-IR can be detected up to 28 days after treatment of wild-type Arabidopsis through NPR1-dependent resistance but this effect disappear by 14 days after treatment when a NPR1-independent resistance is activated. Another work about BABA (Schwarzenbacher et al., 2014) included in this ebook is a commentary about a previously published paper (Luna et al., 2014b) which study the plant perception of BABA mediated by an aspartyl-tRNA synthetase. Using BABA as priming agent in a screening for Arabidopsis mutants against the biotrophic oomycete *Hyaloperonospora arabidopsidis,* authors identify an impaired in BABA-induced Immunity 1 (*IBI1*) gene, encoding an aspartyl-tRNA synthetase (AspRS). This mutation seems to block both priming SA-dependent or SA-independent responses to BABA, indicating unilateral control of BABA-induced resistance by IBI1 (Luna et al., 2014b).

Nitrogen fertilization influences plant-pathogen interactions and elevated levels of nitrogen can promote susceptibility against biotrophs as well as can influence in plant resistance. The disruption of an ammonium transporter involved in the plant immune system, the ammonium transporter AMT1.1, alters basal defenses generating resistance against *Pseudomonas syringae* and *Plectosphaerella cucumerina* (Pastor et al., 2014a). In this work their authors study the role of this ammonium transporter on the basal defenses and the resistance against *P. syringae* and *P. cucumerina* demonstrating that it is a negative regulator of Arabidopsis defense responses (Pastor et al., 2014a).

Cross-talk between different signaling pathways has been reported to generate both synergistic and antagonistic defense responses. In some cases this cross-talk might contribute to fine-tune defense responses against some pathogens according to its mode of infection. Using some resistance elicitors such as acibenzolar-S-methyl (ASM), β-aminobutyricacid (BABA), cis-jasmone (CJ), and a combination of the three compounds, which involve SA and/or JA-dependent signaling pathways, Walters et al. (2014) study if these treatments are capable to control infection of spring barley by *Rhynchosporium commune* under field conditions (Walters et al., 2014).

Heavy metals like cadmium are an important source of contamination and a serious problem in the modern agriculture. We need further research on this abiotic stress. Some important novel insights into cadmium sensing have been studied and it seems to induce a rapid mobilization of defense mechanisms through the activation of specific signaling transduction pathways (Chmielowska-Bak et al., 2014).

Priming phenomena have been widely described, however mechanisms underlying are still unclear. One of the papers included in this ebook study how during the priming phase plant prepares for further challenges by accumulating and storing conjugates or precursors of molecules as well as other compounds that play a role in defense (Pastor et al., 2014b).

An important issue in this topic is the influence of the environment and the genotype in the plant responsiveness to defense elicitors. Interestingly, Bruce (2014) have shown that herbivore and pathogen attack can promote defense induction phenotypes across generations and that epigenetic change may be the basis for its long lasting effects (Bruce, 2014).

Plants are able to respond to biotic or abiotic stresses through a complex network involving phytohormones, a potent secondary metabolism and secondary messengers like calcium, and stress receptors. Light also plays a key role in plant resistance. Protein kinase/phosphatase cascades are another important component of this network. Rasool and co-workers study the effects of the light on these proteins using light-grown *Arabidopsis thaliana* wild type and in mutant lines defective in several protein phosphatase regulatory subunits on aphid fecundity and susceptibility to *P. syringae* infection (Rasool et al., 2014).

Aranega and co-workers update the role of natural compounds as priming agents and focus on the molecule hexanoic acid as a model. They review on the different mode of action of natural compounds that induce resistance by a priming mechanism (Aranega et al., 2014).

Another interesting work included in this topic focuses on the activation of the plant immunity by a pathogen detection system known as pattern-triggered immunity (PTI) response (Huang and Zimmerli, 2014). This system relies on the accurate detection of pathogen-or microbe-associated molecular patterns by pattern-recognition receptors (PRRs). Resistance is the rule in the majority of plants. Huang and Zimmerli suggest that the reinforcement of PTI through genetic engineering may generate crops with broad-spectrum field resistance (Huang and Zimmerli, 2014).

Finally, Borges and co-workers propose priming crops as a way for controlling biotic and abiotic stresses and focus on the effect of the water-soluble vitamin K3 derivative, known as menadione sodium bisulphite (MSB), as a novel priming agent and as a tool for studying priming mechanisms. The work review the priming phenomenon and the importance of reactive oxygen species (ROS) as key signaling molecules that contribute to control of plant development as well as to the sensing of the external environment and priming induction (Borges et al., 2014).

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ORIGINAL RESEARCH ARTICLE
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Role of NPR1 and KYP in long-lasting induced resistance by β-aminobutyric acid

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Priming of defense increases the responsiveness of the plant immune system and can provide broad-spectrum protection against disease. Recent evidence suggests that priming of defense can be inherited epigenetically to following generations. However, the mechanisms of long-lasting defense priming within one generation remains poorly understood. Here, we have investigated the mechanistic basis of long-lasting induced resistance after treatment with β-aminobutyric acid (BABA), an agent that mimics biologically induced resistance phenomena. BABA-induced resistance (BABA-IR) is based on priming of salicylic acid (SA)-dependent and SA-independent defenses. BABA-IR could be detected up to 28 days after treatment of wild-type *Arabidopsis*. This long-lasting component of the induced resistance response requires the regulatory protein NPR1 and is associated with priming of SA-inducible genes. In contrast, NPR1-independent resistance by BABA was transient and had disappeared by 14 days after treatment. Chromatin immunoprecipitation (ChIP) assays revealed no increased acetylation of histone H3K9 at promoters regions of priming-responsive genes, indicating that this post-translational histone modification is not critical for long-term transcriptional priming. Interestingly, the *kyp-6* mutant, which is affected in methyltransferase activity of H3K9, was blocked in long-lasting BABA-IR, indicating a critical requirement of this post-translational histone modification in long-lasting BABA-IR. Considering that KYP suppresses gene transcription through methylation of H3K9 and CpHpG DNA methylation, we propose that KYP enables long-term defense gene priming by silencing suppressor genes of SA/NPR1-dependent genes.

Keywords: priming, induced defense, *Arabidopsis*, NPR1, KYP

INTRODUCTION

Plants can resist pathogen attack by increasing the responsiveness of their immune system. This phenomenon typically occurs after perception of stress-indicating signals and is known as priming of defense. Priming provides non-specific protection against a wide range of biotic and abiotic stresses (Conrath et al., 2006; Pastor et al., 2012; Tanou et al., 2012), which is associated with relatively minor costs on growth and reproduction (Van Hulten et al., 2006). Induction of defense priming results in a faster and stronger expression of basal immune responses upon pathogen attack (Conrath et al., 2006, 2011), and can render plants immune if the augmented defense reaction precedes immune-suppression by pathogen (Ahmad et al., 2010). In most cases, however, defense priming slows down pathogen colonization and reduces disease. Research over the past decades has identified various chemicals that can mimic biologically induced priming responses. These chemicals are often plant-derived signaling metabolites, such as salicylic acid (SA; Shirasu et al., 1997), jasmonic acid (JA; Kauss et al., 1994; Conrath et al., 2002), azelaic acid (Jung et al., 2009), or herbivore-induced volatiles (Ton et al., 2007; Frost et al., 2008). There are also xenobiotic chemicals that can trigger defense priming in plants. Amongst these, the non-protein amino acid β-aminobutyric acid (BABA; Zimmerli et al., 2000; Ton et al., 2005) and benzo-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Kohler et al., 2002) have emerged as popular agents to study the mechanistic basis of defense priming in plants (Conrath, 2011).

BABA-induced resistance (BABA-IR) mimics component of defense priming that are active during pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-induced systemic resistance (ISR; Van der Ent et al., 2009). Consequently, it provides protection against an exceptionally broad range of pathogens and insects. The signaling pathways controlling BABA-IR against the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 relies on production of the plant hormone salicylic acid (SA) and a functional non-expressor of PR GENES (NPR1) protein (Zimmerli et al., 2000). However, BABA-IR against the oomycete pathogen *Hyaloperonospora arabidopsidis* and the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* can function independently from NPR1, but requires components of the abcisic acid (ABA) signaling pathway (Ton and Mauch-Mani, 2004; Ton et al., 2005). Both pathways operate independently from each other and provide different mechanisms of defense priming (Ton et al., 2005). The NPR1-independent pathway primes cell wall defense, which leads to augmented deposition of callose-rich papillae after pathogen attack. On the other hand, the NPR1-dependent pathway controls priming of SA-dependent genes, which is marked by enhanced transcription of NPR1-dependent

transcription factor (TF) genes that control SA-dependent gene induction (Van der Ent et al., 2009). The latter finding suggested that greater abundance of defense regulatory TFs contributes to transcriptional priming of SA-inducible defense genes. However, TFs have limited turn-over times and their enhanced accumulation after application of a single priming stimulus is not a satisfactory explanation for a long-lasting induced resistance response.

Epigenetic mechanisms, such as histone modifications or DNA methylation, have emerged as important regulatory mechanisms in plant immunity (Alvarez et al., 2010). There is ample evidence that post-translational modifications of histone proteins are influences by JA-, SA-, and ABA-dependent signaling pathways (Devoto et al., 2002; Mosher et al., 2006; Walley et al., 2008; Cho et al., 2012). Furthermore, exposure to disease, herbivores and abiotic stresses can have profound impacts on patterns of symmetric and asymmetric DNA methylation (Pavet et al., 2006; Boyko et al., 2010; Verhoeven et al., 2010; Dowen et al., 2012). It is, therefore, not surprising that priming of defense has been associated with epigenetic regulatory mechanisms (Conrath, 2011; Pastor et al., 2012). First evidence for an epigenetic basis of defense priming came from Jaskiewicz et al. (2011), who demonstrated that infection of *Arabidopsis* by *Pseudomonas syringae* pv. *maculicola* primes stress-inducible expression of transcription factor genes via NPR1-dependent modifications of histone H3 at their promoter regions. Furthermore, López et al. (2011) demonstrated that mutants blocked in RNA-directed DNA methylation are primed to activate SA-inducible defense genes, which was associated with H3 modifications marking a facilitated state of gene transcription: acetylation at lysine residue 9 (H3K9ac) and triple-methylation at lysine 4. Hence, defense priming is often associated with post-translational histone modifications at promoter regions of primed defense genes.

Recently, three independent research groups provided evidence that priming of defense can be inherited epigenetically from isogenic plants that had been treated with pathogens, herbivores, or BABA (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012). Although these studies demonstrated an epigenetic component of defense priming, the extent by which epigenetic regulation contributes to long-lasting defense priming within one plant generation remains unknown. In this study, we have investigated the mechanisms controlling durable maintenance of defense priming in individual plants after treatment with the chemical agent BABA. We show that only the NPR1-dependent component of BABA-IR is long-lasting in *Arabidopsis*, which is associated with priming of SA-inducible defense genes. Furthermore, we provide evidence that this long-lasting defense resistance requires the histone methyltransferase KYP.

MATERIALS AND METHODS

PLANT MATERIAL, GROWTH CONDITIONS, AND EXPERIMENTAL DESIGN

Arabidopsis thaliana (Col-0), *npr1-1* (Despres et al., 2003), and *kyp-6* (Alonso et al., 2003; Chan et al., 2006) were cultivated in a growth chamber with a 8-h day (150 μE m^{-2} s^{-1} at 20°C) and 16-h night (18°C) under at 65% relative humidity. Seeds were planted in 60-ml pots containing a 50% (v/v) sand/M3 mixture and kept at 4°C in the dark for 2 days to break dormancy. Five day-old seedlings were soil-drenched with water or BABA solution (Sigma-Aldrich; Cat.: A4, 420–7) to a final concentration of 40 mg/L in the soil. Six days after treatment, seedlings were transplanted to BABA-free soil (**Figure 1A**). At different time-points after treatment, plants were examined for differences in fresh weight (7 and 28 days), inoculated with *H. arabidopsidis* (7, 14, and 21 days), inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 *luxCDABE* (*Pst*-luxCDABE; Fan et al., 2008; 28 days), or examined for priming of salicylic acid (SA)-inducible gene expression and chromatin modification (28 days).

GROWTH ANALYSIS AND INDUCED RESISTANCE BIOASSAYS

Plant growth was analyzed by measuring shoot fresh weights at 6 and 28 days after induction treatment (seedlings were excised from the roots, including hypocotyls). Inoculation and determination of pathogen colonization were performed as described previously (Luna et al., 2012). Colonization by *H. arabidopsidis* was scored at 6 days after inoculation. Colonization by *Pst*-luxCDABE (Fan et al., 2008) was quantified at 3 days after inoculation.

GENE EXPRESSION ANALYSIS

Water- and BABA-treated plants ($n = 30$) were sprayed with 0.5 mM SA (Sodium salicylate; Sigma-Aldrich; Cat.: S3007). At 0, 4, 8, and 24 h after application of SA, at least three biologically replicated samples containing pooled material from individual pots, were snap frozen in liquid nitrogen. RNA extraction, cDNA synthesis, and reverse-transcriptase quantitative PCR (RT-qPCR) with gene-specific primers were entirely performed as described before (Luna et al., 2012). Fold induction values were normalized to average $2^{\Delta Ct}$ values relative to time-point 0 h before SA application of control-treated plants.

CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) assays were carried out as described in the manufacturer's protocol (EpiQuik Plant ChIP kit; Epigentek, Brooklyn, NY, USA), using mature leaves from 5-week-old plants. For each experiment, at least three biologically replicated samples were collected, each consisting of rosettes from five to seven plants. Chromatin samples were immunoprecipitated using antibodies against acetyl-histone H3K9 (Millipore 07-352). Before and after immunoprecipitation, DNA abundance in chromatin extracts was analyzed by quantitative PCR, using the ABI PRISM® 7900 HT sequence detection system. Two technically replicated reactions per sample were performed in a final reaction volume of 25 µl, containing Jump Start SYBR Green (Sigma-S4438). Sequence-specific primers were used to amplify promoter DNA from *PR1*, *WRKY6*, *WRKY29*, *WRKY53* (Jaskiewicz et al., 2011), and *WRKY70* (Fw: AATTAGATTCAAGTCCACAACCAA Rv: ATCAAGAAATTGTCATCCAACAC). Results were normalized to DNA amounts in the input control, as described by Haring et al. (2007) with modifications. To prevent possible bias to inaccurate estimations of input DNA, two independent DNA extractions were performed from each chromatin extract. Only if input values differed less than 0.25 C_t values, samples were considered reliable for further analysis and C_t input

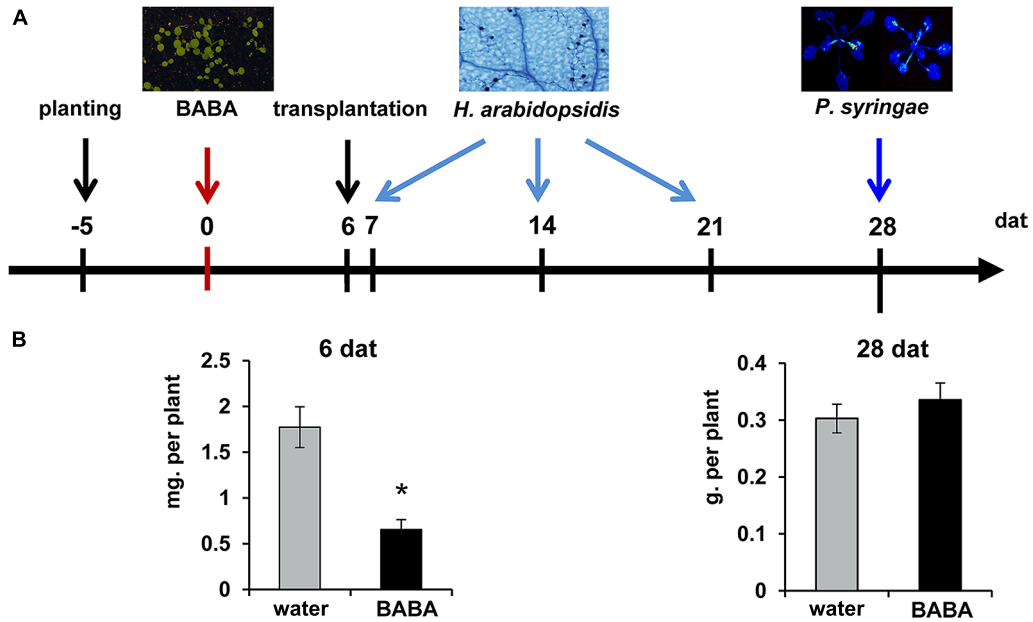

FIGURE 1 | Experimental design to determine durability of induced resistance. (A) Five-day-old seedlings were soil-drenched with water or BABA (40 mg/L) and transplanted to BABA-free soil at 6 days after induction treatment (dat). Plants were examined for shoot fresh weight at 6 and 28 days after treatment (dat), or inoculated with either *Hyaloperonospora arabidopsidis* WACO9 (7, 14, 21 dat), or *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*-luxCDABE; 28 dat). **(B)** Mean shoot fresh weights of water- and BABA-treated plants at 6 and 28 dat. Error bars represent the SEM. Asterisk indicates a statistically significant difference between water- and BABA-treated plants (Student's *t*-test; $\alpha < 0.05$; $n = 15$).

values were averaged for normalization of immunoprecipitated DNA.

STATISTICS

Average shoot fresh weights and % bacterial bioluminescence were based on at least 15 individual plants per treatment and were analyzed for statistical differences by Student's *t*-tests ($\alpha = 0.05$; SPSS, v19.0). *H. arabidopsidis* class distributions were based on 50–100 leaves and differences between treatments were analyzed for statistical significance by $\chi 2$ contingency tests using SPSS, v19.0. Average fold-change values of gene expression and H3K9ac levels were based on three biological replicates per treatment and statistical differences were determined by Student's *t*-tests ($\alpha = 0.05$; SPSS, v19.0). Each experiment was repeated twice from the onset.

RESULTS

NO LONG-LASTING IMPACTS OF BABA ON PLANT DEVELOPMENT

Induction of disease resistance by BABA can reduce growth of *Arabidopsis* (Van Hulten et al., 2006; Wu et al., 2010). To examine the long-lasting impact of BABA on plant development, 5-day-old seedlings were soil-drenched with 40 mg/L BABA. Seedlings were kept in BABA-drenched soil for 6 days, after which they were transferred to un-treated soil in order to exclude ongoing induction by excess amounts of soil-based BABA (**Figure 1A**). Fresh weight analysis of shoots at 6 days after treatment revealed a statistically significant reduction of plant growth in BABA-treated plants (**Figure 1B**). However, no growth differences were apparent by 28 days after treatment, indicating that BABA-treated plants can fully recover from the induction treatment.

INDUCED RESISTANCE BY BABA LASTS UP TO 4 WEEKS AFTER TREATMENT

To determine durability of BABA-IR, plants were infected with *H. arabidopsidis* at 7, 14, and 21 days after BABA application (**Figure 2A**), after which colonization was microscopically analyzed at 6 days after inoculation. Because of age-related resistance against *H. arabidopsidis* at later developmental stages, bioluminescent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*-luxCDABE) was used to determine disease resistance at 28 days after induction treatment. BABA-treated plants express nearly complete levels of resistance against *H. arabidopsidis* when inoculated at 7 days after induction treatment, which declined when plants had been inoculated at later time points. Nevertheless, statistically significant levels of induced resistance were still detectable by 21 days after treatment (**Figure 2A**). Moreover, when plants had been infected *Pst*-luxCDABE at 28 days after induction treatment, BABA-treated plants still allowed lower levels of leaf colonization by these bacteria (**Figure 2B**). Hence, BABA-IR declines during the first 2 weeks after treatment, but remains stable during following weeks. Slaughter et al. (2012) demonstrated that plant-endogenous BABA levels at 3 weeks after soil-drench application (40 mg/L) are 10-fold lower than the threshold level required for induced resistance. Accordingly, we conclude that long-lasting resistance by BABA is not due to lingering traces of BABA in the tissue, but rather due to long-lasting physiological changes in the plant.

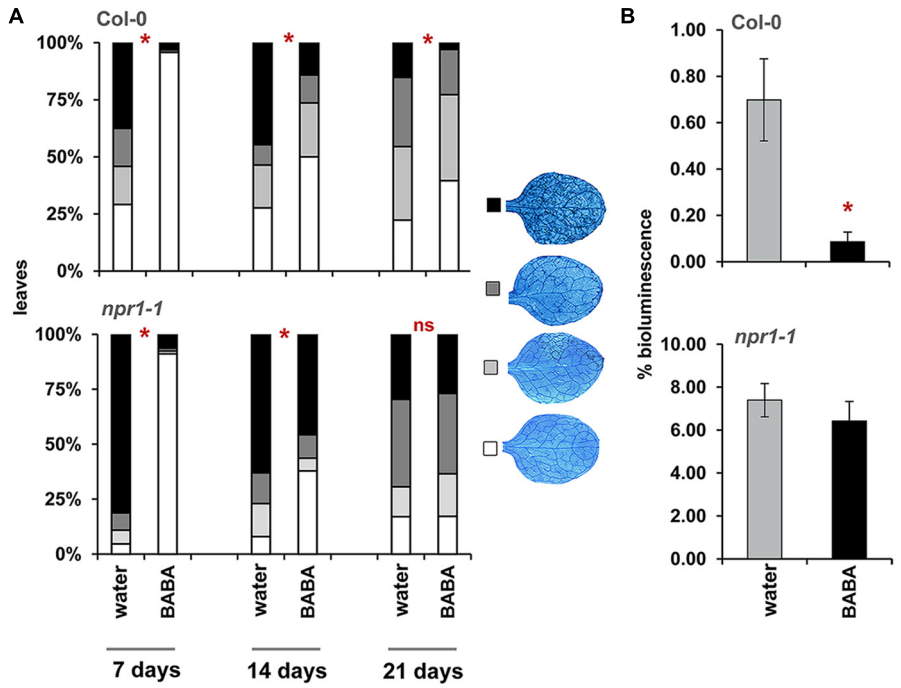

FIGURE 2 | Durability of BABA-IR in *Arabidopsis*. (A) Levels of leaf colonization by *H. arabidopsidis* in wild-type (Col-0) and *npr1-1* plants infected at 7, 14, and 21 days after treatment with 40 mg/L BABA (dat). Shown are % of leaves assigned to four different pathogen colonization classes, based on scoring of 50–100 trypan-blue stained leaves at 6 days after inoculation with 10^5 spores mL^{-1}. Asterisks indicate statistically significant differences between treatments (χ^2 contingency test; $\alpha < 0.05$). **(B)** Bacterial colonization by *Pst*-luxCDABE in wild-type (Col-0) and *npr1-1* plants at 28 dat (40 mg/L BABA). Shown are average values (±SEM) of relative bioluminescence per plant at 3 days after inoculation. Asterisk indicates a statistically significant difference between water- and BABA-treated plants (Student's *t*-test; $\alpha < 0.05$). ns = no significant.

LONG-LASTING BABA-IR REQUIRES NPR1

The *npr1-1* mutant is blocked in SA-dependent defense (Cao et al., 1994). Consequently, this mutant is only capable of expressing the SA-independent component of BABA-IR (Zimmerli et al., 2000). To examine which component is responsible for long-lasting disease protection, we measured durability of BABA-IR in *npr1-1* plants. As observed in wild-type plants, *npr1-1* expressed relatively high levels of BABA-IR when the plants had been inoculated at 7 days after priming treatment, which declined as time progressed (**Figure 2A**). However, unlike wild-type plants, BABA-treated *npr1-1* failed to express induced resistance to *H. arabidopsidis* and *Pst*-luxCDABE when inoculated at 21 and 28 days after induction treatment, respectively (**Figure 2**). These results indicate that long-lasting BABA-IR is regulated by the NPR1-dependent pathway.

LONG-LASTING PRIMING BY BABA IS ASSOCIATED WITH PRIMING OF SA-INDUCIBLE GENES

NPR1 regulates priming of SA-dependent defense (Kohler et al., 2002; Van der Ent et al., 2009). Since our experiments revealed that NPR1 is necessary for long-lasting resistance by BABA, we investigated whether the resistance is associated with transcriptional priming of SA responsive genes (*PR1, PR5, WRKY70, WRKY6, WRKY53,* and *WRKY38*). At 28 days after BABA application, leaves were sprayed with 0.5 mM SA and harvested at different time-points after treatment for RT-qPCR analysis of defense gene expression. As is shown in **Figure 3**, basal transcription levels of all genes before SA application were similar in BABA- and control-treated plants. However, after application of SA, all genes tested showed faster and stronger transcriptional induction in BABA-treated plants compared to control plants (**Figure 3**). Hence, long-lasting induced resistance is not based on enhanced transcription of SA-dependent defense genes, but rather on a transcriptional priming of these genes.

SA-DEPENDENT GENE PRIMING IS NOT MARKED BY INCREASED ACETYLATION OF H3K9

Chromatin remodeling is an epigenetic mechanism that can provide long-lasting changes in the plant's transcriptional capacity (Berger, 2007). Post-translational modifications at the lysine residue 9 of histone 3 (H3K9) have been shown to regulate gene transcription (Li et al., 2007). Acetylation of H3K9 correlates with increased transcriptional capacity, whereas methylation of this residue correlates with gene silencing (Zhou et al., 2010). Previously, we demonstrated that transgenerational priming of SA-dependent defense in progeny from diseased *Arabidopsis* is associated with enrichment of H3K9ac at the promoter regions of the primed genes (Luna et al., 2012). This post-translational histone modification has also been associated to short-term defense gene priming after treatment with BTH and BABA (Jaskiewicz et al., 2011; Po-Wen et al., 2013). Based on these

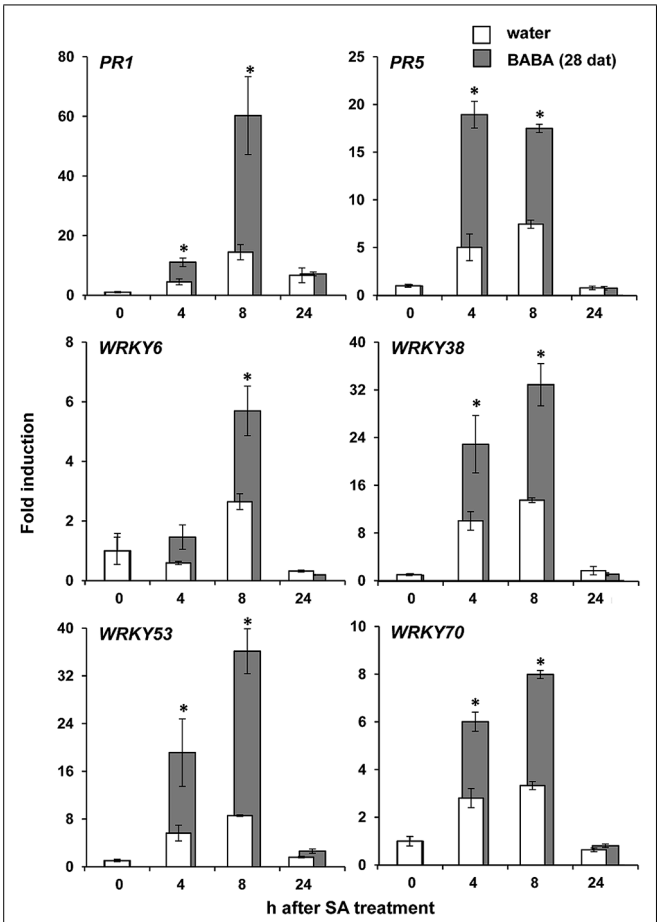

FIGURE 3 | Long-lasting priming of SA-dependent gene transcription. Levels of gene transcription were determined by RT-qPCR analysis of shoot tissues from water- and BABA-treated plants at 28 days after treatment (dat) with 40 mg/L BABA, and different hours (h) after spraying of the leaves with 0.5 mM SA. Data represent average fold-change values (±SEM) relative to expression levels before SA application. Asterisks indicate statistically different levels of expression between control- and BABA-treated plants (Student's *t*-test; $\alpha < 0.05$; $n = 3$).

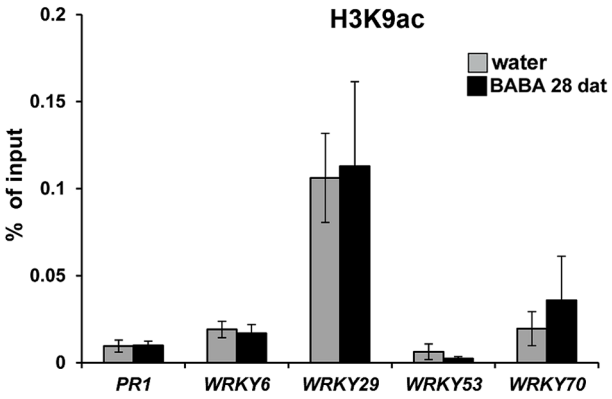

FIGURE 4 | Long-lasting defense gene priming is not marked by increased H3K9ac. Levels of H3K9ac at the promoters of the SA-inducible *PR1, WRKY6, WRKY29, WRKY53,* and *WRKY70* genes in water and BABA treated wild-type plants at 28 dat. Chromatin-immunoprecipitation (ChIP) was quantified by qPCR and expressed relative to DNA amounts in chromatin extracts before immunoprecipitation (input). Data represent mean values (±SEM) from three biologically replicated samples.

observations we assessed levels of H3K9 acetylation in promoters of defense genes displaying long-lasting priming by BABA (**Figure 3**), using similar primer pairs and H3K9ac antibody as described before (Jaskiewicz et al., 2011; Luna et al., 2012). Unexpectedly, these ChIP analyses revealed that BABA did not have long-term impacts on H3K9ac levels at the promoter of *PR1*, nor did it consistently affect H3K9ac levels at promoters of *WRKY* genes (**Figure 4**). These results indicate that H3K9ac is not a *cis*-acting requirement for long-lasting defense gene priming by BABA.

KYP REGULATES LONG-LASTING DEFENSE GENE PRIMING

SUVH4/KRYPTONITE (KYP) is a histone methyltransferase that methylates H3K9 residues and its activity results in gene silencing through the interaction with CHROMOMETHYLASE3 (CMT3) DNA methyltransferase (Jackson et al., 2002). Loss of KYP results in decrease H3K9me2 and DNA methylation levels at CpHpG context (Chan et al., 2006). Previously, in an attempt to decipher the epigenetic mechanisms controlling transgenerational immune priming, we identified KYP as a key regulator of this phenomenon (Luna and Ton, 2012). In order to assess the role of this enzyme in long-lasting priming by BABA, we measured levels of BABA-IR against *H. arabidopsidis* at 7 and 21 days after treatment in the *kyp-6* mutant (Jackson et al., 2002, 2004; Chan et al., 2006; Henderson and Jacobsen, 2008). This mutant displayed wild-type levels of basal resistance against *H. arabidopsidis* and showed significant levels of BABA-IR resistance to *H. arabidopsidis* when inoculated at 7 days after induction treatment (**Figure 5**). However, this mutant had lost its ability to express BABA-IR at 21 days after treatment (**Figure 5**), indicating that KYP acts as a positive regulator of long-term maintenance of BABA-IR,

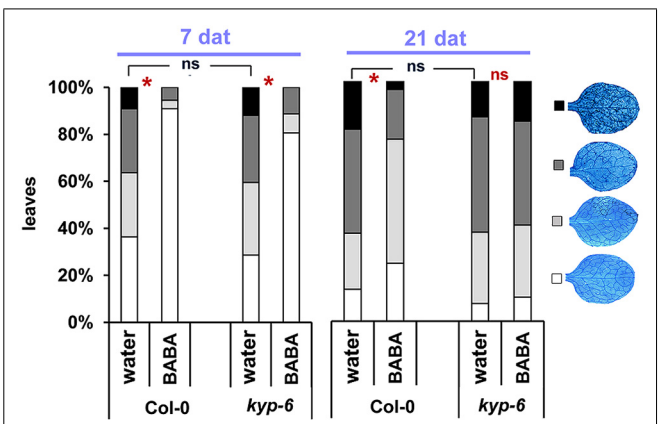

FIGURE 5 | KYP acts as a positive regulator of long-term maintenance of BABA-IR. BABA-IR against *H. arabidopsidis* in wild-type and *kyp-6* plants at 7 and 21 days after induction treatment with 40 mg/L BABA (dat). For details see legend of **Figure 2**. Asterisks indicate statistically significant differences between treatments (χ^2 contingency test; $\alpha < 0.05$). ns = no significant.

possibly by repressing negative regulatory genes of defense priming.

DISCUSSION

We have investigated the durability of induced resistance after seedling treatment with the priming agent BABA. The response of plants to this non-protein amino acid mimics different biologically induced resistance responses in plants (Ton et al., 2005; Van der Ent et al., 2009). Consequently, this agent is effective against an exceptionally wide range of diseases (Jakab et al., 2001; Cohen, 2002). BABA primes the plant immune system via at least two independent signaling pathways, which differ in their dependency for NPR1 (Zimmerli et al., 2000). The NPR1-independent pathway primes pathogen-inducible expression of cell wall defense, whereas the NPR1-dependent pathway primes SA-inducible genes (Ton et al., 2005; Van der Ent et al., 2009). In this study, we showed that NPR1-independent resistance by BABA is transient and disappears within 2 weeks after application (**Figure 6A**). On the other hand, NPR1-dependent BABA-IR is long-lasting and remains up to 28 days after treatment (**Figure 6A**). Consistent with the idea that long-lasting BABA-IR involves epigenetic regulation, Slaughter et al. (2012) demonstrated increased defense phenotypes in progeny from isogenic *Arabidopsis* lines upon treatment with BABA. These phenotypes included reduced disease susceptibility to *H. arabidopsidis* and a sensitization to priming treatment by BABA, i.e., plants were "primed to be primed." Interestingly, transgenerational effects by BABA were largely blocked in the *impaired in BABA-induced sterility1* (*ibs1*) mutant, which had previously been reported to be affected in priming of NPR1-dependent defense by BABA (Ton et al., 2005). Furthermore, we recently demonstrated epigenetic inheritance of NPR1-dependent resistance from *Pst*-luxCDABE-infected *Arabidopsis*, which remained stable up to two generations. We, therefore, conclude that long-lasting NPR1-dependent resistance within the same generation has an epigenetic component, which can, at least partially, be transmitted to following generations.

Previous studies have demonstrated a clear correlation between H3K9 acetylation and transcriptional priming at defense gene promoters (Jaskiewicz et al., 2011; López et al., 2011; Luna et al., 2012; Po-Wen et al., 2013). Using similar gene primers as described earlier (Luna et al., 2012), our ChIP experiments showed that long-lasting priming of SA-inducible defense genes within one generation can occur independently from increased H3K9 acetylation (**Figure 4**). Although we cannot exclude the possibility that flanking promoter regions of these genes did show enhanced H3K9 acetylation, our findings indicate that H3K9 acetylation at the regions analyzed is not a strict requirement for long-lasting priming of SA-inducible genes. The difference between our current H3K9ac results and those reported in progenies from *Pst*-luxCDABE-infected plants (Luna et al., 2012) suggests that the within-generational effects by BABA and transgenerational effects by pathogens are based on different epigenetic mechanisms. This is further supported by the fact that transgenerational resistance by BABA is lost after one stress-free generation (Slaughter et al., 2012), whereas transgenerational resistance by *Pst*-luxCDABE is maintained over 1 stress-free generation (Luna et al., 2012).

Interestingly, the *kyp-6* mutant failed to retain long-lasting induced resistance by BABA (**Figure 5**), suggesting that *KYP*

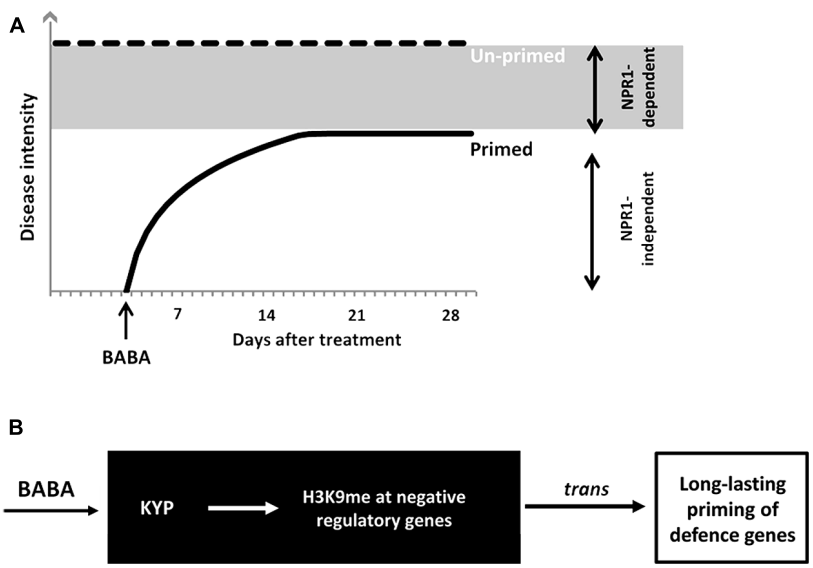

FIGURE 6 | Model of durability of BABA-IR. (A) BABA primes SA-inducible defense genes in a NPR1-dependent and -independent manner. BABA-IR lasts up to 28 days in wild-type plants, but only up to 14 days in *npr1-1* plants. Hence, long-lasting disease protection by BABA acts through a NPR1-dependent signaling pathway. **(B)** KYP-mediated control of long-lasting priming by BABA. The *kyp-6* mutant is blocked in long-lasting induced resistance by BABA, but expressed wild-type levels of disease susceptibility. This indicates that KYP stimulates long-lasting defense gene priming by BABA. Since KYP mediates transcriptional silencing through H3K9 methylation and CpHpG DNA methylation, we propose that KYP promotes long-lasting defense priming through silencing of *trans*-acting genes that encode for negative regulators of defense priming.

enables long-lasting defense priming by BABA. Although the possibility that the *kyp-6* mutant harbors an independent second mutation disrupting long-lasting BABA-IR cannot be excluded, the phenotype of the *kyp-6* mutant strongly suggests that H3K9 methyltransferase activity by the KYP protein is necessary for long-term maintenance of SA-dependent defense priming in BABA-treated plants. Since KYP-dependent H3K9 di-methylation and associated CpHpG DNA methylation are repressive mechanisms of gene expression (Kass et al., 1997; Zhou et al., 2010), we propose that KYP maintains BABA-IR by silencing repressive regulatory genes of SA-dependent defense genes, thereby priming their responsiveness to pathogen infection (**Figure 6B**). Previously, we proposed that disease-induced repression of RNA-directed DNA methylation is responsible for transmission of defense priming (Luna et al., 2012). Further research is required to resolve the interaction between KYP and components of RNA-directed DNA methylation with regards to long-lasting BABA-IR.

There is no evidence to support that post-translational histone modifications themselves can be transmitted through meiosis in plants. It is, therefore, plausible that transgenerational transmission of BABA-IR is determined by differentially methylated DNA regions (DMRs) that can faithfully be transmitted through meiosis. Indeed, we recently reported that transgenerational resistance in progeny from *Pst*DC30000-infected *Arabidopsis* is most likely transmitted through a reduction in non-CpG DNA methylation (Luna and Ton, 2012; Luna et al., 2012). Interestingly, however, Slaughter et al. (2012) did not detect consistent changes in DNA methylation at priming-responsive defense genes in progenies of BABA-treated plants, indicating that transgenerational priming of defense genes by BABA is regulated by *trans*-acting DMRs. Future research is required to decipher the complex interplay between small RNAs, DNA (hypo)methylation, and post-translational histone modifications (Bond and Baulcombe, 2014).

Safeguarding food security represents an urgent challenge in this century, which is further aggravated by climate change that can render agricultural lands less suitable for crop production. Consequently, there is a pressing need to improve the efficiency of sustainable food production, including intensification of durable crop protection strategies (Royal-Society, 2009). Although usage of modern fungicides poses relatively little direct risks on food safety and soil ecology, repeated applications of fungicides demand considerable energy consumption. Integration of long-lasting induced resistance in existing disease management schemes would allow fewer energy costs to reach similar levels of disease protection. Worrall et al. (2012) recently reported that seed treatment of tomato with BABA provides long-lasting protection against powdery mildew. Considering that some crops are cultivated hydroponically, seedling application of BABA would provide another means of achieving long-lasting induced resistance against disease. Our present study has uncovered chromatin remodeling as an important regulatory mechanism of long-lasting induced resistance. Future research is required to identify the *trans*-acting genes that become targeted by chromatin remodeling after priming treatment, which will help to optimize the efficiency of durable induced resistance in plants.

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Preparing to fight back: generation and storage of priming compounds

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Immune-stimulated plants are able to respond more rapidly and adequately to various biotic stresses allowing them to efficiently combat an infection. During the priming phase, plant are stimulated in absence of a challenge, and can accumulate and store conjugates or precursors of molecules as well as other compounds that play a role in defense. These molecules can be released during the defensive phase following stress. These metabolites can also participate in the first stages of the stress perception. Here, we report the metabolic changes occuring in primed plants during the priming phase. β-aminobutyric acid (BABA) causes a boost of the primary metabolism through the tricarboxylic acids (TCA) such as citrate, fumarate, (S)-malate and 2-oxoglutarate, and the potentiation of phenylpropanoid biosynthesis and the octodecanoic pathway. On the contrary, *Pseudomonas syringae* pv tomato (*PstAvrRpt2*) represses the same pathways. Both systems used to prime plants share some common signals like the changes in the synthesis of amino acids and the production of SA and its glycosides, as well as IAA. Interestingly, a product of the purine catabolism, xanthosine, was found to accumulate following both BABA- and *PstAvrRpt2*-treatement. The compounds that are strongly affected in this stage are called priming compounds, since their effect on the metabolism of the plant is to induce the production of primed compounds that will help to combat the stress. At the same time, additional identified metabolites suggest the possible defense pathways that plants are using to get ready for the battle.

Keywords: priming, β-aminobutyric acid (BABA), metabolites, primary metabolism, amino acids, induced resistance

INTRODUCTION

Plants are generally confined to one location for their whole lifetime and had therefore to develop adaptive and defensive mechanisms against biotic and abiotic stresses that take this limitation into account. The first crucial step for a plant is to recognize that it is attacked (Nimchuk et al., 2003). The speed by which the plant senses and accurately recognizes a specific stress(or) determines how appropriate and successful its reaction will be. Failure to mount a timely or fitting response will, in case of pathogen attack, lead to colonization of the host tissues and hence to disease (Ebel and Cosio, 1994; Jones and Takemoto, 2004). This basal immunity of a plant contributes to slowing down the colonization process but is generally too weak to effectively prevent disease (Nürnberger and Lipka, 2005). The level of basal immunity of a plant, however, can be enhanced through application of appropriate stimuli. This is commonly referred to as induced resistance (IR). Plants have acquired the ability to widely improve their defensive capacity against a broad range of pathogens including viruses, fungi, oomycetes, and bacteria toward which they are genetically speaking susceptible (Durrant and Dong, 2004; Hammerschmidt, 2009). This defense has to be triggered by an inducing treatment. Various such treatments have been shown to successfully induce resistance. They consist among others of an inoculation with pathogens, rhizobacteria or a treatment with defined chemicals and lead to horizontal resistance of the plant against a broad range of pathogenic organisms (van Loon et al., 1998; Oostendorp et al., 2001; Cohen, 2002; Hammerschmidt, 2009). This resistance operates in all plant parts distant from the original locus of inoculation and is therefore called systemic resistance (Durrant and Dong, 2004).

The initial phase of resistance induction, where the plant is preparing for a future attack but has not yet been challenged by a pathogen is called the priming phase (Conrath et al., 2002). Priming leads to a physiological state in which a plant responds faster and/or more accurately to an attack (Prime-A-Plant Group et al., 2006). This phase lies between the perception of the priming cue and the first exposure to a future stress. During this time slot the plant has to generate and store information that will enable it to deploy this faster and/or more accurate response to stress. How a plant senses and translates a priming cue is not known, however, there is a lot of information on what stimuli successfully induce the priming state. They reach from avirulent pathogens, insect pests, microbe- and host-derived molecules, synthetic substances up to metabolic disturbances of the plant and abiotic stressors (Prime-A-Plant Group et al., 2006). This large diversity in priming triggers suggests that multiple approaches could

lead to the induction of the priming state. There is evidence that some of these primary stimuli target epigenetic mechanisms (Bruce et al., 2007). It had been suggested that histone modification and histone replacement could take place at the onset of priming (van den Burg and Takken, 2009). In the meantime it has been shown that the induction of JA-dependent defenses through infection *with Alternaria brassicicola* and *Botrytis cinerea* or by JA itself leads to histone methylation in the promotors of JA-inducible genes (Berr et al., 2010). In rice, *JMJ705* codes for a histone lysine demethylase that is induced upon pathogen infection. Overexpression of this histone demethylase results in an increase of resistance against bacterial blight (Li et al., 2013). An association of SA-dependent defenses with NPR1-dependent post-translational modifications of histone tails in promotors of genes coding for defense-related TFs has also been reported (Jaskiewicz et al., 2011). All this hints to an implication of epigenetic mechanisms right at the onset of priming.

There is not much information available on the actual priming phase that follows such epigenetic changes. Possible mechanisms underlying the actual priming have recently been reviewed (Pastor et al., 2013a). The earlier proposition that the priming process is associated with an accumulation of inactive protein kinases has been substantiated by the fact that treatment with the SAR inducer benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) leads to an accumulation of inactive MAP kinase 3 (MPK3) and MPK6 (Beckers et al., 2009). Induction of resistance by β-aminobutyric acid (BABA) and the concomitant priming of SA-dependent defenses correlates with a higher expression of SA-regulatory transcription factor (TF) genes and priming with rhizobacteria (WCS417r) leads to an enhanced expression of jasmonic acid (JA)-TF regulatory genes (Van der Ent et al., 2009). The mentioned inactive protein kinases could then be rapidly activated upon stress exposure and the availability of the TF allow for a more effective defense signaling. Hence, both mechanisms could possibly contribute to a faster reaction of the plant.

Plants have also been shown to accumulate inactive defense-metabolite conjugates in their vacuoles that could be released upon attack. Well-known examples are the phytoanticipins glycosinolates and benzoxazinoids, both released into an active form upon hydrolysation with glucosidases (Morant et al., 2008). Plant hormone conjugates could also play a role in priming. Methylated and glucosylated abscisic acid (ABA)-conjugates are mainly stored in vacuoles (Kaiser et al., 1985) and free ABA is then released upon contact with apoplastic esterases (Sauter et al., 2002). During infection of tobacco plants, the induced salicylic acid (SA) is partially metabolized into SA 2-O- beta-D-glucose (SAG) by a SA glucosyltransferase (SAGT) (Edwards, 1994; Lee and Raskin, 1998, 1999; Dean and Mills, 2004; Dean et al., 2005; Song, 2006). Such bound SA could be rapidly liberated through the action of a beta-glucosidase (Seo et al., 1995) when the plant is challenged (Dean et al., 2005). Interestingly, an Arabidopsis mutant impaired in SAG and SEG (*ugt74f1*) is more susceptible to infection by *Pseudomonas syringae* pv tomato (*Pst*) than its wild type counterpart (Boachon et al., 2014) and is partially blocked in BABA-IR against *Pst* (Flors and Mauch-Mani, unpublished). In maize plants, it was shown that following resistance induction with the hemibiotrophic fungus *Colletotrichum graminicola*, the plants accumulate an array of metabolites comprising, besides the well-described benzoxazinoids, molecules such as apigenin, genkwanin, and chlorogenic acid with known fungicidal effects. Interestingly, the reaction of maize to priming treatment was shown to be organ-specific (Balmer et al., 2012; Balmer and Mauch-Mani, 2013).

The specific events targeted by priming (the primed mechanisms) depend on the attacker or stress to be countered. Among these early events are the control of stomatal closure in defense against pathogens using stomata as their entry point into the plant (Jakab et al., 2005), the generation of reactive oxygen species (Moller et al., 2007; Trouvelot et al., 2008; Pastor et al., 2013b), interference with effector-triggered susceptibility (Jones and Dangl, 2006) or callose deposition at the attempted points of entry (Garcia-Andrade et al., 2011). At later time points, priming can influence hormonal signaling pathways such SA-, JA, ABA-, and ET signaling (Ton and Mauch-Mani, 2004; Flors et al., 2008; Jung et al., 2009; Van der Ent et al., 2009; Conrath, 2011; Rasmann et al., 2012). While interactions with biotrophic pathogens are generally controlled via the SA pathway, necrotrophic pathogens and insects are rather contained by mechanisms depending on JA/ET signaling (Glazebrook, 2005). The ABA pathway on the other hand plays an important role in protective mechanisms involving callose deposition or stomatal closure (Ton and Mauch-Mani, 2004; Jakab et al., 2005; Hamiduzzaman et al., 2005; Ton et al., 2009).

More sensitive chromatographic techniques make it now possible to identify metabolites that accumulate during the priming phase as well as in the phase following challenge by a stressor (priming vs. primed metabolites). Several secondary metabolites that mediate priming have recently been identified. Among them figure azelaic acid (Jung et al., 2009), imprimatins (Noutoshi et al., 2012), indol-3-carboxilic acid (I3CA; Gamir et al., 2012), pipecolic acid (Návarová et al., 2012) or galacturonic acid and hypoxanthine (Gamir et al., 2014).

The aim of the present study was to establish a metabolomic profiling of the priming phase of *Arabidopsis thaliana* following induction of priming by two different priming cues, i.e., priming by inoculation with avirulent Pseudomonas and chemical priming by soil-drench with BABA. The ultimate goal was to determine whether different priming stimulus can prepare the plant during the priming phase activating common/different metabolic pathways.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

Arabidopsis accession Col-0 seeds were germinated in soil and maintained at 21°C day/18°C night, with 9 h of light (125 μE m^{-2} s^{-1}) and 60% of relative humidity. One week after germination seedlings were individually transferred to 33 mL Jiffy pellets and kept in the same conditions until the treatments. The experiments were performed with 4–5 weeks-old plants. The avirulent strain *Pseudomonas syringae* pv tomato DC3000 (*PstAvrRpt2*) was grown overnight in liquid medium King B with antibiotics rifampicin (50 μg × mL^{-1}) and kanamicin (25 μg × mL^{-1}) for selection.

PLANT TREATMENTS, SAMPLING, AND METABOLITE EXTRACTION

Col-0 plants were soil-drenched with 250 μM of BABA (as a final concentration) or water (as control). For the avirulent strain *PstAvrRpt2*, 2–3 leaves of plants of the same age as for the BABA treatments (4–5 weeks old) were dip-inoculated in the bacterial suspension of 10^9 colony-forming units mL^{-1} in 10 mM of $MgSO_4$ and 0.01% of Silwet L-77 for 4 s, or in the same solution without bacteria (as a control). Samples were taken at 24 and 48 hpt, 3–4 h after the onset of the light phase. The material was lyophilized, extracted in MeOH 10% and further processed as described in Gamir et al. (2012).

To ensure that the plants used for the experiments were primed, for each experiment sample plants were challenged with virulent *Pseudomonas syringae* pv tomato (*Pst*) at a concentration of 10^5 colony-forming units (cfu). mL^{-1} as described previously (Slaughter et al., 2012). The disease phenotype was assessed 5 days after challenge and expressed as number of symptomatic leaves.

LC-ESI FULL SCAN MASS SPECTROMETRY (Q-TOF INSTRUMENT)

Metabolome analysis was performed using an Acquity UPLC system (Waters, Mildford, MA, USA) interfaced to hybrid quadrupole time-of-flight (QTOF MS Premier). The LC separation was performed by HPLC SunFire C18 analytical column, 5 μm particle size, 2.1 × 100 mm (Waters). Analytes were eluted with a gradient of methanol and water containing 0.01% HCOOH. Chromatographic conditions and QTOF MS parameters were followed as described in Gamir et al. (2012).

FULL SCAN DATA ANALYSIS

Raw data obtained from Masslynx software was transformed to .CDF using Databrigde provided by Masslynx package. The .CDF data was process with R for statistical computing using XCMS package for relative quantification (Smith et al., 2006). For Principal Component Analysis (PCA), heatmap construction and clustering of metabolite the software MarVis Filter and MarVis cluster (http://marvis.gobics.de/; Kaever et al., 2012) were used. The most prominently induced compounds and others shown in text were subjected to identification (**Table 1**) and the relative intensity of accumulation was analyzed using a paired non-parametric Wilcoxon Mann–Whitney test to confirm a significant difference between the samples ($P < 0.05$).

Table 1 | List of compounds identified by exact mass and fragmentation spectrum.

Compound	Mass (neutral)	Fragments	ESI	RT (min)
Citrate/isocitrate	192.027	111.010, 87.011	–	2.81
Fumarate	116.013	71.0148	–	3.06
S-Malate	134.022	115.005, 89.027	–	1.81
Succinate	118.029	73.032	–	1.43
2-Oxoglutarate	146.023	101.026, 73.016	–	4.30
9(S)-HPOT/2(R)-HPOT	310.214	309.106, 291.185	–	12.90
13(S)-HOT	294.184	236.106, 221.157	–	12.40
OPC-8:0/9(S)-HOT	294.217	275.202, 96.962	–	13.18
13 (S)-HODE	296.234	71.018	–	13.37
Sinapate	224.069	208.03, 93.037	–	8.50
Sinapoyl malate	340.0806	223.063, 133.015	–	9.41
1-O-Sinapoyl-β-D-glucose	386.122	223.065, 190.026	–	3.48
Xanthosine	284.077	151.029	–	1.81
Guanosine	283.093	133.016	–	1.52

9(S)-HPOT, (10E,12Z,15Z)-(9S)-9-Hydroperoxyoctadeca-10,12,15-trienoic acid; 2(R)-HPOT, (2R)-(9Z,12Z,15Z)-2-Hydroperoxyoctadecatri-9,12,15-enoic acid; 13 (S)-HOT, (9Z,11E,15Z)-(13S)-Hydroxyoctadeca-9,11,15-trienoate; OPC-8:0, 8-[(1R,2R)-3-Oxo-2-[(Z)-pent-2-enyl]cyclopentyl]octanoate; 9(S)-HOT, (9S)-(10E,12Z,15Z)-9-Hydroxyoctadecatri-10,12,15-enoic acid; 13 (S)-HODE, (13S)-Hydroxyoctadecadienoic acid.

RESULTS

DIFFERENT PRIMING STIMULI IMPOSE SUBTLE METABOLIC CHANGES IN THE ABSENCE OF CHALLENGE

The primed state can be induced by various agents. In order to differentiate between chemical and biological induction, BABA was used as a chemical inducer (Zimmerli et al., 2000; Jakab et al., 2001; Ton and Mauch-Mani, 2004) and the avirulent bacterium *Pseudomonas syringae* carrying the avirulence gene *AvrRpt2* (*PstAvrRpt2*; Mudgett and Staskawicz, 1999) as a biological inducer of resistance. Since metabolites are the final products resulting from metabolic pathways, the present work aims to identify, the main compounds that could act as signals during the priming phase and the involved pathways. It is presumed that at first contact with BABA or *PstAvrRpt2* a plant response is initiated that will allow it to get ready to combat further stresses. To ensure that the plants used in our experiments were primed, for each experiment sample plants were challenged and the resulting disease phenotype was assessed (**Figure 1**).

To this end *Arabidopsis* Col-0 plants were treated with BABA and *PstAvrRpt2* with water- and mock-treated plants as control, respectively. To gain a global overview of the metabolite balance during the priming phase, i.e., the 48-h time interval before the stress is usually applied (Pastor et al., 2013a and the references within), a metabolic analysis with UPLC coupled to Q-TOF mass spectrometry (quadrupole-time of flight mass spectrometer) was performed. Metabolic and bioinformatic analysis of the signals obtained in positive and negative electrospray ionization (ESI) were done according to the methods of Fernie et al. (2011), Kaever et al. (2012), and Gamir et al. (2014). When comparing the four groups (**Figure 2**; water, BABA, mock, and *PstAvrRpt2*) the principal component analysis (PCA) shows the global behavior of the signals in both modes of ESI at 24 hpt and 48 hpt. BABA and *PstAvrRpt2* both induce qualitative different metabolites, easily visualized in the cluster realized by the Marvis Cluster software. This might point to at least partially different mechanisms of priming action for BABA and *PstAvrRpt2*.

Signals found in BABA-treated plants do not overlap but remain close to control plants. This confirms the previous view that priming does not lead to major changes in a plant (Conrath, 2009). The fact that in positive mode the signals are closer than in negative mode means that in general, the compounds that are negatively ionized could be more important in the establishment of priming, since they present a higher difference in respect to

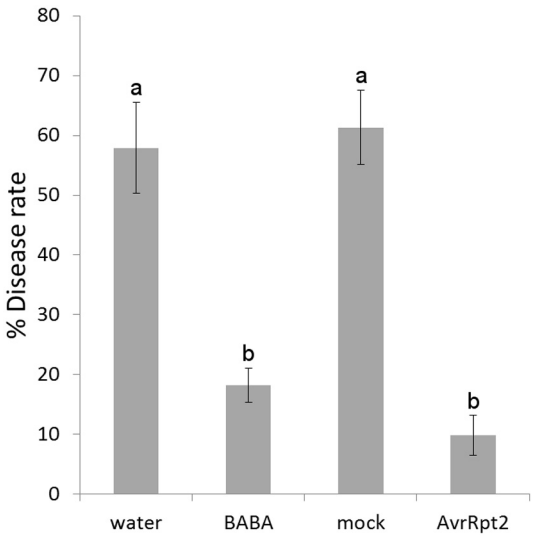

FIGURE 1 | Disease symptoms in Col-0 plants. Arabidopsis plants were inoculated with a bacterial suspension of *Pst* at 10^5 colony-forming units (cfu). mL^{-1}. Disease symptoms were determined after 5 days of inoculation and quantified as the proportion of leaves with symptoms. The values are means of the percentage of diseased leaves per plant ± SD. Data presented are from a representative experiment that was repeated for every experiment with similar results. Different letters indicate statistically significant differences between control and infected plants ($p < 0.05$, $n = 20$–25).

the control. On the contrary, inoculation with avirulent bacteria shows that the action of bacteria is slower than BABA. Separation between bacteria and mock treatment becomes visible only after 48 hpt.

The differences between treatments was visualized through a supervised heatmap analysis using the signals that showed a significant difference (Kruskal–Wallis test $P < 0.05$). At both 24 and 48 hpt, there is more dynamism in the group of compounds stemming from BABA-treated plants than from *PstAvrRpt2*-inoculated plants. However, when clustering water vs. BABA and mock vs *PstAvrRpt2* (**Figures S1**, **S2**) the differences between treated and not treated plants are more visible. All together BABA-treatment leads to a more rapid induction of the priming phase than inoculation with bacteria does.

CHEMICAL AND BIOLOGICAL PRIMING TARGETS PRIMARY METABOLISM DURING PRIMING PHASE: THE ROLE OF CARBOXYLIC ACIDS

Priming is a horizontal phenomenon potentiating basal defenses and further layers of defense in case basal defense is defective (Pastor et al., 2013b). The plant is put in a state of alert allowing a wide elasticity in the use of defenses. Therefore, priming metabolites might be very primary compounds that can be channeled to any direction in order to rapidly strengthen the defensive state of the plant. To determine the most relevant compounds in priming, the most strongly induced signals in the clusters were identified by exact mass, fragmentation spectrum and the retention time of the fragments using the Metlin (http://metlin.scripts.edu) and MassBank (http://www.massbank.jp) databases, and, if necessary, the Kegg and Aracyc databases (http://www.genome.jp/kegg/; http://pathway.gramene.org/gramene/aracyc.shtml). Interestingly, the most induced compounds by BABA belong to the tricarboxylic acid cycle (TCA). These compounds accumulated to a lesser degree following *PstAvrRpt2* infection (**Figure 3**). Citrate/isocitrate production was strongly induced by BABA at 24 and 48 hpt and, to a lesser extent, also a (S)-Malate, 2-Oxoglutarate and fumarate. Interestingly, *PstAvrRpt2* repressed the accumulation of all these compounds. Nevertheless, succinate was less present in plants treated by both types of priming. Hence, BABA-induced priming functions through the potentiation of the TCA cycle, while *PstAvrRpt2*-induced priming acts differently. All this suggests that the primary metabolism plays an important role in priming for defenses, and is a key point in defining the mechanisms operating in compatible interactions.

CHEMICAL AND BIOLOGICAL PRIMING TARGETS PRIMARY METABOLISM DURING PRIMING PHASE: CHANGES IN AMINO ACIDS

The compounds involved in TCA flux are the origin for different amino acids pathways. Defense responses against pathogens and insects induce antimicrobial and signaling compounds such as glucosinolates and their breakdown products (Barth and Jander, 2006; Bednarek et al., 2009), or JA-Ile (Fonseca et al., 2009). These secondary metabolites are derivatives or conjugates of amino acids. The balance of amino acids has a major impact on plant defense and priming (Liu et al., 2010; Singh et al., 2010; Návarová et al., 2012; Zeier, 2013; Gamir et al., 2014). In order to know whether these metabolites play a role during the priming phase after BABA and *PstAvrRpt2* treatment, the amino acids were identified using a library of amino acids set up with standards (Gamir et al., 2014).

After 24 hpt (**Figure 4**) BABA-treated plants accumulated Cys, Met, Glu, Ile+Leu (no distinguishable in our chromatographic conditions), His, Thr, Tyr, and Phe. At the same time, the plants primed by avirulent bacteria produced more Cys, Met, and Trp. At the later time point (48 hpt; **Figure 5**), the levels of Cys, Met, Glu, and Ile+Leu remained high in BABA-treated plants, Thr and Tyr (these last two in lower amounts), but His and Phe were repressed. Other amino acids like Ala, Ser, Asn, and Lys accumulated at this later time point following BABA-priming. On the other hand, plants incoculated with *PstAvrRpt2* kept accumulating Cys, Met, and Trp (this one in higher levels that at 24 hpt), but showed a reduced accumulation of Arg, Pro, and Tyr however were induced at this time point.

Asp, Gln, and Val are less abundant in all time points, independently of the treatment, while Cys, Met, and later, Tyr, were always accumulating under priming conditions. In general, at the beginning, there was depletion in amino acid accumulation during the priming phase, and this effect was more pronounced at 24 hpt.

Thus, the priming state leads to a drop in amino acids during the first hours, and Cys, Met, and Tyr are "priming amino acids" preparing the plant for primed defenses.

PRIMING OF SECONDARY METABOLITES

Since the primary metabolism seems to play a central role in the establishment of priming, the contribution of the secondary

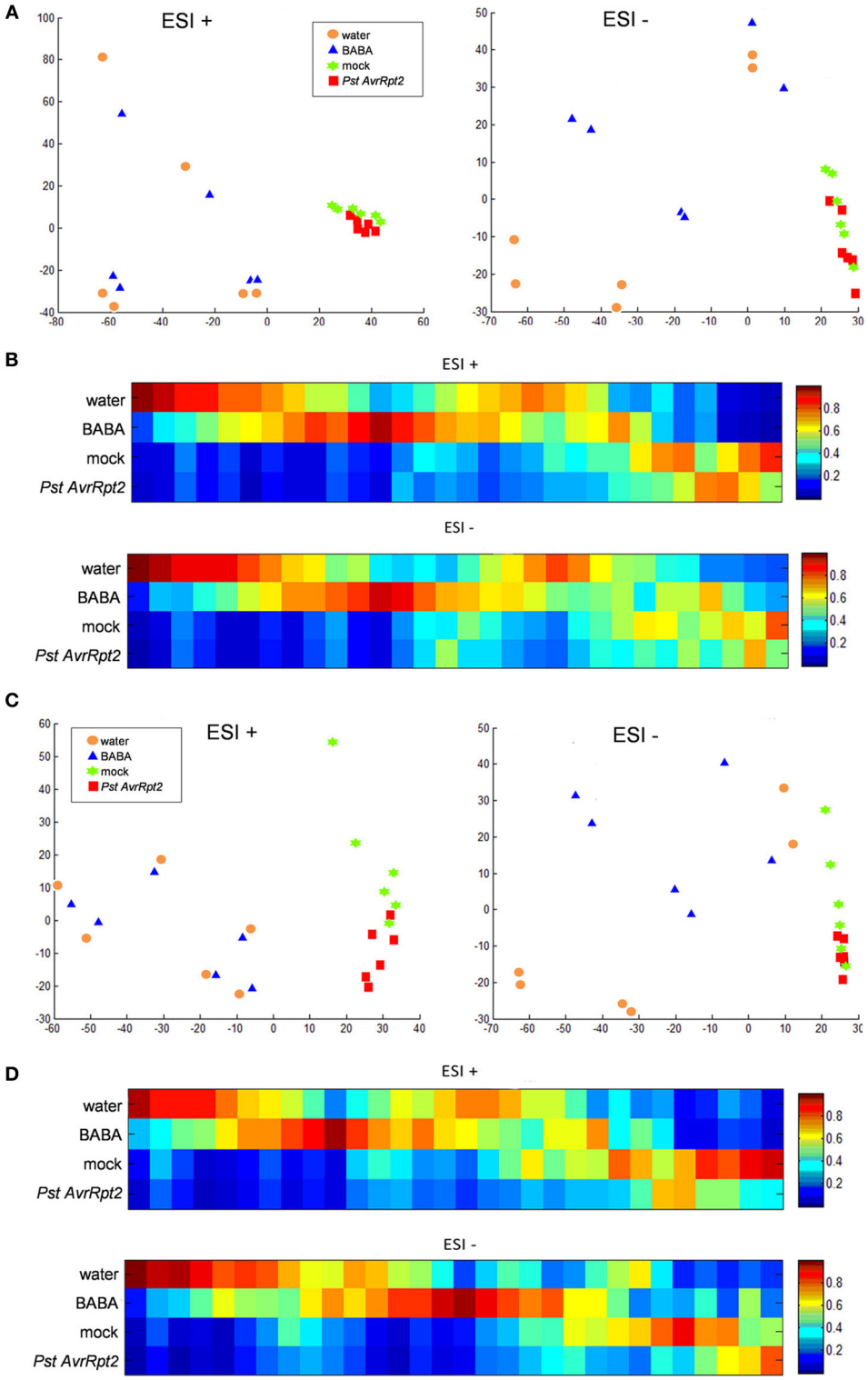

FIGURE 2 | Principal component analysis (PCA) and cluster plots comparing the four groups generated from the major sources of variable signals obtained in ESI+ and ESI− by non-targeted analysis by HPLC-QTOF MS of the four groups water, BABA, mock, and *PstAvrRpt2*. The PCA and cluster analyses were performed using Marvis Filter and Cluster packages, following a Kruskal–Wallys test ($p < 0.05$). **(A)** PCA analysis score plot after 24 hpt. **(B)** Cluster plot of main compounds of the four groups after 24 hpt. **(C)** PCA analysis score plot at 48 hpt. **(D)** Cluster plot of significant compounds after 48 hpt.

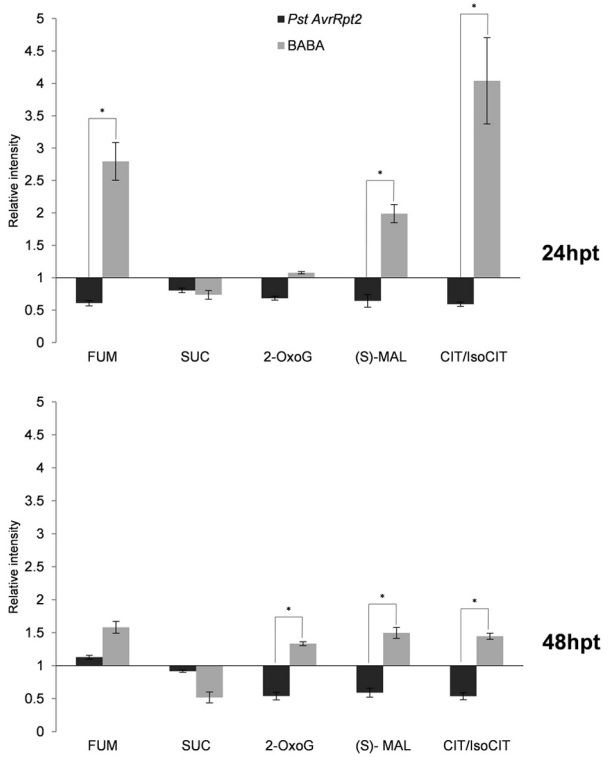

FIGURE 3 | Profile of compounds related to the tricarboxylic acid cycle. Four five-week-old Col-0 plants were processed for relative quantification after 24 and 48 hpt. The concentration of metabolites was obtained by normalizing the chromatographic area of each compound to the dry weight of the sample. The bars show the relative accumulation (relative intensity) of each compound in respect to its control, BABA vs. water, *PstAvrRpt2* vs. mock. Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent the average of three independent experiments.

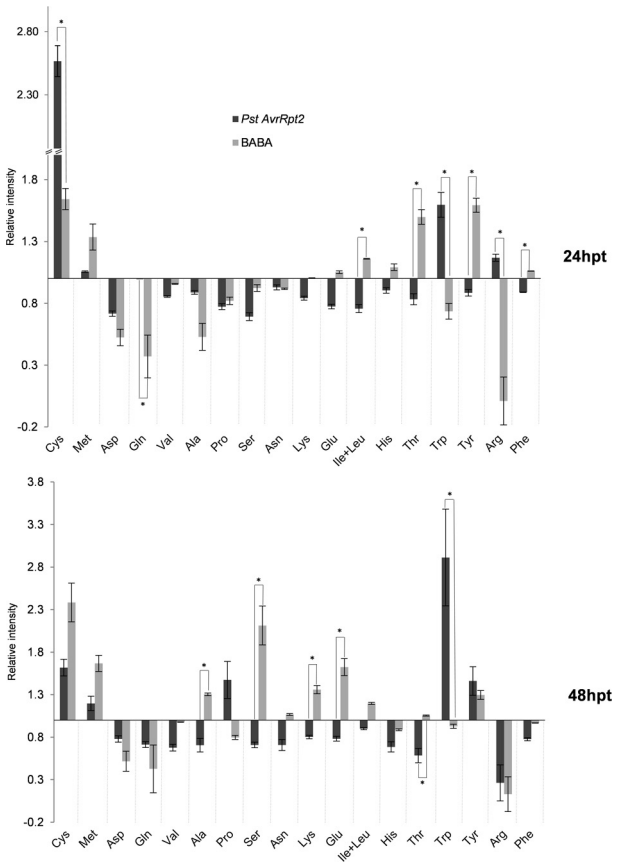

FIGURE 4 | Amino acid profile of plants primed with BABA and *PstAvrRpt2*, respectively. The abundance of each amino acid was calculated as described for **Figure 2**. Col-0 plants, 4–5 week-old, were harvested at 24 and 48 hpt. Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent average of three independent experiments.

metabolism was also investigated. Using the library of metabolites (see above), some hormones (**Figure 5**) and indolic compounds (**Figure 6**) participating in priming were identified.

Free salicylic acid (SA) and glucosides of SA (SAG+SGE) accumulated in both time points and the induction was higher by BABA than avirulent bacteria. Indole-3-acetic acid (IAA) showed higher levels at 24 hpt but decreased afterwards and was repressed by BABA. Additional indolic compounds have been identified, among them indole-3-carboxaldehyde (I3CHO), indole-3-acetamide (IAM), indol-3-pyruvic acid (IPA), indole-3-acetyl-L-Ala (IALA), and indole-3-carboxylic acid methyl ester (I3CAME). At 24 hpt all of them showed elevated levels in comparison to the controls. Interestingly, IALA was significantly more present in *PstAvrRpt2*- treated than in BABA-treated plants. After 48 hpt, the situation was different, since in BABA-treated plants the levels of IALA remained elevated but in *PstAvrRpt2*-inoculated plants the accumulation of this conjugate was repressed. In general, *PstAvrRpt2* treatment represses the accumulation of most these indolic compounds after 48 hpt. When this is not the case, then BABA-treatment leads to a repression of these compounds.

JA levels were repressed at 24 hpt confirming the well-known crosstalk already between SA and JA (Koornneef and Pieterse, 2008). At 48 hpt JA starts to accumulate in BABA-treated plants. OPDA was consistently accumulating over time in BABA-treated plants and depleted by *PstAvrRpt2* treatment in both time points.

Interestingly, Val conjugated to JA (JA-Val) showed a strong accumulation upon BABA treatment and the opposite effect resulted from *PstAvrRpt2* treatment at 24 hpt. Using exact mass, fragment spectrum and retention time of fragments, some components of the α-linolenic and linoleic pathways (octadecanoid pathway) were detected, although in our experimental conditions it was not possible to differentiate between some lipid derivatives due to the similarity between fragments (**Figure 7**). These compounds are described in **Table 1**. All of them are precursors of OPDA and JA. The bacteria repressed the α-linolenic pathway as well as some compound from the linoleic pathway, such as 13 (S)-HODE. After BABA treatment, accumulation was only seen after 48 h except for 9-(S) HPOT/2(R)-HPOT, which accumulated to a lesser extent.

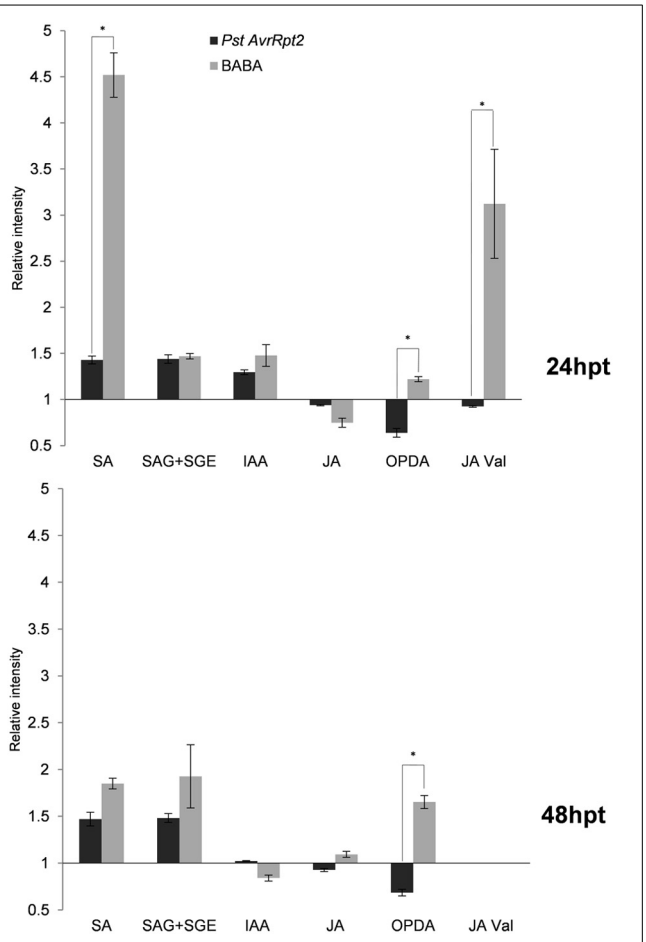

FIGURE 5 | Levels of hormones and conjugates after 24 and 48 hpt. The abundance of every compound was calculated as in **Figure 2**. Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent average of three independent experiments.

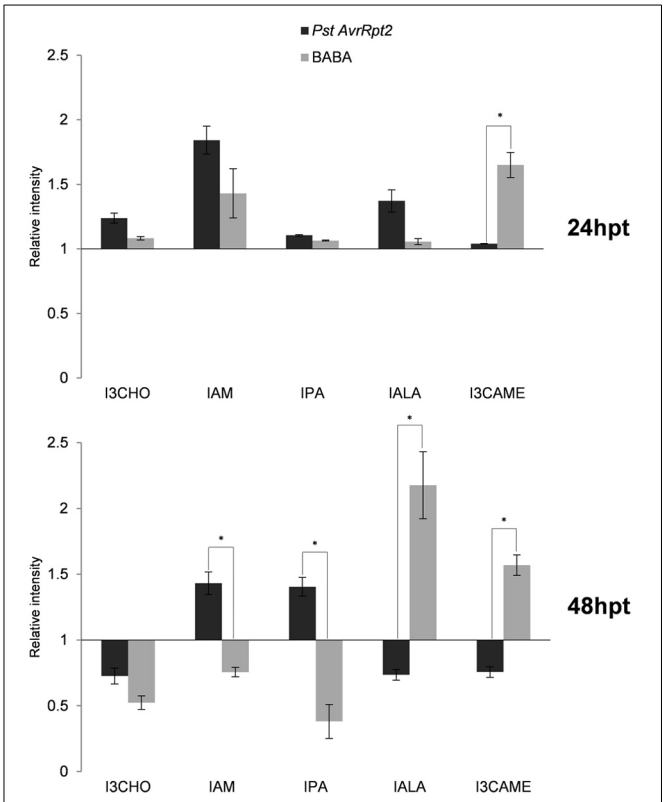

FIGURE 6 | Profile of indolic compounds during the priming phase. The experimental conditions and quantification of the levels were calculated as indicated in **Figure 2**. Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent average of three independent experiments.

Caffeic, cinnamic and ferulic acid were identified using the library of standards (Gamir et al., 2014). At 24 hpt only ferulic acid showed an increase and only upon BABA treatment. At 48 hpt caffeic acid accumulated in BABA-treated plants and the levels of ferulic acid remained as at 24 hpt. The bacteria induced the accumulation of cinnamic acid within 48 hpt. All these compounds are related to phenylpropanoid biosynthesis pathway (**Figure 8**). Additional compounds belonging to this pathway have been identified by exact mass like sinapic acid, 1-O-sinapoyl-β-D-glucose and sinapoyl malate. As shown in **Figure 8**, BABA strongly stimulated the production of sinapic acid and its ester, sinapoyl malate. The glycosylated intermediate compound (1-O-sinapoyl-β-D-glucose) accumulated to a lesser extent following the treatment suggesting that the glycosylated form is feeding into the production of the ester (Milkowski and Strack, 2010).

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Interestingly, an additional compound, xanthosine, was induced by both types of priming. This nucleoside was strongly induced, almost to the same levels as citrate and the levels remained high during the entire priming phase following inoculation with the bacteria (**Figure 8**). The accumulation of xanthosine was inversely correlated to the one of guanosine. Both compounds play role in purine catabolism, where the amino group of guanosine is released by guanosine deaminase to produce xanthosine (Dahncke and Witte, 2013).

DISCUSSION

It is crucial for the survival plants to sense changes taking place in their natural environment. Their cells have to adapt the composition and the levels of metabolites to these changes and if the natural balance of metabolites is disturbed, the plant can answer with massive changes at the transcriptional level to recover the equilibrium (Katagiri, 2004; Kresnowati et al., 2006). An adaptation to such environmental changes is facilitated when the plant has been primed to do so. Priming has been described as a sensitized state in which plants can react more adequately to combat stresses. In a primed plant, the speed and the strength of the answer to a given stress is improved, hence, the primed plant survives better and with less damage. The mechanisms underlying priming are still under discussion. The strategies used by primed plants comprise both, relative early as well as long lasting responses (Pastor et al., 2013a). The latter ones have even been shown to be transferred to the offspring (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012). However, what is

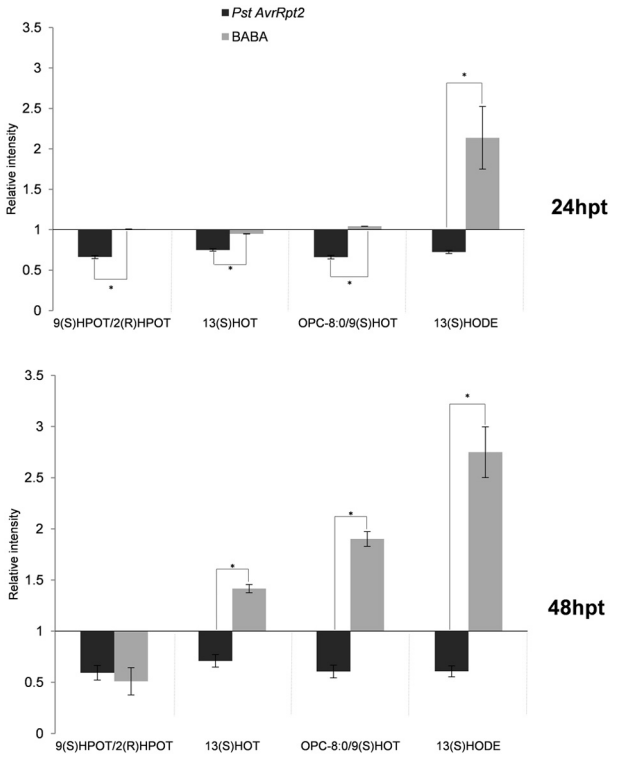

FIGURE 7 | Fatty acid production in priming. The experimental conditions and quantification were performed as explained above (**Figure 2**). Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent average of three independent experiments.

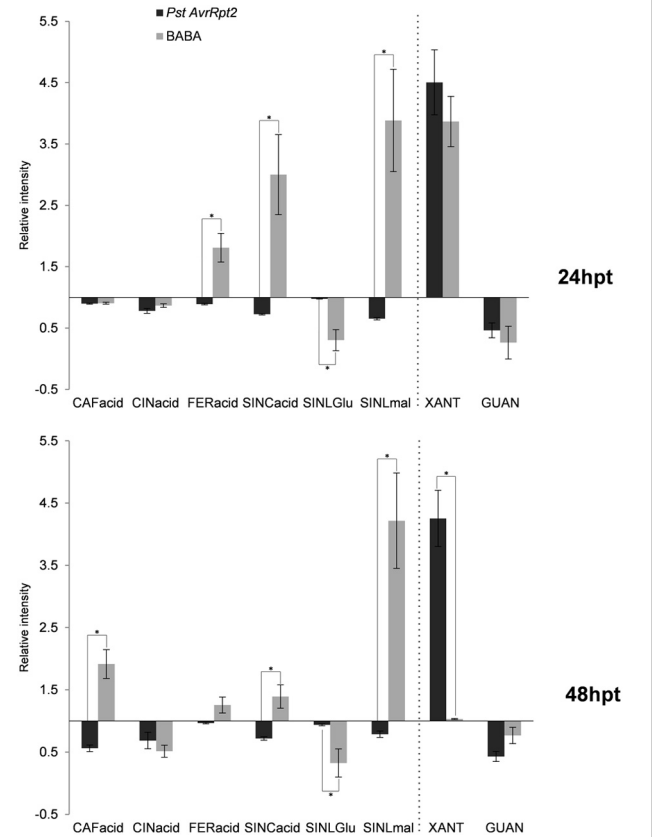

FIGURE 8 | Phenylpropanoid biosynthesis and purine catabolism compounds in priming. The experimental conditions and quantification were performed as explained above (**Figure 2**). Asterisks represent statistically significant differences (Wilcoxon Mann–Whitney test, $p < 0.05$, $n = 6$). Data represent average of three independent experiments.

happening in a plant after the perception of a priming cue but prior to encountering the stress remains to be determined. The aim of the present work was to acquire an overview of the metabolites that act as priming compounds and the pathways implicated in the priming phase following treatment of the plants with BABA as a chemical and *PstAvrRpt2* as a biological priming agent. Given the nature of priming, i.e., the rapid reaction against any type of stress (biotic and abiotic), the compounds accumulating before challenge with an actual stressor are expected to belong to the very early phase in the chain of events leading to the biochemical synthesis of secondary metabolites.

The comparison of the effect of the priming treatments (BABA and *PstAvrRpt2*) and their respective control treatments (water and mock,) on the plants' metabolome revealed a different dynamism when comparing BABA- and *PstAvrRpt2*-primed plants. BABA, as an easily water-soluble chemical, is taken up and distributed rapidly in the plant and might therefore lead to faster changes in the metabolome. On the opposite, the bacteria use quorum sensing to organize and coordinate the interaction between themselves and with their host. *PstAvrRpt2* might need more time to induce changes in the plant since the bacteria have first to multiply in order to colonize the tissues (Miller and Bassler, 2001; Schikora et al., 2011).

The metabolites undergoing the most pronounced changes in BABA-treated plants belong to the tricarboxylic acid (TCA) cycle. Citrate/isocitrate (indistinguishable in our chromatographic conditions), show the highest accumulation followed by other compounds of the cycle. Only the levels of succinate are reduced by BABA. On the opposite, *PstAvrRpt2*-treatment leads to a general repression of the TCA cycle. The primary metabolism appears like a key point in priming and highlights the differences between the two kinds of priming inducers.

It is known that both compatible and incompatible interactions lead to a reduction of photosynthesis (Swarbrick et al., 2006; Berger et al., 2007 and references therein) due to the chlorosis provoked by the infection. The reduction of photosynthesis during the resistance responses is accompanied by an increase of carbohydrates in the apoplast and could act as a signal for repression of photosynthesis. The process needs an income of carbon-skeletons that in turn leads to a reduction in the levels of compounds belonging to the TCA cycle (Chou et al., 2000). On the other hand, BABA seems to help in the accumulation of carboxylic acids, perhaps because the plant can perceive the decrease with no apparent reason (no infection) of amino acids following BABA treatment. This could stimulate the production of carboxylic acids (Bolton, 2009) that in turn are not required for other purposes and can be accumulated in order to fuel other

pathways (Bolton, 2009). It still needs to be investigated whether the accumulation triggered by BABA is a consequence of a minor stress caused by the chemical or if it is a more direct action due to its amino acid nature. The response to stress is accompanied by a wide mobilization of the defense system, nevertheless, not all defense mechanism are used by the plant (Galletti et al., 2008; Rayapuram et al., 2008). In any case, plant response to stress is highly energy-consuming and depends on carbon supply to sustain the biosynthesis of defense compounds. The TCA cycle not only provides such carbon structures for the biosynthesis but also participates in ATP generation. The accumulation of these compounds can help to provide the energy or the carbon skeletons for future demands. The major C sink is to Glu and Gln from citrate and 2-oxoglutarate (Gauthier et al., 2010), but here we found that after BABA-application, only Glu was accumulating over the time of the experiment and this coincided with the induction of 2-oxoglutarate. On the contrary, Gln levels remained low following both types of priming. A possible explanation could be the simultaneous accumulation of the nucleoside xanthosine, a product of the catabolism of purines. The N structure of the purine ring comes from the Glu, Asp and Gly (Stasolla et al., 2003), and the levels of all three amino acids go markedly down following BABA- and *PstAvrRpt2*-treatment. The accumulation of xanthosine coincides with the depletion in guanosine. In Arabidopsis, xanthosine is generated from the deamination of guanosine with the help of a specific guanosine deaminase (Dahncke and Witte, 2013). Xanthosine is a precursor of caffeine in some plant species but has not been reported in Arabidopsis (Mohanpuria and Yadav, 2009), and to our knowledge, there is not any direct evidence for a role of this compound in defense. The catabolism of purines ends with the production of CO_2 and nitrogen. But purines also participate in building other molecules such as DNA, RNA or other secondary metabolites, and significant amounts of xanthosine are used for RNA synthesis (Ashihara, 2012). This again suggests that the metabolism of primed plants is getting ready for a future need for defense compounds or help in the synthesis of nucleic acids (Riegler et al., 2011).

Secondary metabolism is important for the final output of the defense response, which can produce long distance signals or, even, biocidal substances inhibiting the action of pathogens and insects. During the priming phase, the accumulation of SA and its glycosides, as well as a repression in the accumulation of JA is taking place. Interestingly, there is a peak in SA accumulation at 24 hpt in BABA-treated plants, and this accumulation is significantly higher than the one induced by *PstAvrRpt2*. After this time point SA remains at the same level in both types of priming. The levels of SA-glycosides do not change during priming and are potentially ready to release free SA upon encounter with a stress. The accumulation of free and conjugated forms of SA after infection with the pathogen *Pseudomonas syringae* pv tomato DC3000 (*Pst*; Pastor et al., 2012) has been reported, and this accumulation is usually accompanied by the repression of genes in the JA pathway and related genes (Koornneef and Pieterse, 2008; Boachon et al., 2014). The complexity of SA synthesis and signaling makes it difficult to get an exact picture of all the possible effects it might have in the plant (Shah, 2003). On the other hand, although JA levels display a similar profile in BABA- and *PstAvrRpt2*-treated plants, the cyclopentenone ODA shows specific behavior depending of the treatment applied. Despite overlapping gene-induction patterns for JA and OPDA (Stinzi et al., 2001), a distinct role for OPDA in signaling can be expected due to the electrophilic nature of the cyclopentenone

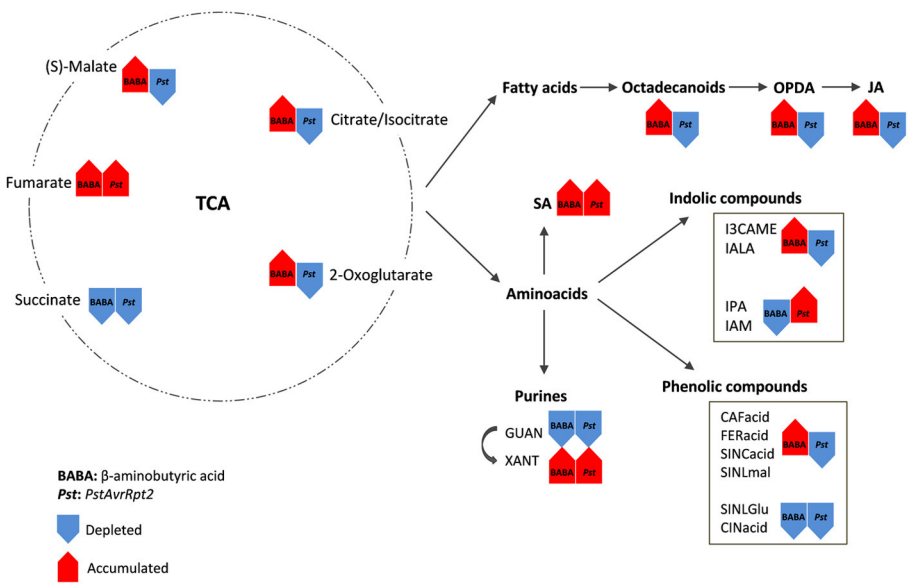

FIGURE 9 | Principal pathways/compounds that show significant changes after the two priming inducers. In priming experiments the stress is usually applied after 48 hpt. Rows represent the general changes after this period of time. Red shows the accumulated compounds and blue rows show the depleted compounds/pathways. TCA compounds are the main source for the synthesis of other metabolites playing a major role in defense. These compounds belong to the octadecanoid pathway (fatty acids) and amino acids. Both pathways are divided in subsequent pathways and compounds that are important in defense signaling and decide the final output when the plant meet the stress.

ring (Farmer et al., 2003). In our experimental conditions, treatment with *PstAvrRpt2* altered the oxylipin pathway through the repression of several precursors of OPDA and JA, while these compounds accumulated at 48 hpt with BABA. The repression of the octadecanoid acid pathway by the *PstAvrRpt2* correlates with the observed depletion of tricarboxylic acids and the accumulation of tricarboxylic compounds by BABA correlates well with the induction of the same pathway. This points to a connection between these two pathways. Interestingly, there is a significant increase of the JA conjugate JA-Val. The (+)-JA-Ile conjugate has been shown to be the active isomer in the signaling pathway, but there is little information about the role of other JA-amino acid conjugates. JA-Ile and JA-Val accumulate in response to oral secretions of *Manduca sexta* mediated by *JAR6*, a homolog of *JAR1,* in *Nicotiana attenuata*,(Wang et al., 2007). In our experimental conditions, an accumulation of JA-Val was only observed at 24 hpt, and JA-Ile was not detected. It is conceivable that the conjugation with amino acids leads to an increase of the bioactivity of JA, but further studies are needed to get a clear picture about the role of amino acid conjugates in general defense responses.

The influence of the TCA cycle on the biosynthesis of other biomolecules also linked to the phenylpropanoid biosynthesis. BABA treatment led to an accumulation of sinapate and its ester, sinapoyl malate, while *PstAvrRpt2* treatment caused depletion in these two phenylpropanoids. Thus, BABA has a major impact on this pathway that is supplying precursers of lignin (Goujon et al., 2003). BABA has been shown to play a major role in the modification of the plant cell walls after pathogen attack (Ton and Mauch-Mani, 2004), and it is tempting to hypothesize that phenolics and sinapates act as priming compounds facilitating the synthesis of molecules needed for the reinforcement of the cell wall. A similar mechanism could be imagined for IAA in priming the accumulation of indolic compounds, although both BABA and the bacteria lead to similar accumulation of IAA and the profile of the indolic compounds identified is more variable. Tryptophan (Trp) metabolism leads to the synthesis of glucosinolates, phytoalexins and auxins, and their regulation is dependent on a branched network that makes it difficult to obtain a clear profile. The induction of the Trp pathway by the bacteria is followed by the accumulation of two compounds that participate in IAA synthesis and depend on the Trp pathway, namely indole-3-pyruvic acid (IPA) and indole-3-acetamide (IAM; Strader and Bartel, 2008; Sugawara et al., 2009). Here again, the plants respond differentially to the treatments. While the accumulation of IAA via the Trp pathway seems to be more relevant following priming by *PstAvrRpt2*, BABA seems to use a Trp-independent pathway (Cohen et al., 2003) since it leads to a depletion of the levels of Trp. Alternatively, it might consume more Trp to produce IAA as well as others conjugates (IALA) or derivatives of indol-3-carboxylates (I3CAME).

The priming state can be installed by certain compounds that supply the material a plant might need when it encounters a stress. Chemical and biological priming each have a certain specificity that prepares the plant in a different manner (**Figure 9**), but the final result is the same: the survival of the plant. In nature a plant has to decide rapidly which is the best way to respond to unexpected stress. Priming plants for defense gives them the tools to do so by taking the right decision.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00295/abstract

Figure S1 | Principal component analysis (PCA) and cluster plots comparing water and BABA groups generated from the major sources of variable signals obtained in ESI+ and ESI− by non-targeted analysis by HPLC-QTOF MS of the four groups water, BABA, mock, and *PstAvrRpt2*. The PCA and cluster analyses were performed using Marvis Filter and Cluster packages, following a Kruskal–Wallys test ($p < 0.05$). **(A)** PCA analysis score plot after 24 hpt. **(B)** Cluster plot of main compounds of the four groups after 24 hpt. **(C)** PCA analysis score plot at 48 hpt. **(D)** Cluster plot of significant compounds after 48 hpt.

Figure S2 | Principal component analysis (PCA) and cluster plots comparing the mock and *PstAvrRpt2* groups generated from the major sources of variable signals obtained in ESI+ and ESI− by non-targeted analysis by HPLC-QTOF MS of the four groups water, BABA, mock, and *PstAvrRpt2*. The PCA and cluster analyses were performed using Marvis Filter and Cluster packages, following a Kruskal–Wallys test ($p < 0.05$). **(A)** PCA analysis score plot after 24 hpt. **(B)** Cluster plot of main compounds of the four groups after 24 hpt. **(C)** PCA analysis score plot at 48 hpt. **(D)** Cluster plot of significant compounds after 48 hpt.

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The discovery of the BABA receptor: scientific implications and application potential

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A commentary on

Plant perception of β-aminobutyric acid is mediated by an aspartyl-tRNA synthetase

by Luna, E., van Hulten, M., Zhang, Y., Berkowitz, O., López, A., Pétriacq, P., et al. (2014). Nat. Chem. Biol. 10, 450–456. doi: 10.1038/nchembio.1520

A significant proportion of global crop production is annually lost to pests and diseases (Savary et al., 2012). While pesticides help to reduce these losses, there is growing concern about pesticide resistance and their impacts on health and environment. Integrated Pest Management (IPM) aims to reduce pesticide usage through a combination of different strategies, including resistant crop cultivars, monitoring disease status, and mechanical and biological control (Birch et al., 2011; Chandler et al., 2011). A potentially novel IPM tool is plant priming agents: stimuli that sensitize the plant's immune system for augmented activation against future pathogen/herbivore attacks. Because priming leads to augmented activation of multi-genic defense mechanisms (Ton et al., 2006; Ahmad et al., 2010), the resulting resistance has the potential to be more durable than protection by single resistance (R-) genes. Despite this advantage, priming agents are not widely used in agriculture, partly because they often do not provide the same level of protection as conventional pesticides and R-genes (Walters et al., 2013). However, the advancement of IPM has spurred increased interest in exploiting priming agents in sustainable crop protection schemes.

Arguably the most effective priming agent is β-aminobutyric acid (BABA). This non-protein amino acid primes defense reactions that are controlled by salicylic acid (SA)-dependent and -independent signaling pathways (Zimmerli et al., 2000; Ton et al., 2005), conferring protection in different plant species against an exceptionally broad spectrum of stresses, including microbial pathogens, herbivores, and abiotic stresses (Jakab et al., 2001; Cohen, 2002). Unfortunately, BABA also has an undesirable side effect: it reduces plant growth (Wu et al., 2010). While this growth penalty is outweighed by its protective effects in environments with high disease pressure, it can be quite severe at higher doses under disease-free conditions (Van Hulten et al., 2006).

Until now, understanding of the molecular mechanisms underpinning the trade-off between BABA-IR and BABA-induced growth repression was limited by insufficient knowledge of how this chemical is perceived in plants. A recent study, however, has provided new insight in this matter (Luna et al., 2014). A screen for Arabidopsis mutants in BABA-IR against the biotrophic oomycete *Hyaloperonospora arabidopsidis* led to the identification of the *Impaired in BABA-induced Immunity 1* (*IBI1*) gene, encoding an aspartyl-tRNA synthetase (AspRS). Unlike previously identified genes controlling either SA-dependent, or SA-independent BABA-IR (Ton et al., 2005), the *ibi1-1* mutation was found to block both priming responses to BABA, indicating unilateral control of BABA-induced resistance by IBI1.

The stereochemical similarity between the amino acid substrate of IBI1 (L-aspartate; L-asp) and the active enantiomer of BABA (R-BABA) suggested that IBI1 might function as the BABA receptor. This hypothesis was supported by several indirect lines of evidence; apart from loss of BABA-IR by independent mutations in *IBI1*, computational models of BABA-binding to AspRS enzymes indicated high-affinity binding of R-BABA in a similar molecular orientation as L-asp, while treatment with active R-BABA caused cellular L-asp accumulation. Further evidence for receptor functionality by IBI1 came from the *in planta* demonstration that R-BABA physically binds to IBI1. It was concluded that R-BABA binds the L-asp-binding domain of IBI1, thereby disrupting canonical AspRS activity and priming the protein for non-canonical defense activity. This model also predicts that R-BABA increases uncharged $tRNA^{asp}$ accumulation. Across eukaryotes, uncharged tRNA serves as a conserved signal for metabolic imbalance by activating the protein kinase GCN2 (Dong et al., 2000; Dever and Hinnebusch, 2005), which in turn inhibits translational activity via phosphorylation of eukaryotic translation initiation factor eIF2α (Wek et al., 2006; Li et al., 2013). Evidence that this stress pathway is activated by BABA came from the demonstration that stress-inducing concentrations of BABA activate GCN2-dependent eIF2α phosphorylation. Moreover, the *gcn2-1* mutant of Arabidopsis was strongly reduced in BABA-induced growth inhibition, while it remained unaffected in BABA-induced

resistance. This latter finding not only confirmed the critical role of GCN2 in BABA-induced stress, it also demonstrated that BABA-IR and BABA-induced stress are controlled by separate pathways.

An important question from the work by Luna et al. (2014) is why plants have evolved a specific receptor protein to a xenobiotic chemical? Does BABA mimic a natural ligand, or does it induce a physiological state that is indicative of pathogen attack? An important clue came from the finding that *IBI1* transcription is increased following pathogen attack, while transgenic overexpression of *IBI1* enhances basal disease resistance through priming of inducible defenses. Hence, IBI1 can contribute to basal resistance in the absence of BABA. One of the emerging scenarios is that IBI1 contributes to basal resistance as a sensor of cellular L-aspartate (**Figure 1**). A sudden decline in cellular aspartate concentrations could indicate parasitization by a biotrophic pathogen and would reduce canonical AspRS activity by IBI1. It was suggested that this deprivation of canonical AspRS activity primes the alternative defense function of IBI1. This situation is mimicked by R-BABA, which blocks L-asp-IBI1 binding, tricking the protein into sensing low L-aspartate levels (**Figure 1**). However, it still takes a secondary stress signal after pathogen attack to fully activate the defense modus of IBI1, resulting in enhanced *IBI1*

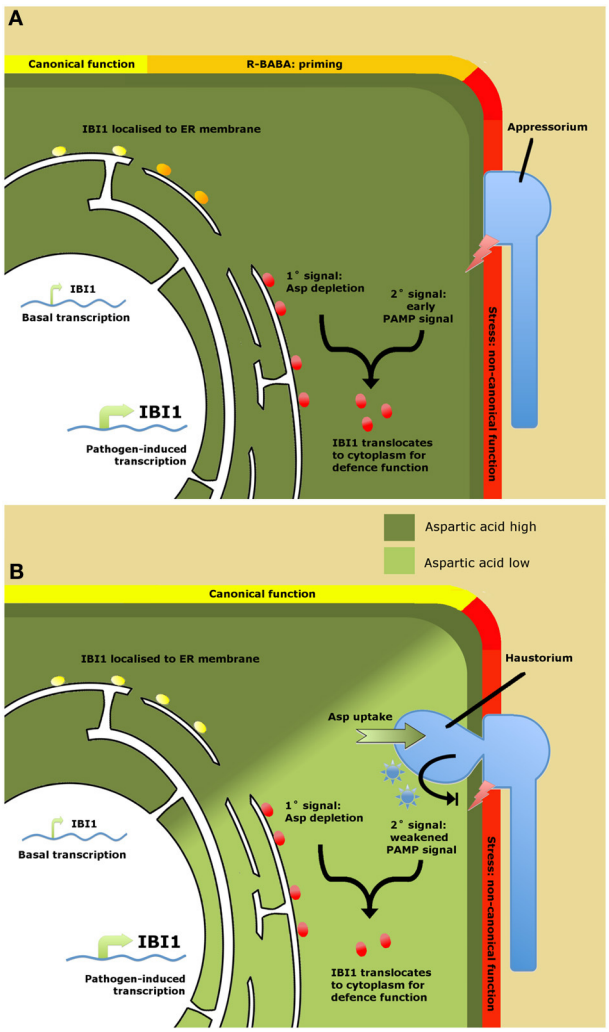

FIGURE 1 | Model of IBI1 as a regulator of BABA-induced resistance (A) and as a "depleted-self sensor" in basal resistance (B). (A) Role of IBI1in BABA-induced resistance (BABA-IR). Binding of R-BABA to ER-localized IBI1 protein (yellow circles) deprives the protein of canonical aspartyl-tRNA synthetase (AspRS) activity, which "primes" the protein's non-canonical defense activity against pathogen attack (orange circles). Detection of pathogen-associated molecular patterns (PAMPs) during the early stages of pathogen infection boosts *IBI1* gene transcription and triggers translocation of IBI1 to the cytosol, where it activates defense activity through interaction with immune-regulatory proteins (red circles). **(B)** Role of IBI1 in basal resistance. Successful pathogen infection leads to suppression of PAMP recognition through the action of pathogen-derived effectors (blue asterisks). Ongoing parasitization and amino acid uptake by the pathogen lowers cellular L-asp levels, depriving IBI1 from its canonical AspRS activity. This, together with effector-weakened PAMP perception, boosts IBI1 transcription and elicits translocation of the protein from the ER to the cytosol, where it activates broad-spectrum defenses (red circles). Hence, IBI1 acts as a "depleted-self" sensor to counteract effector-triggered suppression of "nonself recognition."

transcription, subcellular translocation of IBI1 from the ER to the cytoplasm, and augmented defense induction. Since BABA has been reported to prime PAMP-triggered immunity (PTI; Singh et al., 2012), the logical conclusion is that this secondary signal are PAMPs. Hence, IBI1 primes PTI by means of "depleted-self recognition." This mechanism provides plants with an improved capability to recognize biotrophic pathogens, thereby counteracting their specialist ability to suppress "nonself recognition" of PAMPs (Dodds and Rathjen, 2010).

Luna et al. (2014) also revealed a caveat concerning agricultural exploitation of BABA: because the chemical blocks the conserved aspartate-binding domain of AspRS enzymes, it might also impact human AspRS activity. However, future research might provide opportunities to engineer constitutively primed crop varieties without relying on BABA application. It was already shown that transgenic over-expression of *IBI1* boosts basal resistance without causing phytotoxicity. This level of BABA-independent disease protection might be improved further by engineering constitutively primed IBI1 protein that is of similar configuration as BABA-bound wild-type IBI1. Such approach would not block native AspRS activity and therefore not activate the GCN2-dependent stress pathway. In summary, the study has unveiled a novel concept in plant immune regulation and given new direction toward the exploitation of defense priming in crop protection.

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Control of foliar pathogens of spring barley using a combination of resistance elicitors

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The ability of the resistance elicitors acibenzolar-S-methyl (ASM), β-aminobutyric acid (BABA), *cis*-jasmone (CJ), and a combination of the three products, to control infection of spring barley by *Rhynchosporium commune* was examined under glasshouse conditions. Significant control of *R. commune* was provided by ASM and CJ, but the largest reduction in infection was obtained with the combination of the three elicitors. This elicitor combination was found to up-regulate the expression of *PR-1b*, which is used as a molecular marker for systemic acquired resistance (SAR). However, the elicitor combination also down-regulated the expression of *LOX2,* a gene involved in the biosynthesis of jasmonic acid (JA). In field experiments over 3 consecutive years, the effects of the elicitor combination were influenced greatly by crop variety and by year. For example, the elicitor combination applied on its own provided significant control of powdery mildew (*Blumeria graminis* f.sp. *hordei*) and *R. commune* in 2009, whereas no control on either variety was observed in 2007. In contrast, treatments involving both the elicitor combination and fungicides provided disease control and yield increases which were equal to, and in some cases better than that provided by the best fungicide-only treatment. The prospects for the use of elicitor plus fungicide treatments to control foliar pathogens of spring barley in practice are discussed.

Keywords: *Blumeria graminis* f.sp. *hordei*, disease control, elicitors, induced resistance, *Rhynchosporium commune*, systemic acquired resistance

INTRODUCTION

Application of various agents to plants can lead to the induction of resistance to subsequent pathogen attack, both locally, and systemically (Walters et al., 2013). Such induced resistance can be split into systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is characterized by a restriction of pathogen growth and a suppression of disease symptom development compared to non-induced plants infected with the same pathogen. Its onset is associated with an accumulation of salicylic acid (SA) at sites of infection and systemically, and with the coordinated activation of a specific set of genes encoding pathogenesis-related (PR) proteins. Treatment of plants with SA or one of its functional analogs e.g., acibenzolar-S-methyl (ASM; marketed in Europe as Bion® and in North America as Actigard®), induces SAR and activates the same set of *PR* genes. ISR develops as a result of colonization of plant roots by plant growth-promoting rhizobacteria (PGPR) and has been shown to function independently of SA and activation of *PR* genes, requiring instead, jasmonic acid (JA), and ethylene (ET) (Pieterse et al., 2012; Spoel and Dong, 2012).

Research over the past decade suggests that SA induces defenses against biotrophic pathogens, while JA mediates defenses against necrotrophic pathogens (Glazebrook, 2005). It is thought that cross-talk between the two signaling pathways might help to fine-tune defense responses against a particular pathogen according to its mode of infection (Beckers and Spoel, 2006). Interestingly, Spoel et al. (2007) found that infection with the biotrophic pathogen *Pseudomonas syringae*, which induces SA-mediated defense, rendered *Arabidopsis thaliana* more susceptible to the necrotrophic pathogen *Alternaria brassicicola*, although this trade-off was restricted to plant tissue adjacent to the initial infection site. In terms of plant-insect interactions, JA is known to play a major role in mediating defenses against insect herbivory (Bostock, 2005), although the situation is rather more complex than was once thought, since both SA- and JA-responsive gene expression can be elicited by aphids and whiteflies. In terms of insect herbivory, although there are examples of negative cross-talk i.e., SA-mediated suppression of JA-inducible gene expression, there are also examples of no trade-offs, and even of positive effects (see Walters et al., 2013).

Because induced resistance offers the prospect of broad spectrum disease control using the plant's own resistance mechanisms, there has been great interest in the development of agents which can mimic natural inducers of resistance (Lyon, 2007). These include elicitor molecules released during the early stages of the plant-pathogen interaction and the signaling pathways used to trigger defenses locally and systemically. Examples include ASM, which has been shown to elicit SAR in a wide range of plant-pathogen interactions (Leadbeater and Staub, 2007), the non-protein amino acid β-aminobutyric acid

(BABA), and the oxylipin, *cis*-jasmone (CJ) (Walters et al., 2007).

BABA is known to induce resistance against pathogens in various systems, including tomato, potato, grapevine, and pea (Cohen et al., 1999; Jakab et al., 2001). In field experiments, Cohen (2002) found that BABA provided significant control of late blight of potato, while Liljeroth et al. (2010) showed that BABA used together with a reduced fungicide dose gave the same level of late blight control as a full dose of the standard fungicide treatment.

CJ is structurally related to JA and methyl jasmonate (MeJA), both of which are well known to activate plant defenses (Farmer and Ryan, 1990; Thaler et al., 1996), although CJ activates a unique and more limited set of genes than does treatment with MeJA (e.g., Pickett et al., 2007). CJ is released naturally from plants damaged by insects and when applied artificially, can activate defense against insects (Birkett et al., 2000; Bruce et al., 2003).

The efficacy of induced resistance under field conditions is variable, representing a major obstacle to its use in practical crop protection (Reglinski and Walters, 2009). Induced resistance is a complex plant response to pathogen attack and as such, will be modified by many factors including genotype. However, insufficient attention has been paid to investigating the mechanisms underlying variable efficacy and approaches that might be adopted to incorporate elicitors into crop protection practice, such as use of elicitors and fungicides together in the same disease control program, and use of combinations of elicitors. The latter aspect has received little attention to date, probably because of the trade-offs that might be associated with using elicitors which activate different signaling pathways, as mentioned above. In this paper, we report the results of field experiments over 3 consecutive years, undertaken to determine the potential for use of an elicitor combination to control foliar pathogens of spring barley. Some preliminary data from this study have appeared previously in a conference paper (Walters et al., 2010).

MATERIALS AND METHODS

PLANT GROWTH AND PATHOGEN INOCULATION UNDER GLASSHOUSE CONDITIONS

The spring barley (*Hordeum vulgare* L.) variety Cellar was used for glasshouse studies. Cellar was chosen since it exhibits moderate susceptibility to *Rhynchosporium commune* (HGCA, 2009). Seeds were sown in pots in Fisons Levington compost and grown in a walk-in growth room at 18°C with a 16 h photoperiod (190 μmol m^{-2} s^{-1} provided by 400 W mercury vapor lamps). The experiment was laid out in a randomized block design, with each of the five treatment groups consisting of 15 plants, with 10 plants used for disease assessment and 5 plants for gene expression analysis. Plants were used for efficacy experiments when the sixth leaf was fully formed and the seventh leaf emerging. Leaves 1–4 were sprayed with elicitors using a hand-held sprayer. Two days later, plants were inoculated with the leaf scald pathogen, *Rhynchosporium commune*, by spraying with a suspension of spores (1×10^5 spores/ml) in distilled water containing 0.01% Tween 20. Inoculated plants were then covered with plastic bags for 48 h (the first 24 h in the dark) and kept at 16°C to provide the conditions necessary for spore germination and early fungal development. Thereafter, the temperature of the growth room was increased to 18°C for the remainder of the experiment. Infection intensity on leaves 5–7 was assessed 21 days after inoculation by determining the % leaf area exhibiting symptoms on each of 10 plants. For gene expression experiments, leaves three and four were treated with elicitor and 2 days later were inoculated with *R. commune*. Leaves were harvested 2 days later and frozen at −80°C for gene expression analysis. Data presented are the means of three replicates.

The elicitors used in these experiments were ASM, BABA, and CJ. ASM (Bion®) was a gift from Syngenta, Basel, Switzerland; BABA was purchased from Sigma, Poole, Dorset, UK; CJ was purchased from Sigma Aldrich, Dorset, UK. ASM (1 mM), BABA (1 mM) and CJ (0.625 g/l) were made up in distilled water containing 0.01% Tween 20.

FIELD EXPERIMENTS

Field experiments were conducted in 2007 at Tibbermore, Perthshire, Scotland, and in 2008 and 2009 at Drumalbin, Lanark, Scotland. Total rainfall and average air temperatures at these sites during the period 1 June—1 September were:

Perthshire, 2007: rainfall = 219 mm; average air temperature = 17°C

Lanark, 2008: rainfall = 317 mm; average air temperature = 16°C

Lanark, 2009: rainfall = 338 mm; average air temperature = 17°C.

Two spring barley varieties (Cellar and Optic) used in all field experiments reported here. Cellar has a resistance rating (RR) of 9 for powdery mildew and 4 for *Rhynchosporium commune*, and Optic has a RR of 5 for powdery mildew and 4 for *R. commune* (RR scale: 10 = high resistance, 1 = low resistance; HGCA, 2009). Each variety was sown in a randomized block design at a seed rate of 360 seeds m^{-2} and an individual plot size of 10 × 2 m, using three plots per treatment. For each barley variety, the factor tested was the applied treatment (i.e., elicitor, fungicide, elicitor + fungicide). Plots received standard fertilizer and herbicide regimes and 16 treatment programs were compared (**Table 1**). Spray dates for treatments were based on plant growth stage as described by Zadoks et al. (1974) and were applied with a knapsack sprayer using an equivalent spray volume of 200 l ha-1. Disease symptoms (% leaf area infected) and % GLA were assessed using 10 plants per plot at spray dates and at 14 day intervals after the final spray. Area under the disease progress curves (AUDPC) were calculated using the formula:

$$\Sigma \left(y_i + y_{(i+1)}\right)/2 \times \left(t_{(i+1)} - t_i\right)$$

where y_i is the disease rating at time t_i.

Plots were harvested at the end of the trial and yields expressed as tonnes/hectare at 85% dry matter content.

GENE EXPRESSION

Total RNA was extracted from barley leaves using a RNeasy™ kit (Qiagen, West Sussex, UK) and RNA yield determined using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). In order to remove any remaining trace

Table 1 | Elicitor and fungicide treatments applied in field experiments in 2007–2009.

Treatment	GS24	GS31	GS39
1	Nil	Nil	Nil
2	Elicitors	Nil	Nil
3	Nil	Elicitors	Nil
4	Nil	Nil	Elicitors
5	Elicitors	Elicitors	Nil
6	Elicitors	Nil	Elicitors
7	Fungicide[1]	Nil	Nil
8	Nil	Fungicide[1]	Nil
9	Nil	Nil	Fungicide[2]
10	Nil	Fungicide[1]	Fungicide[2]
11	Fungicide[1] + Elicitors	Nil	Nil
12	Nil	Fungicide[1] + Elicitors	Nil
13	Nil	Nil	Fungicide[2] + Elicitors
14	Elicitors	Fungicide[1]	Fungicide[2]
15	Elicitors	Fungicide[3]	Fungicide[4]
16	Elicitors	Elicitors + Fungicide[3]	Fungicide[4]

Treatments were applied at different growth stages (GS) as described in the Materials and Methods.

GS = growth stage (see Zadoks et al., 1974).

Elicitors = ASM + BABA + C.

Fungicide[1] = prothioconazole at full-rate x 0.4 + cyprodinil and picoxystyrobin at full-rate x 0.5.

Fungicide[2] = prothioconazole at full-rate x 0.4 + chlorothalonil at full-rate.

Fungicide[3] = prothioconazole at full-rate x 0.2 + cyprodinil and picoxystrobin at full-rate x 0.3.

Fungicide[4] = prothioconazole at full-rate x 0.2 + chlorothalonil at full-rate x 0.5.

of DNA likely to interfere with measurements, samples were treated with desoxyribonuclease enzymes using the DNA-*free*™ kit from Applied Biosystems (California, USA). The final quantity and quality of the RNA was tested using a RNA 6000 Nano Chip kit (Agilent Technologies, Santa Clara, CA, USA).

Primer sequences for *PR1-b, LOX2*, and the cyclophilin gene (internal control) are listed in **Table 2**. All sequences were purchased from Eurofins MWG Operon (Ebersburg, Germany) and all primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, California, USA).

Following RNA extraction, cDNA was generated using a SuperScript™ first-strand cDNA synthesis kit (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was then performed with a MX3000P system (Stratagene, CA, USA) using a Brilliant 11SYBR Green QPCR master mix with ROX (Agilent Technologies, Santa Clara, CA, USA). In order to construct standard curves for the genes, six data points were used with a 5-fold dilution series (1:10–1:31,250). A 25 µl reaction for PCR amplification contained 12.5 µl of SYBR Green master mix (see above), 0.75 µl forward primer, 0.75 µl reverse primer, 6 µl sterile distilled water, and 5 µl cDNA. All PCR reactions were

Table 2 | Primer sequences for genes used in this study.

Gene	Primer sequence (F)	Primer sequence (R)
PR1-b	CTACGACTACGGCTCCAACAC	GCATCACGGTTAGTATGGTTTCTG
LOX2	CGGCAGACTCCCTCATCACTAAAG	GGCAGCAACAGGTCGTGGTAG
Cyclophilin	CCTGTCGTGTCGTCGGTCTAAA	ACGCAGATCCAGCAGCCTAAAG

Amplicon lengths: PR1-b = 190 base pairs. LOX2 = 121 base pairs. Cyclophilin = 122 base pairs.

performed in duplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 40 cycles, each consisting of 30 s denaturing at 95°C, 60 s annealing at 57°C, 30 s at 72°C for new strand synthesis. The standard curves were used to calculate the absolute quantity of the product in each sample, Relative expression values were then calculated by normalizing against the cyclophilin gene as an internal control.

STATISTICAL ANALYSIS

All data were subjected to One-Way ANOVA using the GenStat Release 11.1 statistical program. The effect of blocks was considered random and the effect of applied treatments was defined as fixed. % leaf area infected values from glasshouse experiments and % GLA data from field experiments were log-transformed prior to analysis. Comparison of treatment means was performed using Fisher's protected least significant difference (LSD) Test.

RESULTS

EFFECTS OF ELICITORS UNDER GLASSHOUSE CONDITIONS

Initial experiments were conducted under glasshouse conditions to examine the effects of Bion®, BABA, and CJ, singly and in combination, on infection of the barley variety Cellar by *R. commune*. Although BABA reduced infection compared to the untreated control, this difference was not significant. In contrast, treatment with Bion® and CJ led to significant reductions in *R. commune* infection, with Bion® reducing infection by 70% and CJ by 64% (**Figure 1**). The largest reduction in infection (96%) was obtained using a combination of Bion®, BABA, and CJ.

Application of the elicitor combination to leaves 1 and 2 of the barley variety Cellar led to changes in the expression of two defense-related genes in leaves 3 and 4. Thus, elicitor treatment resulted in significant increases in expression of *PR1b* in both leaves 3 and 4 (4.3-fold and 3.8-fold, respectively; **Figures 2A,B**). Expression of *PR1b* was also increased significantly in leaf 3 following inoculation of untreated leaves (2.6-fold increase), but was not affected in leaf 4 (**Figures 2A,B**). However, the largest increases in *PR1b* expression were obtained when leaves 3 and 4 were first treated with elicitor and subsequently inoculated with *R. commune*. Here, *PR1b* expression was increased 7-fold in leaves 3 and 4 compared to the untreated control (**Figures 2A,B**). This suggests that the elicitor combination primes the plant for enhanced expression of *PR1b*.

In contrast to *PR1b*, expression of *LOX2* was reduced by treatment with the elicitor combination, compared to the untreated control. Indeed, all three treatments led to substantial and significant decreases in expression of *LOX2* in leaves 3 and 4 (**Figures 3A,B**).

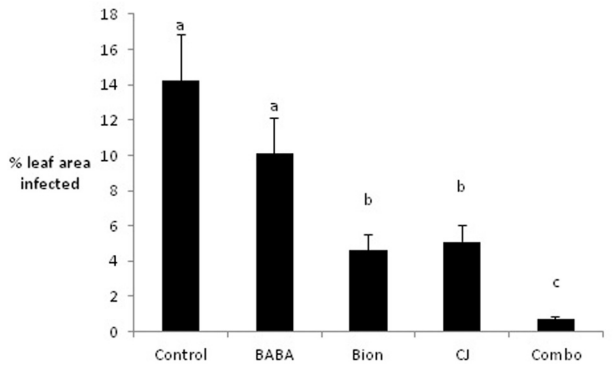

FIGURE 1 | Effects of elicitors, singly and in combination, on infection of barley with *R. commune*. Leaves 1–4 were sprayed with elicitor and inoculated with *R. commune* 2 days later. Infection intensity was assessed 21 days later on leaves 5–7. Bars with a different letter are significantly different at $P < 0.05$ (Fisher's LSD).

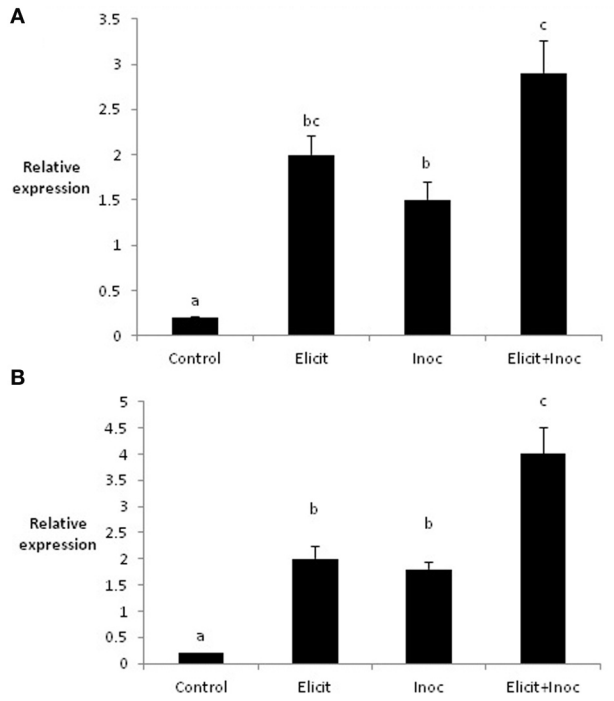

FIGURE 2 | Relative quantity of *PR1-b* in leaf 3 (A) and leaf 4 (B) of barley plants treated with the elicitor combination. Leaves three and four were treated with elicitor and inoculated with *R. commune* 2 days later. Leaves were harvested 2 days after inoculation for analysis of gene expression. Bars with a different letter are significantly different at $P < 0.05$ (Fisher's LSD).

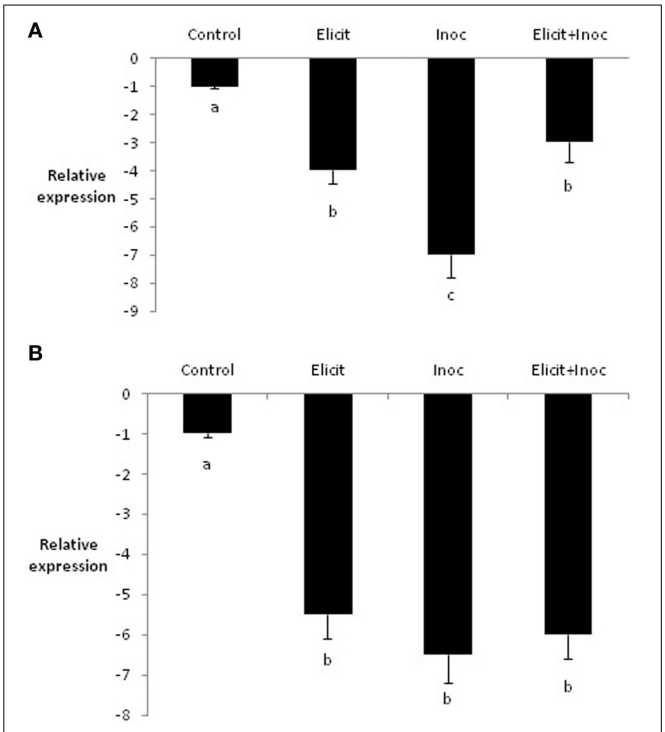

FIGURE 3 | Relative quantity of *LOX2* in leaf 3 (A) and leaf 4 (B) of barley plants treated with the elicitor combination. Leaves three and four were treated with elicitor and inoculated with *R. commune* 2 days later. Leaves were harvested 2 days after inoculation for analysis of gene expression. Bars with a different letter are significantly different at $P < 0.05$ (Fisher's LSD).

FIELD EXPERIMENTS

Since the elicitor combination provided most effective control of *R. commune* under glasshouse conditions, this treatment was chosen for inclusion in field experiments. Over the 3 years of field experiments, the two major foliar diseases detected on the spring barley crops were powdery mildew and *R. commune*. However, disease levels varied between years and in some years (2008 and 2009), only *R. commune* was observed on the variety Cellar. This probably reflected differences in weather at the different sites, since, for example, the 2007 season at the Perth site was considerably drier than the Lanark sites in 2008 and 2009.

The 3 years of field experimentation, involving 17 different treatments on two varieties generated too much data to be shown here. Instead, only data for selected treatments are shown. These treatments are untreated (1), one fungicide-only treatment (10), one elicitor-only treatment (5), and one elicitor + fungicide (reduced rate) treatment (13). The data for all treatments are provided in Supplementary Material.

The efficacy of the elicitor combination was dependent on crop variety and year. Thus, in 2009, the elicitor combination provided significant control of both powdery mildew and *R. commune* on the variety Optic and of *R. commune* on variety Cellar (**Figure 4**). However, on both varieties, most effective disease control was achieved using a combination of the elicitor combination and fungicide. Indeed, the level of disease control achieved with the elicitor and fungicide treatment was at least as good as that obtained using the fungicide only treatment (**Figure 4**). Interestingly, although the elicitor combination on its own provided significant disease control on the two varieties, grain yield remained unchanged compared to untreated plants (**Figure 4**). In contrast, grain yield was increased significantly ($P < 0.05$) in both barley varieties treated with the elicitor plus fungicide treatment (**Figure 4**).

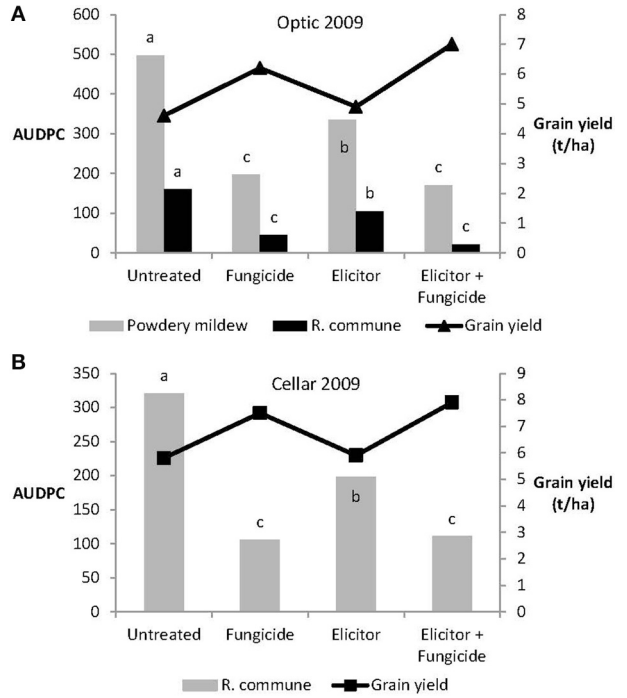

FIGURE 4 | Effects of the elicitor combination and fungicides on (A) AUDPC for powdery mildew and *R. commune,* and grain yield in the spring barley variety Optic, and (B) on AUDPC for *R. commune* and grain yield in the variety Cellar, in a field experiment in 2009. Bars with different letters are significantly different at $P < 0.05$ (Fisher's LSD).

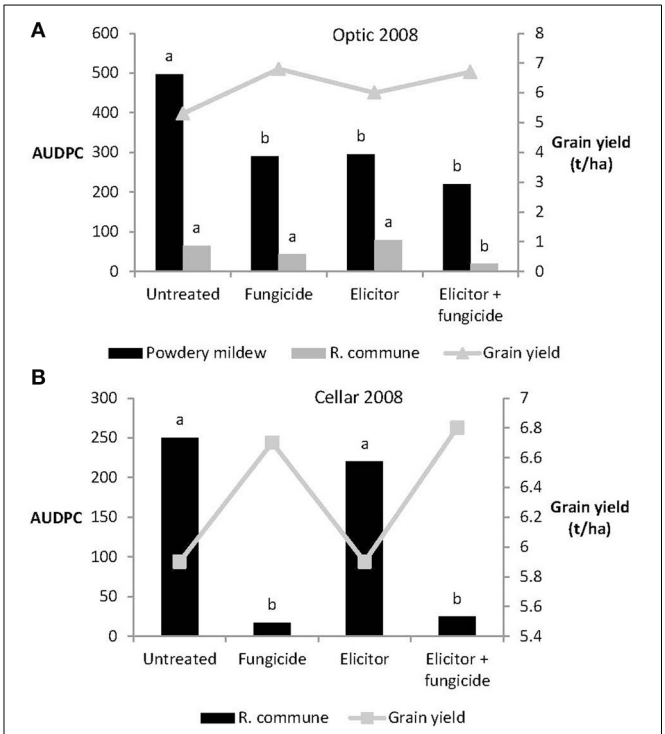

FIGURE 5 | Effects of the elicitor combination and fungicides on (A) AUDPC for powdery mildew and *R. commune,* and grain yield in the spring barley variety Optic, and (B) on AUDPC for *R. commune* and grain yield in the variety Cellar, in a field experiment in 2008. Bars with different letters are significantly different at $P < 0.05$ (Fisher's LSD).

In 2008, the elicitor combination applied on its own reduced levels of powdery mildew significantly on variety Optic, but provided no control of *R. commune* on either Optic or Cellar (**Figure 5**). Treatment with elicitor plus fungicide provided significant control of both diseases on Optic and of *R. commune* on Cellar. In both varieties, highest grain yields were obtained from plants receiving the elicitor plus fungicide treatment (**Figure 5**).

Levels of both powdery mildew and *R. commune* were not significantly affected by treatment of either variety with the elicitor combination on its own in 2007 (**Figure 6**). In contrast, on both varieties, treatment with elicitor plus fungicide provided significant control of both diseases and in most cases the level of disease control achieved was as good as that obtained using the fungicide only treatment (**Figure 6**). Grain yields of both varieties were significantly increased in plants receiving the elicitor plus fungicide treatment compared to the other treatments, while in Optic, the elicitor combination on its own actually reduced grain yield (**Figure 6**).

The absence of any significant effect of the elicitor-only treatment on grain yields in the 2 varieties across the years probably reflects, in part, the lack of a significant effect of this treatment on green leaf area (GLA) (**Figure 7**). This is supported by the fact that the significant reduction in grain yield in the variety Optic in 2007 (**Figure 6**) was associated with a significant reduction in GLA (**Figure 7E**). This contrasts with the elicitor plus fungicide treatment, where, in most cases, increased grain yields (**Figures 4–6**) were associated with significantly increased GLAs (**Figure 7**). The only exception was the variety Cellar in 2007, where increased grain yield in the elicitor plus fungicide treatment (**Figure 6B**) was accompanied by a small, but statistically non-significant increase in GLA (**Figure 7F**).

DISCUSSION

Under glasshouse conditions, ASM and CJ reduced *R. commune* infection of barley by 64–70%, while BABA had much less effect. Although there are many examples of disease control provided by ASM and BABA (Cohen et al., 2010; Walters et al., 2013), to our knowledge, this is the first report of disease control provided by CJ. Interestingly, applying the three elicitors together provided the best levels of disease control, reducing infection by 96%, and confirms previous reports from this laboratory (Walters et al., 2011a,b). Reports of the use of elicitor combinations to control plant disease are rare, although combinations of ASM and Milsana® (extract of *Reynoutria sachalinensis*) were found to control powdery mildew on cucumber (Bokshi et al., 2008).

Molecular studies on tobacco and *Arabidopsis* have shown that ASM activates the SAR pathway by mimicking the activity of SA (Gaffney et al., 1993; Friedrich et al., 1996; Lawton et al., 1996). The situation with BABA is more complex and seems to involve SA-dependent, SA-independent, and abscisic acid (ABA)-dependent mechanisms, with the relative importance of the different signaling pathways depending on the particular

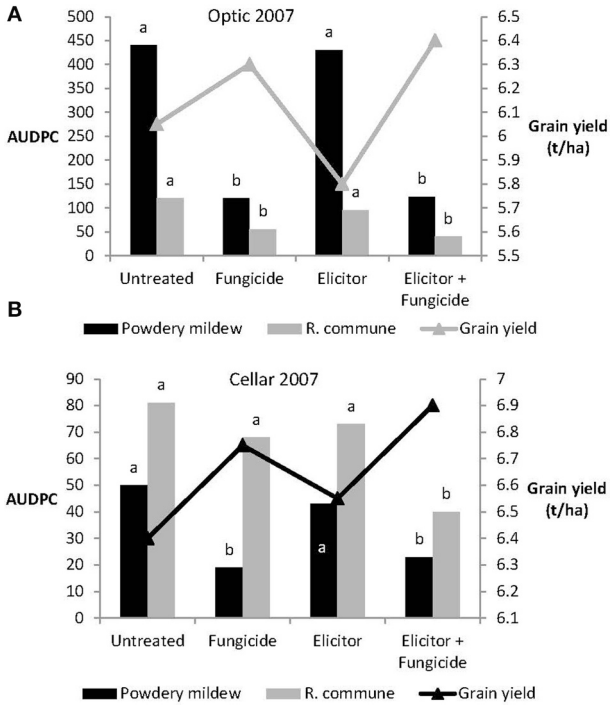

FIGURE 6 | Effects of the elicitor combination and fungicides on (A) AUDPC for powdery mildew and *R. commune,* and grain yield in the spring barley variety Optic, and (B) in the variety Cellar, in a field experiment in 2007. Bars with different letters are significantly different at $P < 0.05$ (Fisher's LSD).

host-pathogen interaction (Ton et al., 2005). As indicated earlier, CJ is structurally related to JA and MeJA, although it up-regulates a unique set of genes compared to MeJA (Birkett et al., 2000; Pickett et al., 2007). Treatment of leaves one and two of barley with the combination of ASM, BABA, and CJ led to an approximately 6-fold up-regulation of *PR-1b* in leaves three and four, confirming previous reports on the effects of the elicitor combination on defense-related gene expression (Walters et al., 2011a,b). Expression of a *PR-1* gene is usually considered to be a molecular marker for SAR (van Loon et al., 2006) and therefore, these data suggest that the elicitor combination activated SAR in barley. *PR-1b* was also up-regulated by inoculation with *R. commune* (∼4-fold increase in leaf 3, for example). However, the largest up-regulation of *PR-1b* was obtained when elicitor-treated plants were subsequently inoculated with *R. commune*. This suggests that the elicitor combination primed *PR-1b* gene expression in these plants. In contrast to *PR-1b,* the expression of *LOX2* exhibited a completely different trend. Treatment of barley plants with the elicitor combination resulted in a substantial down-regulation of *LOX2* (4-fold in leaf 3 and 5.5-fold in leaf 4). Inoculation with *R. commune* led to a greater down-regulation of *LOX2* in the treated leaves, while application of the elicitor combination followed by inoculation had no further effect on gene expression than the elicitor combination only. The *LOX2* gene, which is involved in the octadecanoid pathway, is auto-regulated by JA, thereby controlling a feed-forward loop in JA biosynthesis (Bell et al., 1995). Suppression of *LOX2* in transgenic *Arabidopsis* was shown to block JA biosynthesis during pathogen infection (Spoel et al., 2003), and the present data on *LOX2* in barley suggests that the elicitor combination might suppress activity of the JA pathway in barley. Concomitant activation of SAR and suppression of the JA pathway might be expected to enhance defense against biotrophic pathogens and increase susceptibility to necrotrophic pathogens (Glazebrook, 2005). *R. commune* is a hemibiotrophic pathogen, possessing an initial biotrophic phase followed by a prolonged necrotrophic phase (Walters et al., 2008). Perhaps it is no surprise therefore, that the elicitor combination reduces infection by *R. commune*. It is possible that the elicitor combination might compromise the ability of the plant to defend itself against necrotrophic pathogens and indeed, the elicitor combination was shown to provide control of powdery mildew and *R. commune*, but was associated with increased symptoms of leaf spot caused by the necrotrophic pathogen *Ramularia collo-cygni* on spring barley in the field (Walters et al., 2011b). Plant defense against chewing insects is mediated by JA signaling (Pieterse et al., 2012), as is establishment of functional arbuscular mycorrhizal (AM) symbioses (Pozo and Azcón-Aguilar, 2007). In view of the down-regulation of *LOX2* in barley treated with the elicitor combination, it would be prudent to examine the effects of treated plants on defense against herbivorous insects and on the establishment of AM symbiosis.

In all 3 years of field experiments, the elicitor combination applied on its own was either partially effective or ineffective at controlling powdery mildew and *R. commune* on the two barley cultivars. In 2009, the elicitor combination provided significant control of both diseases on both spring barley varieties, and also controlled powdery mildew on Optic in 2008. However, the elicitor treatment did not control *R. commune* on either variety in 2008 and provided no disease control in 2007. Perhaps unsurprisingly therefore, given the poor levels of disease control provided by the elicitor-only treatment, grain yield was not significantly affected, apart from 2007, when grain yield of Optic was significantly reduced compared to the untreated control. Here, the reduced grain yield probably reflected the significantly reduced GLA in the elicitor-only treatment. Infection by many pathogens, including biotrophic pathogens such as powdery mildews, results in chlorosis and reduced photosynthetic rates (e.g., Walters and McRoberts, 2007) and the failure of the elicitor-only treatment to control infection would probably have affected rates of photosynthesis. Whether the elicitor combination affects photosynthesis in barley is not known, but should be examined.

The data on disease control presented in this paper highlight two typical problems associated with the practical use of elicitors on certain crops under field conditions, namely inconsistency and poor levels of disease control (Walters and Fountaine, 2009). In contrast to the elicitor-only treatments, the performance of the elicitor plus fungicide treatment was better both in terms of disease control and consistency. The acid test for such combined treatments is whether the performance of the combination is superior to that of the fungicide treatment on its own. Unfortunately, in most cases, although the combined elicitor and fungicide treatment performed as well as the fungicide-only treatment in terms of disease control, only in one case (control of

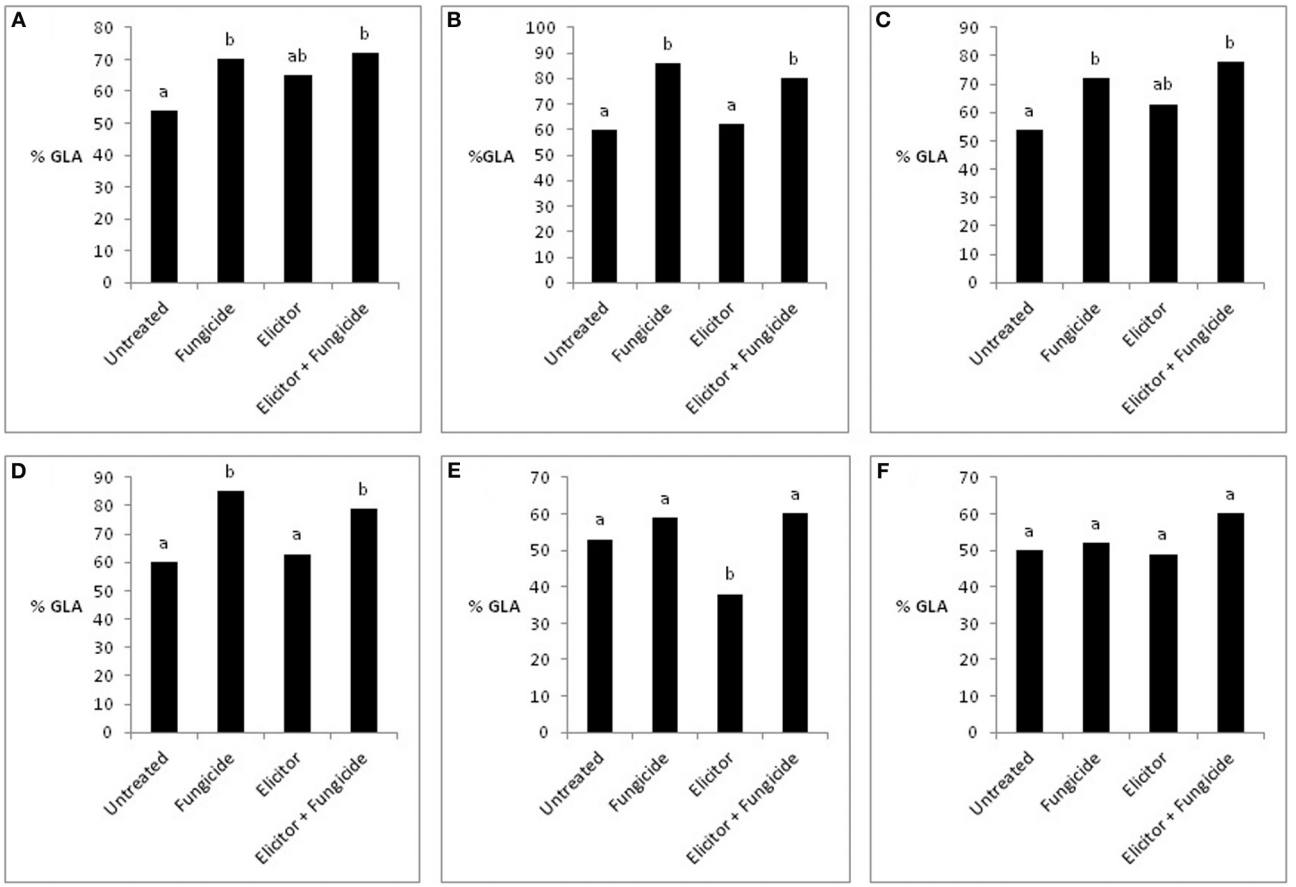

FIGURE 7 | Effects of the elicitor combination and fungicides on percentage green leaf area (% GLA) in Optic and Cellar in 2009 (A,B), Optic and Cellar in 2008 (C,D), and Optic and Cellar in 2007 (E,F). Bars with different letters are significantly different at $P < 0.05$ (Fisher's LSD).

R. commune on Optic in 2008) did the elicitor plus fungicide treatment out-perform the fungicide-only treatment. A similar situation was found for grain yield. Here, although grain yield tended to be increased by application of the elicitor plus fungicide treatment, most of these increases were not significantly different from the fungicide-only treatment. The lack of consistency in terms of disease control shown by the elicitor combination in barley, contrasts with the situation in oilseed rape (*Brassica napus*). Here, application of the elicitor combination to winter oilseed rape provided better control of light leaf spot caused by *Pyrenopeziza brassicae*, than standard fungicide treatments (Oxley and Walters, 2012).

Over the 3 years of field experiments, the elicitor plus fungicide combinations providing most consistent disease control were treatments 13, 14, and 16. As indicated earlier, only data for treatment 13 are shown, since this treatment performed most consistently throughout the study. Treatment 13 involved application of a combination of fungicide at reduced rate plus elicitor at GS39, with no control treatments applied at earlier growth stages. It has been suggested that application of elicitors earlier in the season might reduce inoculum levels, thereby requiring less fungicide to be applied later (Walters et al., 2013). On the basis of the results obtained in the present paper, this suggestion does not appear to work for spring barley. This suggests that, at least for spring barley, protecting later stages of crop growth is important in maintaining grain yield.

In some crops, use of elicitors and fungicides (or bactericides) can be effective. For example, the use of ASM (as Actigard®) in combination with fungicides and bactericides was recommended in tomato spray programs in North Carolina, USA (Ivors and Louws, 2007). The rationale here was that the elicitor would increase plant resistance, while the fungicides and bactericides would reduce pathogen inoculum levels. On mandarins (variety Murcott), tank-mixing ASM with azoxystrobin improved the efficacy of the fungicide by more than 50% (Miles et al., 2005), although this effect was clearly variety-specific, since no extra benefit of tank-mixing the elicitor and fungicide was obtained with the mandarin variety Imperial (Miles et al., 2004).

It has been suggested that one of the reasons for the relatively poor performance of elicitors is due to the likelihood that under field conditions, plants are already induced (Walters, 2009). Indeed, Herman et al. (2007) found that in tomato under field conditions, some defense genes were already expressed prior to ASM application. Nevertheless, the expression of these genes was increased further following ASM application. In preliminary work, examination of CAD activity in leaves from the field

experiment in 2007 indicated that activity of the enzyme was already high prior to elicitor treatment (Paterson and Walters, unpublished results). Although it is tempting to suggest that CAD activity was increased further following elicitor application, any increases observed were not significant. It is possible that in barley, unlike tomato, prior induction of resistance compromises the ability of the plant to respond effectively to elicitors. Indeed, this was reported for barley treated with the elicitor combination, where prior inoculation with *R. commune* compromised the ability of the plant to respond to subsequent elicitor treatment (Walters et al., 2011a). It was suggested that this might help to explain the relatively poor performance of induced resistance in the field, particularly in cereals, compared to plants grown under controlled conditions (Walters et al., 2011a).

The results presented in this paper indicate quite clearly that use of a combination of elicitors alone does not provide effective disease control in spring barley. In contrast, using the elicitor combination and fungicides, even at half-rate, can provide levels of disease control and yield increases that are equal to the best fungicide-only treatment. From a practical perspective, an elicitor plus fungicide program is only likely to be attractive to a grower if it is cost-effective i.e., it provides levels of disease control and yield increases above that achieved using the fungicide on its own. This suggests that barley growers are unlikely to find the elicitor plus fungicide treatments examined in this work an attractive proposition. This might change however, if fungicide availability is further reduced through legislation. Indeed, elicitor/fungicide combinations could be valuable in reducing fungicide use, and prolonging the useful life of certain fungicides.

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SUPPLEMENTARY MATERIAL

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Priming of plant resistance by natural compounds. Hexanoic acid as a model

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Some alternative control strategies of currently emerging plant diseases are based on the use of resistance inducers. This review highlights the recent advances made in the characterization of natural compounds that induce resistance by a priming mechanism. These include vitamins, chitosans, oligogalacturonides, volatile organic compounds, azelaic and pipecolic acid, among others. Overall, other than providing novel disease control strategies that meet environmental regulations, natural priming agents are valuable tools to help unravel the complex mechanisms underlying the induced resistance (IR) phenomenon. The data presented in this review reflect the novel contributions made from studying these natural plant inducers, with special emphasis placed on hexanoic acid (Hx), proposed herein as a model tool for this research field. Hx is a potent natural priming agent of proven efficiency in a wide range of host plants and pathogens. It can early activate broad-spectrum defenses by inducing callose deposition and the salicylic acid (SA) and jasmonic acid (JA) pathways. Later it can prime pathogen-specific responses according to the pathogen's lifestyle. Interestingly, Hx primes redox-related genes to produce an anti-oxidant protective effect, which might be critical for limiting the infection of necrotrophs. Our Hx-IR findings also strongly suggest that it is an attractive tool for the molecular characterization of the plant alarmed state, with the added advantage of it being a natural compound.

Keywords: priming, natural inducers, hexanoic acid, vitamins, oxidative stress, *Botrytis cinerea*

PRIMING PLANT DEFENSES

Plants are subjected to a variety of external factors that adversely affect their growth and development, and are often divided into biotic (insect herbivores and microbial pathogens) and abiotic (extreme temperature, inappropriate water supply, etc.) stresses. Adaptation to these environmental stresses is essential for survival and propagation (Rasmann et al., 2012). Among the plethora of defense strategies that plants have evolved, some are constitutive, but the majority are induced in response to stimuli, thus they are more specific (Frost et al., 2008). Recognition of different elicitors leads to the activation of diverse subsets of defense responses. Central regulatory hormones are salicylic acid (SA) and jasmonic acid (JA), although ethylene and abscisic acid (ABA), among others, also play key roles (Denance et al., 2013). For a plant, successfully tackling certain stress or a simultaneous group of stresses is a complex task, and responses largely overlap and can be interconnected positively and negatively (Ahmad et al., 2010). Induced resistance (IR) leads to various types of systemic resistance throughout the plant. IR is based on two general mechanisms: direct activation of defense responses in systemic tissue after local stimuli and priming, which implies activation of systemic responses, but only when the pathogen reaches these sites. The best characterized type of IR is systemic-acquired resistance (SAR), which is mostly dependent on SA, unlike the less understood JA-dependent defense (Conrath, 2009).

Priming is a mechanism which leads to a physiological state that enables plants to respond more rapidly and/or more robustly after exposure to biotic or abiotic stress (**Figure 1**). The "primed" state has been related to increased, more efficient activation of the defense response and enhanced resistance to challenging stress (Conrath, 2009). This increased alertness correlates with no or minimal gene induction (Slaughter et al., 2012). The primed state results from the improved perception and/or amplification of defense response-inducing signals, rather than from the direct activation of these defense responses. Wide-ranging ways of inducing priming are known: infection by pathogens, colonization of roots by beneficial microbes, treatment with natural or synthetic chemicals, primary metabolism alteration and perception of certain volatile organic compounds (VOCs; Conrath et al., 2006). The molecular basis of priming has recently started to be unraveled, but is still poorly understood. Accumulation of both inactive mitogen-activated protein kinases (MAPKs) and transcription factors and certain epigenetic marks is best characterized (Conrath, 2011). The link between priming and epigenetic changes is further supported

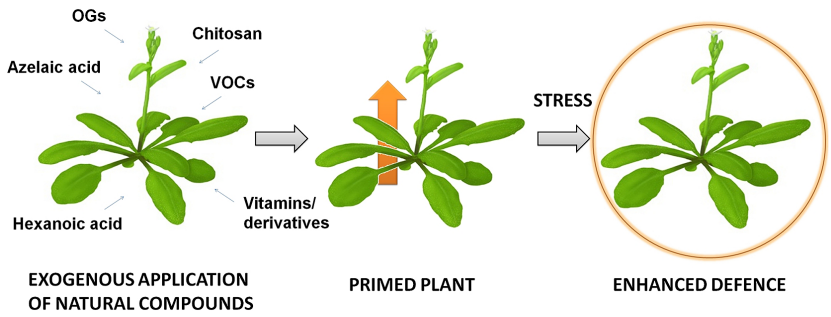

FIGURE 1 | Treatment with natural compounds increases plant protection against future stresses.

by the transgenerational priming phenomenon when the progeny of primed plants shows an enhanced defense response (Luna and Ton, 2012).

Induced resistance enables plants to activate the appropriate set of defenses in each situation to avoid misuse of resources and to minimize tradeoffs between defenses against different enemies. However, the time required to implement the response can mean that plants are subjected to considerable damage before the defense response acts. Priming probably evolved to compensate for this vulnerability, and to also allow plants to sense environmental cues and to promote a state of readiness to enable a quick, strong response upon pathogen attack (Frost et al., 2008). Priming is, indeed, the common theme underlying plant responses against both biotic and abiotic stress (Bruce et al., 2007). In this context, it is also worth noting that priming compounds do not tend to be highly specific, which can be an advantage or a disadvantage depending on the situation.

Over the years, a range of chemical treatments has proven capable of triggering IR, mostly through the priming mechanism. The first to be identified were synthetic SA analogs, such as 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA), and benzo (1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), which triggers SAR (Oostendorp et al., 2001; Conrath et al., 2002). A wide range of cellular responses has been reported to be potentiated by these compounds, including alterations in ion transport across the plasma membrane, synthesis and secretion of antimicrobial secondary metabolites (phytoalexins), cell wall phenolics and lignin-like polymers, and activation of various defense genes (Conrath, 2009). Non-protein amino acid β-aminobutyric acid (BABA) has received plenty of attention given its versatility, and its priming for different defense responses dependent on distinct hormones pathways and upon different challenging stresses (Conrath, 2009). This is remarkable because synthetic chemicals tend to prime SA-dependent immunity, as illustrated by the identification of priming-active compounds called imprimatins in synthetic library screening (Noutoshi et al., 2012).

PRIMING BY NATURAL COMPOUNDS

Many natural compounds have been claimed to be plant growth promoters, plant activators or plant defense inducers, among other names. A large portion of them has already been related to priming, including oligosaccharides, glycosides, amides, vitamins, carboxylic acids, and aromatic compounds. In general, natural compounds tend to be better tolerated by plants than most of the synthetic compounds tested, but there is still concern about toxicity (Iriti et al., 2010; Noutoshi et al., 2012). Most mimic pathogen interaction by acting as (endogenous or exogenous) elicitors, and are able to induce or prime defense in a concentration-dependent fashion. However as Ahn et al. (2007) pointed out, the mode of action of priming agents is eventually determined by hosts and the stress challenging them. This makes it difficult to decipher the molecular bases underlying the priming mechanism. In addition, they usually show antimicrobial activities at higher concentrations than those required for priming. Overall they represent an active area of research in pest and disease management because of their versatility, their ability to prime JA-dependent defense and their general low toxicity, which allows better crop tolerance and fewer human health concerns usually associated with conventional strategies.

One group of successfully recently tested natural inducers in *Arabidopsis thaliana* consists in redox active compounds. Among them, thiamine (vitamin B1; Ahn et al., 2007), riboflavin (vitamin B2; Zhang et al., 2009), and quercetin (Jia et al., 2010) are all capable of inducing resistance by potentiating *Arabidopsis* sensitivity to *Pseudomonas syringae* elicitors. This leads to the activation of various plant defenses, such as the hypersensitive response, callose deposition and defense-related gene expression. The H_2O_2 burst seems to play a critical role as it acts as a signal to trigger the whole response. Although NPR1 is also required for priming by these compounds, the mechanism in this plant–pathogen interaction seems to act independently of classical defense pathways and is, perhaps, similar to the oxidative stress response. Recently, it has been demonstrated that thiamine can modulate the cellular redox status to protect *Arabidopsis* against *Sclerotinia sclerotiorum* at early stages of infection (Zhou et al., 2013). Early in the pathogenesis, thiamine can effectively alleviate the inhibition of host reactive oxygen species (ROS) generation by *Sclerotinia*-secreted oxalate. Thiamine can also induce cell wall fortifications with callose/lignin to prevent oxalate diffusion. Further reports in other plants are consistent with the central role of ROS, particularly H_2O_2 in vitamin-IR. The exogenous application of riboflavin primed bean, but not tomato plants,

accelerates H_2O_2 generation after *Botrytis cinerea* infection. H_2O_2 is a signaling molecule involved in cell wall modification, gene expression regulation and cross-talk with various defense pathways (Azami-Sardooei et al., 2010). Riboflavin-IR also correlates with JA-dependent pathway activation by priming for enhanced lipoxygenase (LOX) activity. LOX enzymes are involved in the first steps of the octadecanoid pathway, which leads to oxypilin synthesis, like JA, and renders various intermediate compounds with defense implications (Azami-Sardooei et al., 2010; Taheri and Tarighi, 2010). The up-regulation of the phenylalanine ammonia-lyase (*PAL*) gene and peroxidase (*cprx1*) genes implicated in the phenolic metabolism has also been observed in sugar beet (both) and rice (only *PAL*) after riboflavin application and challenging with *Rhizoctonia solani*. Phenolics play a role in cell wall fortification, and also show antimicrobial and antioxidant activity (Taheri and Tarighi, 2010, 2011).

Para-aminobenzoic acid (PABA) is a cyclic amino acid belonging to the vitamin B group. Field experiments have proven that it is capable of enhancing resistance against Cucumber mosaic virus and *Xanthomonas axonopodis* by inducing SAR, while simultaneously improving plant yield (Song et al., 2013). This contrasts with BTH which, in the same study, reduced disease severity, but produced shoot length shortening and significant fruit weight reduction when compared to PABA and control treatments.

Menadione sodium bisulfite (MSB) is a vitamin K3 derivative known to be a growth regulator (Rama Rao et al., 1985). Borges et al. (2003a) found that MSB protects rape plants (*Brassica napus*) from the fungus *Leptosphaeria maculans* by stimulating ROS production, but without inducing *PR1*. Other authors have shown that MSB has a systemic effect (Liu et al., 2007) and reported H_2O_2 production induced by the compound through gene induction (Benitez et al., 2005). Borges et al. (2003b, 2004) also demonstrated that MSB protects banana from Panama disease caused by *Fusarium oxysporum* and that MSB primes phytoalexin accumulation. Later on, these authors demonstrated that MSB induces *Arabidopsis* resistance against *P. syringae* via a priming mechanism as MSB induces only ROS and *PR1* accumulation on post-inoculation day 3 (Borges et al., 2009). In their study, the authors analyzed gene expression profiling after menadione treatment by microarray technology. MSB produced a unique molecular footprint, but most up-regulated genes have been previously connected to stress. Furthermore, the G-box in their promoters was over-represented, and, interestingly, other up-regulated genes coded for transcription factors, including the putative regulators of the G-box (Borges et al., 2009). It is remarkable that a menadione derivative (Param-A) has been commercially launched to induce resistance against Panama disease in bananas because when this derivative is sprayed, can significantly reduce disease occurrence and delay symptom appearance in the field (Fernández-Falcón et al., 2009).

Chitosan is a polymeric deacetylated derivative of chitin that is naturally present in some fungi cell walls, and has various deacetylation degrees and molecular weights. Although it performs several antimicrobial activities, its main contribution to reduce plant disease is to enhance plant defenses (El Hadrami et al., 2010). Chitosan has also been reported to improve growth and yield (Reddy et al., 1999; Kim et al., 2005; Cho et al., 2008). It is a potent general elicitor of proven efficiency in a wide range of experiments with different host plants and pathogens (Iriti et al., 2010). Iriti and Faoro (2009) pointed out that chitosan can directly activate systemic resistance or can prime the plant for a more efficient defense response upon challenge, depending on dose, by considering the different cytotoxicity thresholds for each chitosan derivative and plant. The diverse mechanisms of action of chitosan have been studied, which include oxygen-species scavenging and antioxidant activities, as well as octadecanoid pathway activation (reviewed in El Hadrami et al., 2010). Despite these studies however, experiments which specifically address the role of priming in the complex chitosan-plant interaction framework are still scarce.

There is evidence to support that the wound signal from the local attack site is transmitted to systemic undamaged regions, where priming or the direct induction of defense responses takes place. Signal transmission can occur either internally, probably through the phloem and xylem, or externally via VOCs (Frost et al., 2008). In the internal signaling mechanism, pathogen-induced damage in the plant cell wall can be the starting point. Cell wall degrading enzymes, such as Endo-1,4-β-glucanases, have also been found to be implicated in IR defense pathways (Flors et al., 2007; Cantu et al., 2008; Finiti et al., 2013). It is widely accepted that the plant cell wall is a dynamic functional structure involved in several plant processes, including response to stress (Huckelhoven, 2007). The elicitors released from it during pathogen infection contribute to basal resistance against fungal pathogens via a signaling pathway, which is also activated by pathogen-associated molecular pattern molecules. However, the actual components and pathways remain largely unidentified (Osorio et al., 2008).

Oligogalacturonides (OGs) are plant cell wall pectin-derived oligosaccharides which consist in linear chains of α-(1-4)-linked D-galacturonic acid with a degree of polymerization between 10 and 25, which can be methyl-esterified or acetylated depending on the source plant. They are considered endogenous elicitors, and the degree of methylation and acetylation has been found to affect the activation of defense responses (Osorio et al., 2008; Randoux et al., 2010). OG treatment has been reported to induce a range of defense responses, like accumulation of phytoalexins, β-1,3-glucanase and chitinase, or generation of ROS by triggering nitric oxide (NO) production (Rasul et al., 2012). Interestingly, some evidence indicates the involvement of OGs signaling in the octadecanoid pathway, whereby LOX activities are enhanced (Randoux et al., 2010). Exogenous treatments with OGs protect grapevine leaves against necrotrophic pathogen *Botrytis cinerea* infection in a dose-dependent manner (Aziz et al., 2004). In *Arabidopsis*, OGs increase resistance to *Botrytis cinerea* independently of JA-, SA-, and ethylene (ET)-mediated signaling. A microarray analysis has shown that about 50% of the genes regulated by OGs display a similar change of expression during *Botrytis cinerea* infection (Ferrari et al., 2007).

Azelaic acid (AA) has been suggested to be a phloem-mobile signal that primes SA-induced defenses (Jung et al., 2009; Shah, 2009). The AA biosynthesis pathway is largely unknown, although recent evidence indicates that it is a derivative of oleic acid or its desaturated derivatives, linoleic and linolenic acids (Yu et al.,

2013). Lipid peroxidation has been proposed as being responsible for AA formation and can proceed by LOX activities or the fragmentation pathway triggered by ROS. In addition, other stress-signaling molecules are generated (Zoeller et al., 2012). AA primes plants for more rapid SA accumulation by inducing glycerol-3-phosphate (G3P) biosynthesis (Yu et al., 2013). G3P levels have been proposed to modulate primary and secondary metabolic pathways, and to contribute to major physiological responses in defense (Chanda et al., 2008). So both AA and G3P seem to be implicated with phytohormones SA and JA. A synergy between AA and dehydroabetinal (DA) signaling has been suggested. DA is an abietane diterpenoid released upon wounding that is induced locally by insect infestation. There is evidence to suggest that it translocates rapidly through the plant and acts as a SAR inducer (Chaturvedi et al., 2012). Further research is required to address the implication of priming in this interaction and in DA-IR.

Along with AA, G3P and DA, pipecolic acid (L-Pip) a Lys-derived non-protein amino acid has been recently implicated as pivotal regulator of SAR, and possibly as the long-distance phloem-mobile SAR signal compound (Navarova et al., 2012; Shah and Zeier, 2013). Amino acids (aa) metabolism plays an increasingly wide range of roles in plant immunity. For example, proline metabolism has been related to oxidative burst and to the establishment of the hypersensitive response; branched chain aa catabolism mediates the cross-talk between SA and JA defenses; acetylated aa form phytohormone-aa conjugates (Zeier, 2013). Apart from these, L-Pip has been identified as a central node in SAR. L-Pip acts as an endogenous mediator of defense amplification in SAR, and also in BABA-IR. L-pip activates SA biosynthesis and its own biosynthesis via a positive feedback loop to thus orchestrate the whole SAR response (Navarova et al., 2012). Interestingly, the exogenous application of L-Pip primes *Arabidopsis* plants for more rapid SA biosynthesis, phytoalexin camalexin accumulation and defense gene expression (Navarova et al., 2012). It also primes tobacco plants for quicker SA biosynthesis and nicotine accumulation (Vogel-Adghough et al., 2013).

Another carboxylic acid with demonstrated inducer activity is hexanoic acid (Hx; Vicedo et al., 2009). We focus on this natural compound as a model for priming by natural compounds in the section below.

Volatile organic compounds play key roles in plant–plant communication as they act as airborne signals by enhancing disease resistance in the plant itself and in neighboring plants, and by also attracting parasitic or predatory insects, these being the enemies of attacking herbivores (Arimura et al., 2010). This multifunctional role makes them desirable for sustainable pest control strategies (Oluwafemi et al., 2013). A subset of VOCs emitted in response to insect attack is called herbivore-induced plant volatiles (HIPVs). Some HIPVs are known to be green leafy volatiles (GLVs), and they usually form from linolenic and linoleic acids as one of the oxypilin pathway branches. GLVs are first emitted upon wounding or herbivore attack (Hirao et al., 2012). They induce a wide range of defense reactions, probably almost entirely based on priming under field conditions, and they also display antibacterial and antifungical activities. The defense reactions linked to VOCs effects include enhanced phytoalexins secretion, incorporation of hydroxycinnamic acid esters and "lignin-like" polymers into the cell wall, enhanced oxidative burst, augmented induction of defense genes, emission of aromatic compounds and quicker trypsin inhibitors production (Conrath, 2009). GLVs prime plants for a more robust defense response by increasing the total VOCs emission and endogenous JA content after detecting an elicitor (Engelberth et al., 2004; Kishimoto et al., 2005). They also seem to increase sensitivity to methyl jasmonate (MeJA), the methyl ester of JA (Hirao et al., 2012). Interactive effects of different VOCs have been described for (Z)-3-hexen-1-ol and ethylene, although ethylene does not seem to have any effect on its own, which implies that it is worth investigating the role of each VOC and its interactive effects (Frost et al., 2008). (Z)-3-hexen-1-ol apparently plays a twofold role by priming and modulating the behavior of herbivorous insects (Wei and Kang, 2011).

Cis-Jasmone (CJ) is a highly volatile compound product of further catabolism of JA, which is known to induce the release of defense VOCs that attract predatory/parasitic insects (Birkett et al., 2000). A transcriptomic analysis has shown that CJ treatment triggers the up-regulation of a unique subset of genes, including cytochrome P450 family members. It has also been indicated that the CJ-induced expression acts independently of COI1, which is the F-box protein mediating the MeJA-induced gene expression (Bruce et al., 2008). Recently, this oxypilin has been demonstrated to enhance defense by priming plants for the quicker induction of VOCs release. The same study has suggested that CJ primes JA-induced pathways, such as the sesquiterpene synthase gene expression, despite its independent signaling roles (Oluwafemi et al., 2013).

HEXANOIC ACID PRIMING AGENT

We have previously demonstrated that root treatment with natural 6C monocarboxylic acid Hx protects tomato plants against necrotrophic fungi *Botrytis cinerea* (Leyva et al., 2008; Vicedo et al., 2009). Root treatment of 4-week-old plants with Hx at concentrations below 1 mM for 48 h prior to infection significantly reduced the incidence of the disease as other well known natural (SA) and non-natural (BABA) inducers did (**Figure 2**; Vicedo et al., 2009). At these concentrations Hx had no antimicrobial effect on *Botrytis cinerea* and shorter conditioning times were not sufficient to protect the plant against this pathogen, which strongly supports the inducer effect of this treatment. In addition, Hx did not accumulate in the aerial part of the plant, suggesting that protection might result from specific interactions with plant defense systems. Hexanoic treatment induced callose accumulation upon *Botrytis cinerea* infection. Cell wall fortification by callose deposition is a key component of resistance induced by chemical inducers like BABA or BTH (Kohler et al., 2002). Hx treatment also increased caffeic acid levels after fungal infection, which further supports the role of a reinforced cell wall in Hx-IR. Callose priming forms part of Hx-IR in different cultivars against *Botrytis cinerea* (Ailsa Craig, Moneymaker and Rheinlands Ruhm). However, the plants from Castlemart are protected by Hx in the absence of callose priming (Vicedo et al., 2009). This result indicates that additional mechanisms are involved in Hx-IR.

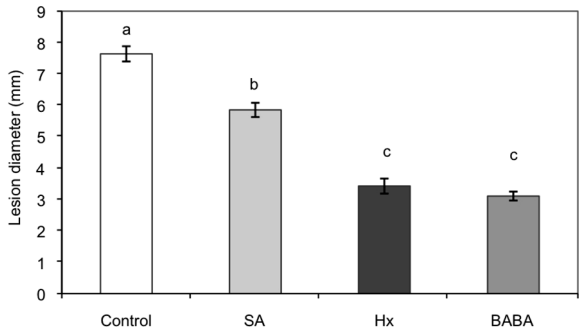

FIGURE 2 | Root treatment with hexanoic acid protects tomato plants against *Botrytis cinerea* infection. Four-week-old tomato plants (cv. Ailsa Craig) were treated with 0.6 mM hexanoic acid, 0.5 mM SA, and 0.5 mM BABA under hydroponic conditions. Control plants were treated with water. Lesion diameter was measured at 72 h after inoculation. Data show the lesion diameter (mm) ± SE ($n = 20$). Different letters represent statistically significant differences ($p < 0.05$; least significant difference test). Figure modified from Vicedo et al. (2009).

Bioactive signal jasmonoyl-isoleucine (JA-Ile; Chico et al., 2008) increased sharply in Hx-primed tomato plants after *Botrytis* inoculation. In addition, Hx-IR was blocked in the JA-insensitive mutant *jai1*, a *coi1* homolog (Vicedo et al., 2009), which is impaired in receptor COI1 (Yan et al., 2009). Oxylipin12-oxo-phytodienoic acid (OPDA) was also primed by Hx treatment after fungal infection (Vicedo et al., 2009). OPDA, a precursor of JA, is partly a regulator of plant defenses in a JA-independent manner and is also active against microorganisms, including *Botrytis cinerea* (Stintzi et al., 2001; Prost et al., 2005). The increase in JA observed in water-treated plants upon infection was not detected in Hx-treated plants, which suggests that this hormone recirculates into its conjugated forms, like JA-Ile (Vicedo et al., 2009). The analysis of tomato genes induced in response to *Botrytis* showed that, consistently with metabolic changes, Hx pre-treatment significantly primes *LoxD*, a LOX involved in the oxylipins pathway leading to OPDA and JA synthesis (Cohn and Martin, 2005; Flors et al., 2007).

Abscisic acid-deficient mutant *flacca* (flc) was also impaired in Hx-IR in three different backgrounds (Vicedo et al., 2009) and correlated with the absence of callose priming upon infection. Therefore, ABA can act as a positive regulator of Hx-IR by enhancing callose deposition, as previously reported for BABA-IR in *Arabidopsis* (Ton and Mauch-Mani, 2004).

HEXANOIC ACID IS A BROAD-SPECTRUM NATURAL INDUCER

Hexanoic acid treatment has also been found to protect *Arabidopsis* plants against *Botrytis cinerea* (Kravchuk et al., 2011). In this case, Hx-IR has also been associated with changes in the JA-signaling pathway upon infection. The JA and ET defense-response marker gene *PDF1.2* (Penninckx et al., 1996), the JA-regulated hevein-like protein gene *PR4* (Van Damme et al., 1999) and the specific JA-inducible marker gene *VSP1* (Norman-Setterblad et al., 2000) were primed in Hx-treated plants upon *Botrytis* infection. The JA and OPDA levels were induced at early stages in Hx-treated plants upon infection, as previously observed in tomato. Accordingly, JA-impaired mutant *jar1* and *jin1-2* were unable to display Hx-IR. JAR1 encodes an enzyme that conjugates JA with Isoleucine (Staswick et al., 2002), while JASMONATE-INSENSITIVE1 (JAI1/JIN1) encodes AtMYC2, which is a nuclear-localized transcription factor whose expression is rapidly up-regulated by JA content (Lorenzo et al., 2004). Thus, the metabolic switch for hexanoic must act upstream of both genes. Further analyses have demonstrated that the plants impaired in the ET, SA, and ABA pathways show intact protection by Hx upon *Botrytis cinerea* infection. Accordingly, no significant changes in SA marker gene *PR1* and in the SA or ABA hormone balance were observed in infected and treated plants. The *eds1-1* mutant (Zhou et al., 1998; Falk et al., 1999) was unable to display Hx-IR. EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) is a nucleocytoplasmic lipase-like involved in plant defense signal transducing (Brodersen et al., 2006; Heidrich et al., 2011). Future research will clarify the possible role of this key regulator in Hx-IR.

Callose accumulation is also primed in Hx-treated *Arabidopsis* plants upon infection. However, it is not essential to express Hx-IR in *Arabidopsis* since the *pmr4-1* mutant (Powdery Mildew Resistant 4-1; Nishimura et al., 2003) is protected by Hx, despite it being unable to synthesize callose. This differs from Hx-IR in tomato, where callose plays an important role in resistance against *Botrytis*. The main function of callose is to act as a physical barrier against pathogens, but it may also reduce the permeability of the toxins secreted by pathogens, and of other molecules. This scenario has been suggested in *Arabidopsis* plants primed by thiamine against *Sclerotinia sclerotiorum* (Zhou et al., 2013). Hx-IR against *Botrytis cinerea* also seems to be independent of ABA production in *Arabidopsis* since the mutant partially impaired in ABA synthesis *npq2-1* (Niyogi et al., 1998) was fully protected by Hx (Kravchuk et al., 2011). All these results indicate that Hx priming against *Botrytis cinerea* is based on JA- and other oxylipin-related defenses in both tomato and *Arabidopsis* by activating additional responses in each background.

Hexanoic acid has also been seen to increase resistance against necrotrophs *Alternaria brassicicola* in *Arabidopsis* (Kravchuk et al., 2011) and *Alternaria alternata* in Fortune mandarin (Llorens et al., 2013). In this case, both JA-signaling and callose priming were required for Hx-IR. Furthermore, a more rapid accumulation of ABA was observed, which could act as a positive regulator of callose deposition, as described in tomato, thus reinforcing the fact that both enhanced physical barriers and the JA-signaling pathway are involved in Hx-IR against necrotrophic pathogens.

Hexanoic acid has been reported to protect tomato plants against hemibiotrophic bacterium *P. syringae* pv tomato DC3000 (Vicedo et al., 2009). In this case, Hx-IR seems to counteract the negative effect of the pathogen coronatine (COR) and JA-Ile on the SA pathway (Scalschi et al., 2013). Hx treatment reduced JA-Ile content upon infection at the expense of an increased expression of jasmonic acid carboxyl methyltransferase (JMT) and of SA marker genes *PR1* and *PR5*, which indicates a boost in this signaling pathway. Interestingly, Hx treatment prompted OPDA accumulation, as seen in tomato and in *Arabidopsis* against *Botrytis cinerea*, suggesting that this molecule might play a role *per se* in Hx-IR. Hence the obtained results support a positive relationship between the SA

and the JA pathways in Hx-primed plants. Hx also seems to inhibit stomatal opening in tomato plants in the presence of COR, which implies that this treatment suppresses pathogen effector action to prevent bacterial entry into the mesophyll (Scalschi et al., 2013). Therefore, Hx induces plant responses in different host plants and against pathogens with distinct lifestyles through a common strategy based on the priming of OPDA accumulation and JA-signaling by diverting it toward the accumulation of different JA-conjugates that depend on the attacking pathogen.

HEXANOIC ACID REGULATES AND PRIMES *Botrytis*-SPECIFIC AND NON-SPECIFIC GENES

We recently analyzed the gene expression profile of *Botrytis*-infected tomato plants 24 hpi by microarray analysis (Finiti et al., 2014). The results indicated that tomato plants respond early to *Botrytis* inoculation by activating a large set of genes, which are mainly related with the biotic stress response, and interestingly with the oxidative stress response. The most induced genes were proteinase inhibitors, defense genes (especially fungus and chitin activated-genes), transcriptional factors, and signaling and hormone-related genes. Oxylipins-, ethylene-, and auxin-related genes were induced, which corroborate the involvement of these pathways in the early response to *Botrytis*. Remarkably a set of redox-related genes was also induced, which evidences the involvement of oxidative stress mechanisms in this plant–pathogen interaction.

The microarray gene expression profile of Hx-treated plants revealed the induction and priming of many *Botrytis*-induced genes (Finiti et al., 2014). This means that Hx preventively activates these genes, thus preparing plants for an alarmed state, which would facilitate a quicker, better response against pathogen attack. Hx is also capable of priming and enhancing the expression of many of those genes after fungus inoculation. This confers increased resistance to treated plants without wasting resources until infection occurs. It is noteworthy that Hx activated a set of genes, which was not induced by the fungus at 24 hpi. These specific Hx early induced genes may prove advantageous for treated plants, and could be further studied as targets of new preventive defense strategies (Boyd et al., 2013).

The microarray technique revealed a high induction in infected Hx-treated plants of the genes encoding for proteinase inhibitors responsive to JA, wounding and insect feeding (Farmer et al., 1992). This evidences the relevance of the JA-pathway in Hx-IR, and how proteinase inhibitors might play a key role in the tomato-*Botrytis* interaction. Genes coding for proteinase inhibitors could also represent new targets for treatments and genetic engineering to increase plant resistance.

The genes involved in the oxylipins pathway, such as *LoxD*, *DES* (divinyl ether synthase) and *Dox1* aplha-dioxygenase, were induced and primed by Hx (Finiti et al., 2014), thus supporting the priming of this metabolic pathway as part of Hx-IR (Vicedo et al., 2009). Notably, the 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene was also primed by Hx. According to Diaz et al. (2002), early ET synthesis activation prior to pathogen attack can increase plant resistance against *Botrytis cinerea*. Hence, the pre-activation and boost of the *ACC* oxidase gene expression found in treated plants likely contributes to increased resistance. SA-responsive defense genes, like *PR1a* and *endochitinase 3*, were also induced in treated plants. This shows the complex effect of Hx priming on the hormonal balance, with a likely positive effect on both the JA and SA pathways, traditionally considered antagonist, as previously observed in Hx-IR against *Pst* (Scalschi et al., 2013).

Many genes encoding for WRKY family members, which modulate the defense response, were induced and primed in Hx-treated plants (Finiti et al., 2014). Huang et al. (2012) reported the involvement of SlWRKYs (*Solanum lycopersicum* WRKYs) in responses to different abiotic and biotic stresses, including *Botrytis cinerea* infection. Among the Hx-induced WRKYs, there are orthologs of *Arabidopsis WRKY18*, *WRKY33*, *WRKY40*, *WRKY53* and *WRKY75*, which form part of the *Arabidopsis* defense response, especially against *Botrytis cinerea* (AbuQamar et al., 2006; Pandey et al., 2010; Birkenbihl et al., 2012). Hx's capability of acting on these regulatory multi-response factors probably contributes considerably to its broad-spectrum IR.

It is particularly noteworthy that Jaskiewicz et al. (2011) have recently reported how *WRKY53* is a specific target of BTH priming in *Arabidopsis*, and that it might be considered a priming marker gene. According to the above-cited authors, priming occurs through changes in histone acetylation before pathogen inoculation. Then the pre-acetylated gene is induced more quickly upon pathogen recognition, leading to higher expression levels and a better defense response. Hence, the over-induction of *WRKY53* observed in Hx-treated plants upon infection supports Hx playing a priming agent role, and being capable of preparing the plant in a silent mode without wasting too much energy until a pathogen is detected.

Hx TREATMENT ALLEVIATES OXIDATIVE STRESS

Oxidative burst and ROS accumulation are critical factors in plant responses to *Botrytis* infection (Heller and Tudzynski, 2011), but the contribution of these factors to plant defense is complex because *Botrytis* stimulates ROS production to its own benefit (Temme and Tudzynski, 2009). Microarray data have indicated that the response of Hx-treated tomato plants is similar to the response of those inoculated with *Botrytis*, which reveals the activation of many redox status-related genes (Finiti et al., 2014). Several of the genes induced by *Botrytis* are over-induced and primed in Hx-treated plants, including peroxidase, glutathione reductase, NADPH quinone reductase, and several glutathione *S*-transferases (GSTs). Other genes, like GST and glutaredoxin, are induced only in treated plants and represent specific targets of the inducer treatment. The early boosting of detoxifying and redox-balance-related genes achieved by Hx supports the direct effect of this inducer on the control of these processes. The analysis of oxidative stress markers confirmed that Hx treatment protects plants by providing a less oxidized cellular environment after infections (Finiti et al., 2014). Superoxide ion (O_2-) and peroxide hydrogen (H_2O_2) accumulation reduced and was more restricted around the infection site. The ascorbate and glutathione reduced/oxidized ratios rose in treated plants at 72 hpi, while the activities of glutathione reductase and catalase remained closer to those of healthy plants. No changes were detected in Hx-treated, but not-infected, plants.

Therefore, Hx primes the transcription of the genes controlling the redox metabolism, which is fully activated and shown only after pathogen recognition (Finiti et al., 2014), just as the priming definition establishes (Conrath et al., 2002). Hence Hx treatment can limit oxidative stress in infected plants by damping the fluctuations of the redox equilibrium and preventing its harmful effects in later infection steps. Interestingly in *Arabidopsis*, wound-IR against *Botrytis cinerea* also requires glutathione and the priming of the gene encoding *GST1* in leaves inoculated with the fungus (Chassot et al., 2008). Studies carried out with the tomato ABA-deficient mutant *sitiens* have also revealed that the timely hyperinduction of H_2O_2-dependent defenses on the epidermal cell wall can effectively block early *Botrytis cinerea* development (Asselbergh et al., 2007), which further demonstrates the importance of oxidative stress in this plant–pathogen interaction.

As previously mentioned, other natural priming agents which promotes defense response like thiamine, riboflavin, MSB, VOCs, OGs, and chitosan also affect the oxidative balance contributing to reduce the symptoms and damages associated to biotic stresses (**Table 1**). Thiamine can modulate the cellular redox status by activating the NADPH oxidase and promoting early ROS generation, which confers resistance against *Sclerotinia sclerotiorum* in *Arabidopsis* (Zhou et al., 2013). Riboflavin promotes the H_2O_2 burst independently of the known hormonal pathways in *Arabidopsis*, suggesting a distinct signaling process for this priming compound. MSB is a ROS generator too, but it also induces detoxification genes like several GSTs and ABC transporters that may scavenge toxic compounds generated during oxidative stress (Borges et al., 2009). In recent years attention has been directed toward the antioxidant activity of chitosan. It promotes ROS generation mainly through the plasma membrane NADPH oxidase, inducing the hypersensitive response and programmed cell death. However, the water-soluble chitosan is an excellent scavenger of hydroxyl radicals, H_2O_2 and anion superoxide, revealing the diverse properties of this compound (El Hadrami et al., 2010).

Priming agents that increase abiotic stress tolerance have been also associated with oxidative stress control. Sodium hydro-sulfide (NaHS) protects plants from salinity and non-ionic osmotic stress by altering the redox machinery in a similar way than Hx (Christou et al., 2013). Treatment with NaHS maintains low ROS concentration in stressed strawberry plants activating enzymatic

Table 1 | Natural inducers and their effects on plant defensive mechanisms reported in this work.

Inducer	Plant	Pathogen	SA*	JA* oxilipins	ET*	SAR	Defense effectors	Cell wall tightening	Oxidative balance	Reference
Hexanoic acid	*Tomato*	*Botrytis cinerea*	+	+	+	Nd	+	+	+	Vicedo et al. (2009), Finiti et al. (2013)
	Arabidopsis	*Botrytis cinerea*	Nd	+	Nd	Nd	+	+	Nd	Kravchuk et al. (2011)
	Tomato	*P. syringae*	Nd	+	Nd	Nd	+	+	Nd	Scalschi et al. (2013)
Thiamine	*Rice, Arabidopsis*	Fungal, bacterial, viral infections	+	−	−	+	−	+	+	Ahn et al. (2007)
Riboflavin	*Arabidopsis*	*P. syringae*	−	−	−	+	+	+	+	Zhang et al. (2009)
	Tomato	*Botrytis cinerea*	Nd	+	−	+	Nd	Nd	−	Azami-Sardooei et al. (2010)
PABA	*Pepper*	CMV, *Xanthomonas*	+	−	Nd	+	+	Nd	Nd	Song et al. (2013)
MSB (k3)	*Arabidopsis*	*P. syringae*	Nd	Nd	Nd	−	+	Nd	+	Borges et al. (2009)
VOCs	*Maize, Bean, Arabidopsis*	Insects	Nd	+	+	Nd	+	+	+	Frost et al. (2008)
OGs	*Arabidopsis*	*Botrytis cinerea*	−	−	−	−	+	Nd	+	Ferrari et al. (2007)
Azelaic acid	*Arabidopsis*	*P. syringae*	+	−	−	+	+	Nd	Nd	Jung et al. (2009)
Pipecolic acid	*Arabidopsis*	*P. syringae*	+	Nd	Nd	+	+	Nd	Nd	Vogel-Adghough et al. (2013)
Chitosan	*Soybean, tomato, maize*	Fungal, bacterial, viral infections	Nd	+	Nd	Nd	+	+	+	El Hadrami et al. (2010)
		Colletotrichum sp	Nd	+	Nd	Nd	+	+	+	
		Xanthomonas	Nd	+	Nd	Nd	+	+	+	
	Broccoli	*P. fluorescens*	Nd	+	Nd	Nd	+	+	+	Li et al. (2010)

Abbreviations: (+) activates, (−) does not activate, (Nd) not determined.
**SA, JA, and ET are figured as dependency (+) of the pathway, or not (−).*

antioxidants such as superoxide dismutases, catalases and ascorbate peroxidases. It also increases the ascorbate and glutathione redox states and induces the expression of key genes for ascorbate and glutathione biosynthesis.

In conclusion, microarray data of Hx-treated plants have revealed the induction of many genes that help characterize the Hx priming effect, especially those related with defense, the signaling network and oxidative stress control, which are over-induced in Hx-treated plants upon fungal infection. The activation and priming of different defense genes responding to the SA and JA pathways match the broad-spectrum action of this natural inducer. This agrees with the present conception that the effectiveness of the plant response against biotrophic and necrotrophic pathogens is much more complex than the classical dichotomy between the SA and JA pathways antagonism. Finally, Hx priming of redox-related genes produces an anti-oxidant protective effect, which might be critical for limiting necrotroph infection. These findings back the importance of controlling oxidative stress to improve plant protection against different pathogens, and suggest that this natural inducer is an attractive tool to further study this topic.

ANTIMICROBIAL ACTIVITY OF Hx

Some natural compounds, which act as inducers, may also have a direct antimicrobial effect under certain conditions. Among these we find chitosan (Ben-Shalom et al., 2003) and some plant volatile compounds (He et al., 2006). This is the case of Hx as it also inhibits *Botrytis cinerea* growth at higher concentrations than those which allow the priming of plant defenses (Leyva et al., 2008). Hx blocks spore germination at a very early stage, prevents germ-tube development and also inhibits *in vitro* mycelia growth of germinated spores. Once again, this reflects the remarkable versatility of this natural compound to act on the plant and pathogen in a concentration-dependent manner.

The characterization of the mechanisms underlying the antimicrobial effect of Hx shows a retraction of the cytoplasm in treated spores, as previously demonstrated for other natural compounds with antifungal properties against *Botrytis cinerea,* such as resveratrol (Gonzalez Ureña et al., 2003) and synthetic compound adipic acid monoethyl ester (AAME; Vicedo et al., 2006). Hx treatment of previously germinated spores has altered fungal membrane permeability by producing a phosphate efflux with no lytic activity. A similar effect has been observed for not only several diterpenoids with antifungal activity against *Botrytis cinerea* (Cotoras et al., 2004), but also for a series of aliphatic (2 E)-alkenals, from C5 to C14, characterized as antimicrobial agents (Kubo et al., 1995). In addition, Hx treatment has been reported to increase the levels of spermine, spermidine, putrescine, and cadaverine in *Botrytis cinerea* mycelia. Polyamine metabolism is a target of other antifungal compounds (Walters, 1995) and plays a main role in programmed cell death (Walters, 2000).

The twin effect of Hx on both germination and mycelia growth has been observed only in highly efficient synthetic fungicides, such as phenylpyrroles and hydroxyanilides (Rosslenbroich and Stuebler, 2000). This twin effect was also confirmed *in planta.* Spraying Hx at fungicidal concentrations (16 mM) on tomato plants prior to fungal inoculation reduces the diameter of necrosis by ∼60%. Application on previously infected plants further reduces necrotic expansion by around 30% (Leyva et al., 2008). We have also observed this preventive and curative effect on mature green fruits and its curative effect on fruits in different ripening stages (**Figure 3**; Leyva Pérez, 2008). The antifungal properties of Hx have also been demonstrated in Micro-Tom tomato plants (Leyva Pérez, 2008) by making the most of its susceptibility to a wide range of pathogens (Takahashi et al., 2005) and the possibility of infecting fruit-producing plants with *Botrytis cinerea* under laboratory-controlled conditions. Spraying Hx at fungicidal concentrations on 2-month-old plants prior to fungal inoculation reduces the diameter of necrosis by ∼15%. Application of Hx on previously infected plants further reduces necrotic expansion by around 60% (Leyva Pérez, 2008). These treatments have no phytotoxic effects and demonstrate the ability of Hx to prevent and reduce *Botrytis* infection in tomato plants and fruits. This feature makes Hx a good candidate to protect tomato crops and for post-harvest application at either fungicide or inducer concentrations.

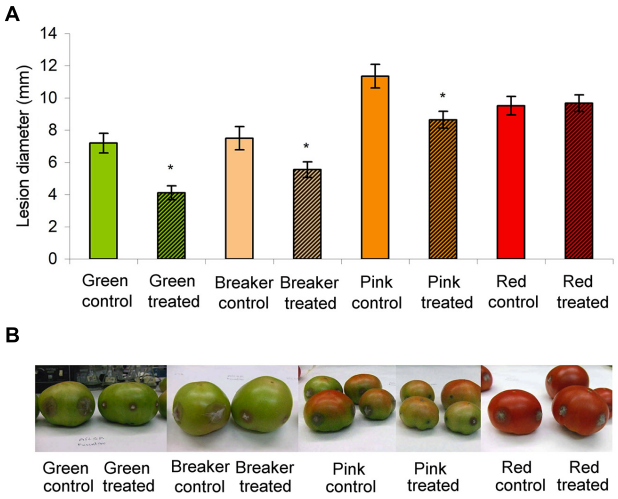

FIGURE 3 | Treatment with hexanoic acid reduces the disease symptoms in tomato fruits infected with *Botrytis cinerea*. Tomato fruits (cv. Ailsa Craig) harvested at different ripening stages were wounded and inoculated with 5 μL of a *Botrytis cinerea* conidia suspension in each wound. 24 h later, when the first symptoms of infection are already visible, twenty fruits were sprayed with Hx 20 mM. Control fruits were sprayed with sterile water. Three days after treatment fruits were scored for symptoms by measuring the lesion diameter. Statistically significant differences are indicated with (*p-value < 0.05). Representative images of the infected fruits are also provided. Figure from Leyva Pérez (2008).

CONCLUSION AND FUTURE PERSPECTIVES

The study into natural plant inducers has helped unravel the complex mechanisms underlying the IR phenomenon (**Table 1**).

As this review shows, Hx-IR shares the protection strategies and mechanisms promoted by several vitamin treatments, like JA dependent pathway activation. Hx can modulate the cellular redox status to protect tomato plants against *Botrytis cinerea* in an early infection stage, as demonstrated for thiamine against *Sclerotinia sclerotiorum* in *Arabidopsis.* In addition, both natural priming agents induce cell wall fortifications with callose. However, the

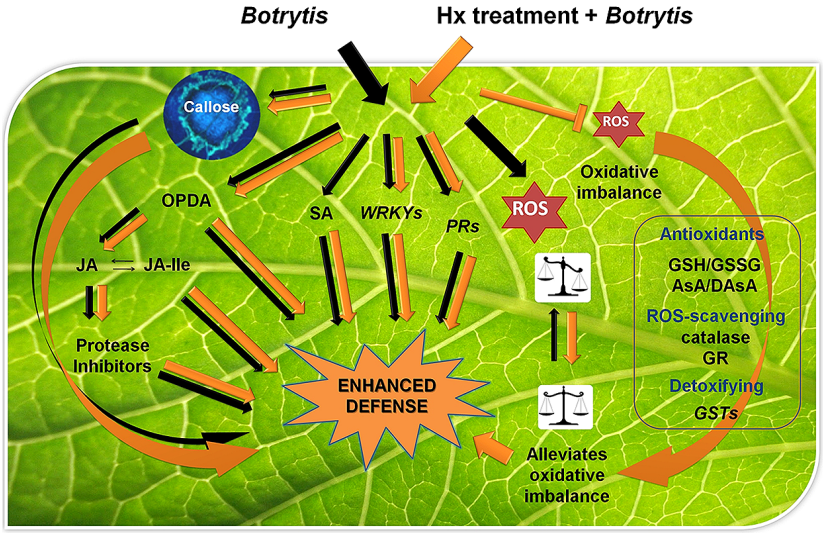

FIGURE 4 | Model for the Hx priming effect on plant defense mechanisms against *Botrytis cinerea*. Black arrows indicate responses in untreated plants upon *Botrytis* infection. Orange arrows indicate induced responses in Hx-treated plants upon *Botrytis* infection. Hx-treatment increases *Botrytis*-induced responses enhancing callose, OPDA, JA and JA-Ile accumulation; potentiating transcript accumulation of genes like *WRKYs*, protease inhibitors and *PRs*, and inducing anti-oxidant, ROS-scavenging and detoxifying mechanisms. Hx, by counteracting the massive ROS accumulation induced by the fungus, alleviates the oxidative imbalance associated with *Botrytis* infection. Abbreviations: JA-Ile, jasmonoyl-isoleucine; GSH/GSSG, reduced/oxidized glutathione ratio; AsA/DAsA, reduced/oxidized ascorbate ratio; GR, glutathione reductase; GSTs, glutathione-S-transferases.

fact that Hx-IR primes some genes like GST and glutaredoxin, which are not early induced by *Botrytis* in tomato, indicates that these genes can be the direct targets of this natural inducer. It is noteworthy that one of the most highly induced genes by MSB, a water-soluble derivative of vitamin K3, encodes for a GST, which is highly induced by H_2O_2 and NO treatments. Hence, a common priming mechanism can relay the more efficiently toxic compounds generated during oxidative stress in scavenging.

Another interesting contribution that stems from studying resistance induced by natural compounds, including Hx, is the evidence found that oxylipins are involved in the priming mechanism in a JA-independent manner. Hx-IR correlates with OPDA accumulation in all the studied pathosystems. It is stressed that the transcriptome analysis of the *Arabidopsis* response to OPDA revealed that 17% of induced genes are related to detoxification processes (Mueller et al., 2008). The most relevant OPDA-induced genes encode GSTs, cytochrome P450s and UDP-glucosyltransferases, and various transporters. Detoxification genes constitute the first line of defense against different stresses, so it is not surprising that they are induced by priming agents like Hx.

It is worth mentioning the similarity of the target genes activated in tomato in Hx-IR against *Botrytis cinerea* to those activated in *Arabidopsis* in menadione-IR against *Pst.* Genes like *GST*s, *WRKY18* and *WRKY40* can modulate the plant response in accordance with the challenging pathogen's lifestyle. This can explain why the mode of action of priming agents is determined eventually by hosts and by the nature of the stress challenging them (Ahn et al., 2007). This evidence suggests that the regulation of the strategic components of plant signaling crosstalk is a key target of naturally priming agents, probably through the induction of epigenetic changes like histone acetylation. Further research using Hx as a model, and with other natural inducers, will elucidate the nature of these putative epigenetic changes.

We propose Hx, a potent natural priming agent in a wide range of host plants and pathogens, as a model tool in this field of research (**Figure 4**). It can early activate broad-spectrum defenses by inducing callose deposition, in addition to the SA and JA pathways. Later it can prime pathogen-specific responses in each particular case according to the pathogen and its lifestyle. Interestingly, Hx primes redox-related genes and has an anti-oxidant protective effect, which might be critical for limiting the infection of necrotrophs.

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Priming crops against biotic and abiotic stresses: MSB as a tool for studying mechanisms

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INTRODUCTION

Biotic and abiotic stresses are the main problems affecting agricultural losses. Consequently, understanding the mechanisms underlying plant resistance or tolerance helps us to develop fruitful new agricultural strategies. These will allow us to face the challenges of producing food for a growing human population in a sustainable and environmentally friendly way.

To compensate for their sessile life and face a broad range of biotic and abiotic stresses, plants have evolved a wide range of survival and adaptation strategies. Amongst them, higher plants are capable of inducing some stress "memory," or "stress imprinting." Bruce et al. (2007) define stress imprinting as genetic or biochemical modifications induced by a first stress exposure that leads to enhanced resistance to a later stress. This phenomenon also known as "priming" results in a faster and stronger induction of basal resistance mechanisms upon subsequent pathogen attack, or greater tolerance against abiotic stresses (Pastor et al., 2013). Basal resistance by itself is too weak to protect against virulent pathogens, since it constitutes a residual level of resistance after immune suppression by the pathogen through co-evolution (Walters and Heil, 2007; Conrath, 2011). However, Ahmad et al. (2010) proposed that priming-inducing stimuli can provide more effective basal resistance, particularly when an earlier defense response precedes immune suppression by the invading pathogen.

Following perception of microbe-associated molecular patterns (MAMPs), recognition of pathogen-derived effectors or colonization by beneficial microbes, priming can also be induced by treatment with some natural or synthetic compounds or even by wounding (Conrath, 2011). Through priming plants are able to induce responses to a range of biotic and abiotic stresses, providing low-cost protection in relatively high stress-pressure conditions. Despite priming phenomena having been widely described, the molecular mechanisms of defense priming are still unclear. Such techniques are now starting to emerge as a promising alternative for sustainable modern pest management in the field, since some pesticides have been shown to actually exert their known plant health- and yield-increasing effects through priming (Beckers and Conrath, 2007). From an ecological point of view, the benefits of priming are clear: rather than leading to the costly and potentially wasteful activation of defenses, a metabolic state of alert is induced after an initial infection, enabling a rapid intense resistance response to subsequent attacks. Thus, this strategy appears promising for crop protection purposes (Walters and Heil, 2007).

REACTIVE OXYGEN SPECIES: KEY MOLECULES IN PRIMING

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radicals are inherent by-products of aerobic metabolism. ROS have not only the potential to cause oxidative damage by reacting with biomolecules, but it is widely accepted that they also have key roles as signaling molecules that contribute to control of plant development and to the sensing of the external environment (Smirnoff, 2005; del Río and Puppo, 2009).

ROS metabolism includes a complex network that interacts closely with hormonal signaling systems and allows plants a subtle regulation of developmental events as well as biotic and abiotic stress responses. Oxidative stress is the term widely used to define the imbalance between ROS production and scavenging or detoxification (Pastor et al., 2013). Recently, a mechanism to explain the role of ROS in cell signaling has been reported. This model proposes that changes in redox homeostasis generate specific ROS signals or ROS waves that, next to other signals such as hormones and small peptides, can prime neighboring cells to defense (Mittler et al., 2011). The afore-mentioned ROS signal waves are sensed by specific receptors that can transfer the message to activate other networks through phosphorylation cascades using mitogen-activated

protein kinases (MPKs) (Colcombet and Hirt, 2008). ROS have been involved in priming events induced by biotic and abiotic stimuli, although the mechanisms are so far not well established. One of the challenges in ROS research is to identify specific ROS receptors and to establish how the cell is able to decode endogenous ROS signals and discriminate between different stimuli giving rise to a very specific defense response. In addition to ROS, nitric oxide (NO) is another key signaling molecule involved in different cellular process (Romero-Puertas et al., 2013). It can induce a priming protective effect against biotic and abiotic factors through a complex network, probably involving ROS by inducing antioxidant systems (Sun and Li, 2013), calcium ions and hormones. This area deserves further research.

MSB: A NOVEL PRIMING AGENT

Menadione sodium bisulphite (MSB) is a water-soluble addition compound of vitamin K3, or pro-vitamin K. Menadione, previously thought to be synthetic, has been isolated from fungi and phanerogams (Binder et al., 1989). Moreover, it is a redox-active compound widely used in the study of oxidant stress in plants (Sun et al., 1999), mammals (Shi et al., 1996), fungi (Emri et al., 1999), and bacteria (Mongkolsuk et al., 1998). It is promptly subjected to cell-mediated one-electron reduction, generating superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Hassan and Fridovich, 1979). The physiological function of vitamin K in plants is associated directly with its redox properties. Quinones, benzoquinones, and naphthoquinones such as menadione have two major chemical properties that render them reactive in biological systems. They may attract electrons acting as oxidant agent or electrophile, and in turn also donate electrons, acting in this case as reducing agent or nucleophile. The grade to which these properties contribute to overall toxicity is highly dependent on the concentration, and the chemical and cellular exposure conditions (Castro et al., 2007). This property can induce an increased production of ROS in which vitamin K3 (within the group formed by vitamins K1 and K2) seems to be more active in the induction of oxidative stress. It has been proposed that vitamin K3 could be converted once metabolized into vitamin K1, but this has not yet been demonstrated (Manzotti et al., 2008). The most studied of such compounds, vitamin K1 or phylloquinone, has been detected inside thylakoid membranes as an electron carrier and key element within the photosystem I redox chain. A recently published review suggests the role of vitamin K as mobile electron carrier in the transport chain transferring electrons across the plasma membrane, and the possibility that this molecule contributes to the maintenance of a suitable redox state of some important proteins embedded in the plasma membrane with protective functions against stress (Lüthje et al., 2013). Phylloquinone is a metabolite of the shikimate pathway widely used by plants and bacteria but not by animals, for this reason they must obtain some compounds including vitamin K through their diet. The physiological function of vitamin K in plants is directly linked to its redox properties deriving from the presence of a double quinone functional group on the naphthalenic ring. In fact, similarly to many other quinones and naphthoquinones, vitamin K can be reduced and reoxidized cyclically by several substances and enzyme pools (Döring and Lüthje, 1996; Lütthje et al., 1998). Given its hydrophobic nature, menadione can easily cross biological membranes, allowing it to enter organelles and and catalyze superoxide, hydrogen peroxide, and hydroxyl radical production (Hassan and Fridovich, 1979; Lehmann et al., 2012). A recent study in Arabidopsis roots using menadione as oxidant showed that ROS are produced by an electron transport chain via mitochondria and plastids (Lehmann et al., 2012). Furthermore, De Nisi et al. (2006) observed that menadione is capable of increasing the activity of H^+-ATPase. This enzyme uses energy derived from ATP hydrolysis to pump protons from the cytoplasm to the apoplast, which creates and maintains a negative membrane potential and an acid pH in the extracellular space. This electrochemical gradient can control many aspects of transport through the plasma membrane, such as secondary transport control of cell turgor, stomatal closure (Elmore and Coaker, 2011) or the movement of sucrose and amino acids to the cytoplasm by symport transporters (Morsomme and Boutry, 2000). This latter might be involved in regulating the activity of this H^+-ATPase of the plasma membrane during the defensive response against pathogens (Elmore and Coaker, 2011).

MSB, first studied as a plant growth regulator (Rama-Rao et al., 1985), has been widely demonstrated to function as plant defense elicitor against several pathogens in a number of different plant species (Borges et al., 2003, 2004, 2009; Liu et al., 2006; Pushpalatha et al., 2007; ShengYi et al., 2007). Changes in gene expression in response to 0.2 mM MSB at different timepoints post-treatment, using microarray technology, show that MSB leads to a unique molecular mark by inducing differentially the expression of 158 genes. More up-regulated genes were included in categories such as "response to stress" than the background, and the behavior of these genes in different treatments confirmed their role in response to biotic and abiotic stress (Borges et al., 2009). Different applications of MSB in agriculture have been patented (Borges-Pérez and Fernández-Falcón, 1996; Borges-Rodríguez et al., 2008; Borges-Rodríguez and Borges-Pérez, 2010) and several MSB-based commercial formulations have been marketed.

MSB was capable of inducing resistance by priming in Arabidopsis against the virulent strain *Pseudomonas syringae* pv. tomato DC3000 (Borges et al., 2009). Previous studies in oilseed rape plants (*Brassica napus* cv Bristol) showed that MSB-pretreatment 24 h before inoculation with *Leptophaeria maculans* exhibited rings of necrotic mesophyll cells surrounding the invasive hyphae of *L. maculans*, after staining with aniline blue in lactophenol. In water pre-treated control plants, unobstructed *L. maculans* hyphal growth was observed at infection sites, with no visible host reaction (Liu et al., 2006). However, staining assays of MSB-treated Arabidopsis plants did not fit with the generation of ROS or SAR *in planta*, despite the fact that a significant up-regulation of genes involved in ROS detoxification was found in the microarray. In this interaction, MSB induced resistance by priming without inducing necrosis or visible damage (Borges et al., 2009). Furthermore, a western blot analysis of the known SA signaling pathway marker

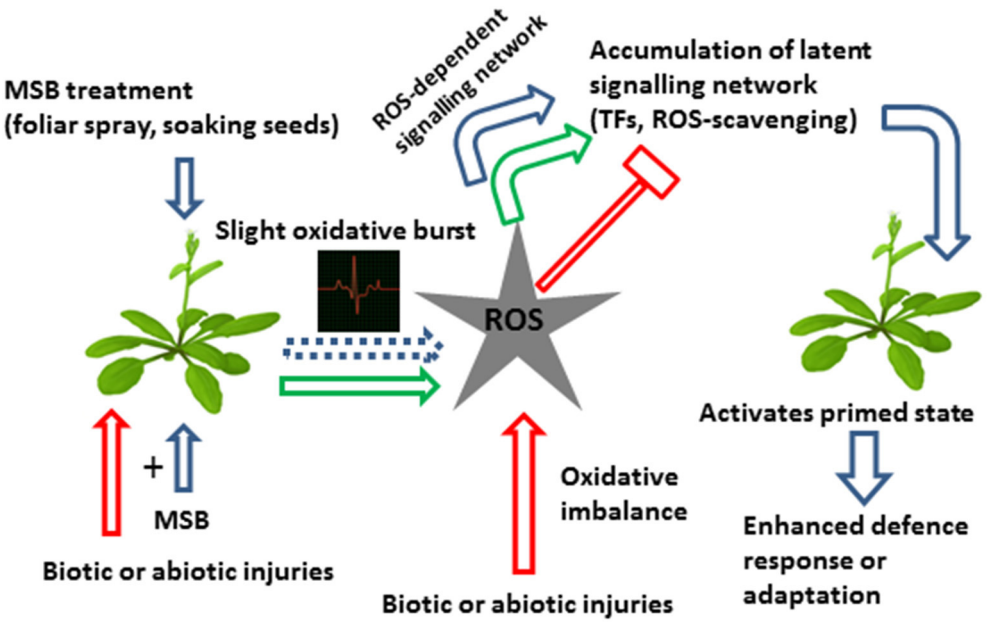

FIGURE 1 | Hypothetical model on the effects of MSB on plant defense mechanisms against biotic and abiotic stresses. MSB treatment is capable of inducing resistance by priming through of a slight oxidative burst which develops a ROS-dependent signaling network and inducing the accumulation of latent defense proteins such as ROS-scavenging and transcription factors, among others, resulting in a primed state and an enhanced stress response. Abbreviations: TFs, transcription factors; ROS-scavenging, reactive oxygen species-scavenging.

PR1 (Dong, 2001) showed that MSB does not itself induce PR1 protein expression. Contrastingly, 3 days after inoculation, MSB-pretreated plants enhanced more than two-fold PR1 expression as compared with mock plants (Borges et al., 2009). Finally, the promoter analysis of MSB-induced *cis*-elements in the microarray clearly showed that most of the genes up-regulated by MSB contain the G-box in their promoter regions. Some interesting functions were represented among the individual up-regulated genes, such as glutathione S-transferases, transcription factors (including putative regulators of the G-box) and cytochrome P450s (Borges et al., 2009). In **Figure 1** we propose a hypothetical model that summarizes the possible mode of action of MSB as priming agent in planta.

Another notable effect of MSB is its capacity to induce a reduction in insect growth rate (*unpublished*). Interestingly, a recent MSB application has been patented for controlling *Trioza erytreae* and *Diaphorina citri*, the psyllid vectors carrying the genus *Candidatus Liberibacter* that are bacterial causal agents of the most serious citrus disease known as Huanglongbing (HLB) (Borges-Rodríguez and Borges-Pérez, 2010).

Another effect of menadione on abiotic stresses is to induce tolerance to chilling stress in maize seedlings (Prasad et al., 1994). These authors suggested that exogenous application of menadione and H_2O_2 to the seedlings might induce a mild oxidative stress leading to chilling tolerance (Prasad et al., 1994). A very recently published work from our laboratory has focused on the MSB effect at the seed stage (Jiménez-Arias et al., forthcoming). Firstly, we found that soaking Arabidopsis seeds in 20 mM MSB induces salt tolerance by priming an early plant adaptation and proline accumulation. In addition, it was found that MSB primes the expression of key transcription factors such as Zat12, one of the key zinc-finger proteins encoded by a multi-gene family and involved in a ROS-dependent signaling network against abiotic stress (Mittler et al., 2011). Interestingly, it was also found that MSB leads to a hypomethylation state in the promoter region of genes involved in the biosynthesis (PYRROLINE-5-CARBOXYLATE SYNTHETASE 1, *P5CS1*) and degradation (EARLY RESPONSIVE TO DEHYDRATION 5, *ERD5*) of proline, demonstrating that one of the mechanisms underlying this early adaptation to salt stress is an epigenetic mark (*submitted for publication*).

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The new insights into cadmium sensing

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Cadmium (Cd) is non-essential heavy metal, which in excess, exhibits deleterious effects to the most of the organisms. Mobilization of defense mechanisms against this toxic agent requires rapid activation of signaling pathways. The article presents recent advances in the research concerning cadmium signal transduction in plants. New insights into the involvement of reactive oxygen species (ROS), nitric oxide (NO), plant growth regulators, and Cd-induced protein modifications are reviewed. Moreover, the role of recently recognized Cd-associated signal elements, including micro RNAs and several *cis*- and *trans*-acting elements is discussed.

Keywords: cadmium, plant signaling, reactive oxygen species, nitric oxide, plant hormones, transcription factors, micro RNA

INTRODUCTION

Contamination of the environment with heavy metals, including cadmium, is a serious problem of the modern world. It is estimated that annually around 30,000 tones of cadmium are released into the environment, of which 13,000 tones result from human activity (Gallego et al., 2012). As sedentary organisms, plants cannot move actively from a contaminated environment. Therefore, their only chance to survive unfavorable conditions is the mobilization of defense mechanisms, which requires the activation of a complex signaling network. The first barriers to the most of the stress factors are cell walls and cell membrane. Numerous studies indicate that cadmium causes stimulation of membrane-localized NADPH oxidase and, in consequence, augmentation of ROS production (Olmos et al., 2003; Garnier et al., 2006; Maksymiec and Krupa, 2006; Rodríguez-Serrano et al., 2006, 2009; Yakimova et al., 2006; Hsu and Kao, 2007; Ortega-Villasante et al., 2007; Yeh et al., 2007; Heyno et al., 2008; De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012). Early reaction to this heavy metal also includes the accumulation of other signaling molecules, namely calcium ions (Garnier et al., 2006; Yeh et al., 2007) and nitric oxide (NO) (Besson-Bard and Wendehenne, 2009; Mahmood et al., 2009; Arasimowicz-Jelonek et al., 2012). The cadmium signal might also be transmitted by polyamines and plant hormones such as ethylene, auxins, and jasmonic (JA), salicylic (SA), and abscisic acid (ABA) (Rodríguez-Serrano et al., 2006; Yakimova et al., 2006; Al-Hakimi, 2007; Maksymiec, 2011; Wen et al., 2011; Kumar et al., 2012; Masood et al., 2012; Stroiński et al., 2013). Within the cytoplasm, response to this heavy metal is, at least in part, mediated by mitogen-activated protein kinases (MAPKs), which are stimulated by Cd on the transcriptional and post-translation levels (Agrawal et al., 2002, 2003; Kim et al., 2003; Jonak et al., 2004; Liu et al., 2010; Chmielowska-Bąk et al., 2013b; Ye et al., 2013). The last stages of the signal transduction pathways include the regulation of genes expression. Several studies report that this heavy metal modulates the expression of transcription factors (TFs) belonging to the MYB, HSF, bZIP, WRKY, and DREB families (Suzuki et al., 2001; Yanhui et al., 2006; Ogawa et al., 2009; Shim et al., 2009; Farinati et al., 2010; Wang et al., 2010). In the past few years, significant progress has been made in understanding the cross talk between these elements and their role in the transduction of the cadmium signal. The present review focuses on the latest insights into the role of reactive oxygen species (ROS), nitrogen oxide, and hormones in plant response to this heavy metal. The most recent findings concerning Cd-dependent regulation of genes expression are also discussed.

REACTIVE OXYGEN SPECIES

ROS are regarded as molecules causing damage to cells as well as ubiquitous signaling molecules participating in the recognition of and response to stress factors (Wrzaczek et al., 2013). It has often been postulated that ROS themselves are signal molecules. It seems that among various ROS, hydrogen peroxide (H_2O_2) acts as the primary messenger, in part because of its relative stability and in part because it can cross membranes through aquaporins (Møller and Sweetlove, 2010).

Reactive oxygen species, including hydrogen peroxide, seem to be important players in plants response to cadmium (**Table 1**). An abundance of published data indicate that Cd can promote the generation of H_2O_2 in both plants and plant cell cultures (Olmos et al., 2003; Garnier et al., 2006; Maksymiec and Krupa, 2006; Rodríguez-Serrano et al., 2006, 2009; Yakimova et al., 2006; Hsu and Kao, 2007; De Michele et al., 2009; Lehotai et al., 2011; Vestena et al., 2011; Arasimowicz-Jelonek et al., 2012; Zhao et al., 2012). Cd-induced H_2O_2 might be produced by plasma membrane NADPH oxidase or originate in mitochondria

as well as in peroxisomes and then diffuse to other parts of cells and to the apoplastic space (Romero-Puertas et al., 1999, 2004; Olmos et al., 2003; Garnier et al., 2006; Maksymiec and Krupa, 2006; Rodríguez-Serrano et al., 2006, 2009; Yakimova et al., 2006; Hsu and Kao, 2007; Ortega-Villasante et al., 2007; Yeh et al., 2007; Heyno et al., 2008; De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012). It has been reported that extracellular NADPH oxidase-dependent generation of H_2O_2 may be followed by increased production of superoxide anion ($O_2\bullet-$) in mitochondria, which in turn, causes fatty acid hydroperoxide accumulation (Garnier et al., 2006). NADPH oxidase generates superoxide by transferring electrons from

Table 1 | Summary of Cd-induced signaling events mediated by reactive oxygen species (ROS) in different plant species.

Plant species (References)	Cd concentration	Time of treatment	Signaling events
Nicotiana tabacum (cell suspension) (Olmos et al., 2003)	5 mM $CdCl_2$	15 min	Oxidative burst mediated by Ca^{2+}, calmodulin and protein phosphorylation
Pisum sativum (Rodríguez-Serrano et al., 2006, 2009)	50 μM $CdCl_2$	15 days	Accumulation of $O_2\bullet-$ and H_2O_2, Ca^{2+}-dependent decrease in NO levels, activation of peroxidases and NADPH oxidase
Arabidopsis thaliana (Maksymiec and Krupa, 2006)	100 μM $CdSO_4$	15 h	Strong, transient $O_2\bullet-$ and H_2O_2 accumulation connected with changes in the activity of NADPH oxidase and superoxide dismutase
Nicotiana tabacum (cell suspension) (Garnier et al., 2006)	3 mM $CdCl_2$	8 h	Three waves of oxidative stress: (1) transient, NADPH oxidase-dependent accumulation of H_2O_2 (2) increased production of $O_2\bullet-$ in mitochondria (3) fatty acid hydroperoxide accumulation concomitant with necrotic type of cell death Regulation of NADPH oxidase activity involving Ca^{2+}-mediated signaling and protein phosphorylation
Lycopersicon esculentum (cell suspension) (Yakimova et al., 2006)	100 μM $CdSO_4$	24 h	Programmed cell death mediated by caspases and accompanied by transient, NADPH-oxidase dependent H_2O_2 accumulation ROS production involving NADPH-oxidase activity as well as phospholipase C and phospholipase D signaling pathways
Oryza sativa (Hsu and Kao, 2007)	5 mM $CdCl_2$	24 h	H_2O_2 accumulation dependent on NADPH-oxidase and phosphatidylinositol 3-phosphate
Oryza sativa (Yeh et al., 2007)	100, 200, 400 mM $CdCl_2$	1 h	Regulation of MAP kinase activity by: non-enzymatic ($OH^\bullet$) and enzymatic ROS production ($O_2\bullet-$ or H_2O_2) involving NADPH oxidase, CDPKs, PI3 kinase, and closing of the mitochondrial pore Regulation of NADPH oxidase and CDPKs activity by Ca^{2+}
Arabidopsis thaliana (cell suspension) (De Michele et al., 2009)	100, 150 μM $CdCl_2$	14 days	The concomitant presence of high levels of both NO and H_2O_2 triggering programmed cell death
Arabidopsis thaliana (Liu et al., 2010)	1, 10, 50, 150, 300 μM $CdCl_2$	12 h	ROS-triggered activation of MPK3 and MPK6
Pisum sativum (Lehotai et al., 2011)	100 μM $CdCl_2$	48 h	Necrotic cell death associated with NO and H_2O_2 generation
Lupinus luteus (Arasimowicz-Jelonek et al., 2012)	89 mM $CdCl_2$	24 h	Programmed cell death related to $O_2\bullet-$ and NO production PCD-initiated signal transduction between roots and leaves
Oryza sativa (Zhao et al., 2012)	100 μM $Cd(NO_3)_2$	13 days	Accumulation of H_2O_2 and modification of the auxin signaling pathway and/or cell-cycle gene expression
Glycine max (Pérez-Chaca et al., 2014)	40 μM $CdCl_2$	6 days	Antioxidative response induced by increased levels of H_2O_2 and NO

NADPH to molecular oxygen to produce $O_2\bullet-$, which is subsequently dismutated to O_2 and H_2O_2 by superoxide dismutase enzymes (SOD). Strong superoxide accumulation (Rodríguez-Serrano et al., 2006, 2009; Lehotai et al., 2011; Arasimowicz-Jelonek et al., 2012) correlated with SOD activity (Maksymiec and Krupa, 2006) was found in plants treated with Cd.

NADPH oxidase-dependent generation of H_2O_2 appeared to be regulated by cytosolic free calcium (Garnier et al., 2006; Yakimova et al., 2006) and ethylene (Yakimova et al., 2006). Furthermore, it has been shown that a rapid increase in cytosolic calcium levels, essential for stimulation of the NADPH oxidase, requires phospholipase C (PLC) activity, and most likely involves inositol-3-phosphate (IP3)-stimulated calcium channels as well as ADPribose-gated channels (Garnier et al., 2006). Apart from calcium (Rodríguez-Serrano et al., 2006, 2009), calmodulin and protein kinases play a key role in the signaling cascade that leads to a Cd-induced oxidative burst (Garnier et al., 2006). According to Yakimova et al. (2006) PLC and phospholipase D (PLD) signaling is also involved in the production of ROS. Cadmium may stimulate phospholipases and initiate further signaling through increased levels of phosphatidylinositol-triphosphate (IP3), phosphatidic acid, and cytosolic calcium (Yakimova et al., 2006). It is suggested that the downstream targets of PLC- and PLD-derived second messengers may be a variety of lipid and protein kinases, including phosphatidylinositol 3-kinase (PI-3-kinase), MAPKs, and calcium-dependent protein kinases (CDPKs) (Yakimova et al., 2006; Hsu and Kao, 2007).

The MAPK cascade is one of the important pathways involved in the transduction of external stimuli into cells. These enzymes are able to phosphorylate a wide range of substrates, including other kinases and/or TFs (Colcombet and Hirt, 2008). It has been found that two kinases, MPK3 and MPK6, exhibit much higher activity after Cd treatment. Pre-treatment with the ROS scavenger glutathione effectively inhibited their activation. These results support the hypothesis that the Cd sensing signaling pathway use a build-up of ROS to trigger activation of MAPKs (Liu et al., 2010). It was reported that Cd-induced activation of MAP kinases may involve not only ROS, including hydroxyl radicals ($OH^{\bullet}$), but also CDPK and PI3 kinase, and may be triggered by mitochondrial dysfunction resulting from the closure of the mitochondrial permeability transition pore (Yeh et al., 2007).

It has recently been suggested that ROS-induced signal transduction may occur by means of oxidized fragments of proteins damaged by oxidative stress. The derived peptides could act in a more a specific way, as they contain information about the organelle subjected to stressful conditions and the type of ROS produced (Møller and Sweetlove, 2010). However, in respect to cadmium, this mechanism requires further experimental research.

Hydrogen peroxide and ROS-induced secondary messengers may affect the expression of plant genes (Møller and Sweetlove, 2010). Cd-induced accumulation of H_2O_2 modifies the auxin signaling pathway, including auxin distribution (*DR5-GUS*), biosynthesis (*OsYUC*s), and transport (*OsPIN*s), auxin-responsive (*OsARF*s/*OsIAA*s) gene expression, and/or cell division (cell-cycle genes). However, the possibility that auxin functions in parallel to H_2O_2 cannot be excluded (Zhao et al., 2012).

A growing body of evidence suggests that ROS in interaction with reactive nitrogen species (RNS) are required to induce signal transduction leading to cell death in plants exposed to Cd (Yakimova et al., 2006; De Michele et al., 2009; Lehotai et al., 2011; Arasimowicz-Jelonek et al., 2012). Depending on the concentration of metal in the medium, diverse forms of cell death may be observed, ranging from apoptosis to necrosis. It was found that programmed cell death (PCD) associated with increased H_2O_2 production was mediated by proteases with caspase-like activity (Yakimova et al., 2006). De Michele et al. (2009) postulated that PCD is initiated by the rapid production of phytochelatins and NO, whereas H_2O_2 accumulation appears later on. This sequence of events actually precedes the rise of PCD in Cd-treated plants. In another model system, it was found that the generation of Cd-induced H_2O_2 was correlated with a significant increase in NO content. It was concluded that cell viability decreased when NO and H_2O_2 levels were simultaneously high in the same tissues (Lehotai et al., 2011). Arasimowicz-Jelonek et al. (2012) revealed that the generation of NO was accompanied by the activation of plasma membrane NADPH-oxidase and subsequent superoxide anion accumulation. The lack of simultaneous H_2O_2 accumulation during the experiment suggests that $O_2\bullet-$ rather than H_2O_2 cooperate with NO to induce PCD. In this report, the effect of Cd on post-stress signaling molecules in different plant parts was investigated. When Cd was applied to the roots, NO synthesis was not accompanied by statistically significant H_2O_2 accumulation in this organ. Nevertheless, in leaves an approximately two-fold increase in H_2O_2 was concomitant with enhanced levels of NO. An accumulation of NO and H_2O_2 in leaves was correlated with PCD symptoms in roots, which led to the assumption that PCD initiate signal transduction between various seedling organs that induce plant defense mechanisms (Arasimowicz-Jelonek et al., 2012).

It has recently been reported (Pérez-Chaca et al., 2014) that Cd leads to a rise in H_2O_2 and NO, and to a lesser extent $O_2\bullet-$ content, after few hours of exposure. Accumulation of these molecules triggers the induction of antioxidative defenses, ASC-GSH cycle, and NADP-dehydrogenases. A second, higher wave of $O_2\bullet-$ production, observed after 3-day treatment, might participate in the reinforcement of antioxidant response. The described participation of reactive oxygen species in the transduction of cadmium signal and its cross talk with other signaling elements is presented in **Figure 1**.

NITRIC OXIDE

A growing body of evidence indicates that cadmium stress modulates NO generation in plants (**Table 2**). This gaseous cell-signaling molecule is involved in many plant growth and development processes, as well as in the regulation of multiple responses to biotic and abiotic stress factors. The signaling mode of NO action at the molecular level includes protein modification by binding to critical Cys residues, heme or iron-sulfur centers, and Tyr residue nitration via peroxynitrite formation ($ONOO^-$) (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011).

The production of NO has been demonstrated *in vivo* in various plant tissues exposed to Cd stress, but the time and intensity of NO generation seems to be strictly dependent on the form

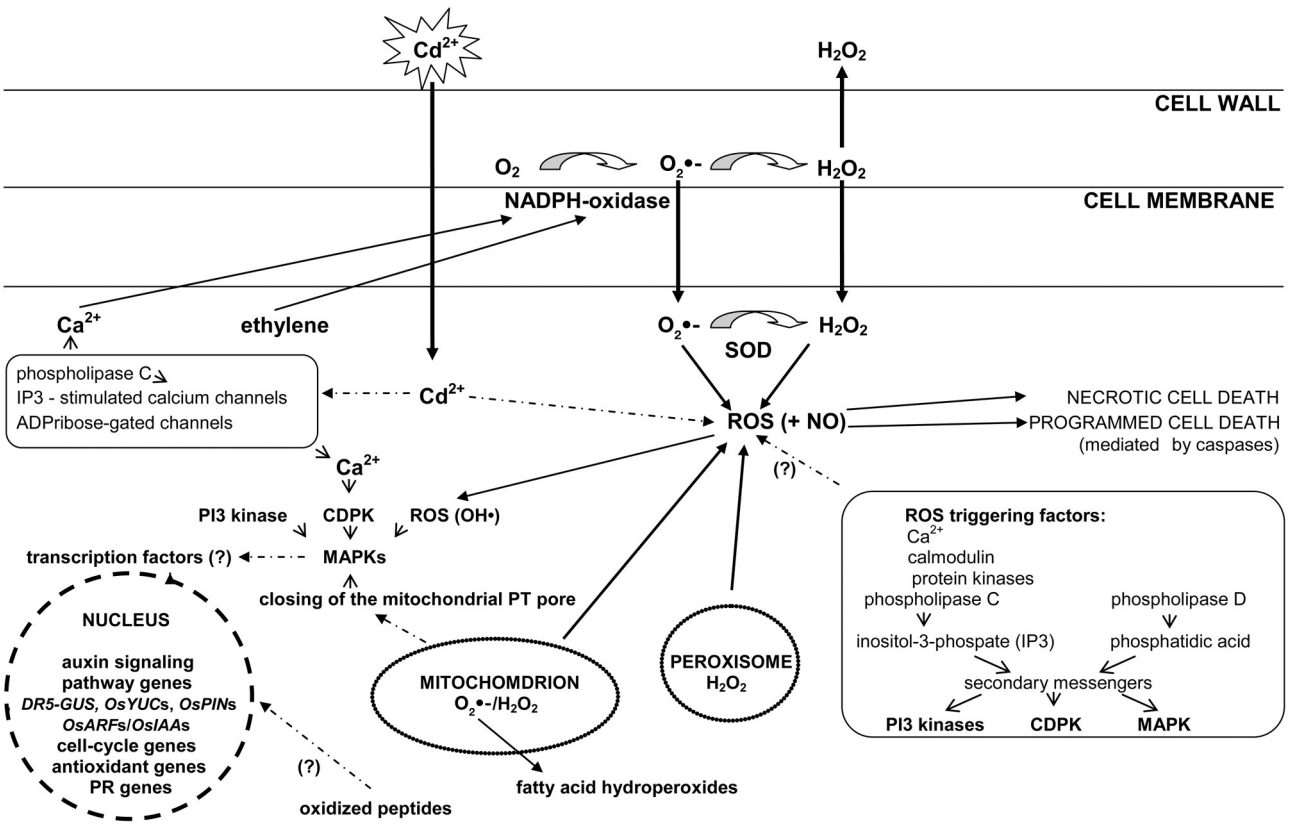

FIGURE 1 | The role of reactive oxygen species in cadmium signal transduction. In response to Cd reactive oxygen species (ROS) can be formed in non-enzymatic and enzymatic reactions (for e.g., catalyzed by NADPH oxidase, superoxide dismutase enzymes) in various cell compartment such as: plasma membrane, mitochondria, or peroxisomes. NADPH oxidase-dependent generation of ROS is regulated by cytosolic free calcium and ethylene. Increase in calcium levels requires phospholipase C activity as well as involves inositol-3-phosphate (IP3)-stimulated calcium channels and ADPribose-gated channels (box on the left). Apart from calcium Cd-induced ROS production is triggered by calmodulin, protein kinases, phospholipase C, and phospholipase D (box on the right). Phospholipases initiate further signaling through increased levels of phosphatidylinositol-triphosphate (IP3) or phosphatidic acid. These molecules activate secondary messengers such as lipid and protein kinases, including phosphatidylinositol 3-kinase (PI-3-kinase), mitogen-activated protein kinases (MAPKs), and calcium-dependent protein kinases (CDPKs). Cd-induced activation of MAP kinases requires not only ROS, but also Ca-dependent protein kinase (CDPK) and PI3 kinase, and may be triggered by closing of the mitochondrial PT pore. ROS-induced (for e.g., by means of MAP kinases activity) secondary messengers may modify transcription factors hence affect the expression of plant signaling and defense genes. ROS in interaction with nitric oxide (NO) are involved in signal transduction leading to necrotic and programmed cell death.

and concentration of metal used, the duration of stress treatment, the plant species and developmental phase of the model plant, as well as the plant tissue or organ analyzed (Xiong et al., 2010). Enhanced NO synthesis has been observed in plant roots even within the first several hours of Cd exposure (Besson-Bard et al., 2009; Mahmood et al., 2009; Arasimowicz-Jelonek et al., 2012). In turn, prolonged treatment to the metal visibly diminishes NO content in roots (Rodríguez-Serrano et al., 2006; Xu et al., 2010). Endogenous NO was found to also be involved in distal signaling during Cd stress, since Cd application at the root level triggered NO formation in leaves, mainly in the vascular bundles and surrounding cells (Besson-Bard et al., 2009; Arasimowicz-Jelonek et al., 2012).

Many experimental designs using NO donors have revealed that exogenous NO might alleviate cadmium toxicity in plants. The application of NO in different forms and doses induced a decrease in Cd accumulation (Xiong et al., 2009) or activated the enzymatic antioxidant system, preventing metal-evoked oxidative stress in plant cells (Kopyra and Gwóźdź, 2003; Laspina et al., 2005; Singh et al., 2008). Moreover, exogenous NO was able to improve Cd tolerance by maintaining auxin equilibrium and enhancing ion absorption (Xu et al., 2010). Most recently, a pharmacological approach performed by Shi et al. (2014) demonstrated interaction between NO and another gaseous signal molecule, H_2S, during Cd stress, which may be essential for plant stress response to the heavy metal. Finally, NO can regulate cellular response via the induction of Cd-dependent signaling-associated genes, including *ACS*, *MAPKK2*, *DOF1*, and *MYBZ2* (Chmielowska-Bąk and Deckert, 2013).

An early endogenous NO accumulation in response to Cd was found to be implicated in PCD induction in both a cell suspension (De Michele et al., 2009; Ma et al., 2010) and a whole plant model system (Arasimowicz-Jelonek et al., 2012; Ye et al., 2013). Most probably NO participates in controlling the threshold for

Table 2 | The effects of various Cd doses on NO generation in plants.

Species/organ	Cadmium concentration	Time of treatment	Changes in NO level	References
White poplar (*Populus alba* L.)/suspension culture	150 μM	30 min	↑	Balestrazzi et al., 2009
Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2)/BY-2 cells	150 μM	2–12 h	↑	Ma et al., 2010
Wheat (*Triticum aestivum* L.)/roots	10 μM	3 h	↑	Mahmood et al., 2009
Soybean (*Glycine max* L.)/roots	40 μM	6 h	↑	Pérez-Chaca et al., 2014
Arabidopsis thaliana/roots	200 μM	7 h	↑	Besson-Bard et al., 2009
Yellow lupine (*Lupinus luteus* L.)/roots	89 μM	12 and 24 h	↑	Arasimowicz-Jelonek et al., 2012
Barley (*Hordeum vulgare* L.)/root tips	1 mM	24 h	↑	Valentovičová et al., 2010
Rice (*Oryza sativa* L.)/roots	100 μM	24 h	↓	Xiong et al., 2009
Pea (*Pisum sativum* L.)/roots	100 μM	24 and 48 h	↑	Lehotai et al., 2011
Arabidopsis thaliana/suspension culture	150 μM	48 h	↑	De Michele et al., 2009
Soybean (*Glycine max* L.)/suspension culture	4 μM 7 μM	72 h	↑	Kopyra et al., 2006
Wheat (*Triticum aestivum* L.)/roots	100 μM	5 days	↑	Groppa et al., 2008a
Pea *(Pisum sativum* L.)/leaves	50 μM	14 days	↓	Rodríguez-Serrano et al., 2009
Pea (*Pisum sativum* L.)/roots	50 μM	14 days	↓	Rodríguez-Serrano et al., 2006
Pea l (*Pisum sativum* L.)/leaves	50 μM	14 days	↓	Barroso et al., 2006
Wheat (*Triticum aestivum* L.)/roots	1 μM	28 days	↑	Mahmood et al., 2009

triggering PCD in plants. In *Arabidopsis* cells, both H_2O_2 and NO were necessary to trigger PCD, whereas in lupine roots O_2^- rather than H_2O_2 functions as the molecule that synergizes with NO to unlock the PCD program under Cd stress (De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012). Additionally, Ye et al. (2013) documented that the mechanism of NO function in Cd-induced PCD in *Arabidopsis* involved MPK6-mediated caspase-3-like protease activation.

Recent published reports have demonstrated that endogenously produced NO plays a key role in the regulation of Cd cytotoxicity (Groppa et al., 2008a; Besson-Bard et al., 2009; De Michele et al., 2009; Elviri et al., 2010; Valentovičová et al., 2010). NO formation during Cd stress may be strictly related to iron deficiency caused by the metal (Besson-Bard et al., 2009; Besson-Bard and Wendehenne, 2009). In *Arabidopsis* roots, NO initiated the Fe-starvation pathway, promoting up-regulation of the expression of iron acquisition-related genes (*IRT1, FRO2*, and *FIT*) and, in consequence, amplifying Cd accumulation and the subsequent inhibition of root growth (Besson-Bard et al., 2009). In barley root tips, NO was associated in the metal toxicity mechanism through ectopic and accelerated differentiation, causing a shortening of the root elongation zone (Valentovičová et al., 2010). Cd-induced NO formation was also directly correlated with wheat root growth inhibition (Groppa et al., 2008a). What is more, in an *Arabidopsis* culture, enhanced NO production reduced the efficiency of Cd ion detoxication through direct S-nitrosylation of phytochelatins, promoting the deleterious effects of Cd (De Michele et al., 2009; Elviri et al., 2010).

PLANT GROWTH REGULATORS

Plants exposed to abiotic stress often resemble plants with an altered phytohormone metabolism (Pasternak et al., 2005). Numerous papers clearly indicate that plant growth regulators are substantially involved in the perception of and downstream response to cadmium treatment. Changes in the hormonal balance are potential signals initiating plant responses to cadmium stress, including hormone crosstalk with the whole plant signaling network, such as the MAPK (Zhao et al., 2013), ROS (Liptáková et al., 2012; Yuan et al., 2013), and NO signaling pathways (Xu et al., 2011; Wang et al., 2013). Unfortunately, the exact nature of these relations remain somewhat obscure and largely dependent on the experimental background, i.e., the species, the plant organ, the concentration of metal used, and the duration of metal treatment (**Table 3**). The majority of experimental data indicate that stress growth regulators such as ethylene, SA, JA, and ABA are involved in the signaling and defense response, but the contribution of other hormones (auxin, cytokinins) cannot be excluded (Al-Hakimi, 2007). In fact, an increase in ethylene (ET) biosynthesis under cadmium treatment was observed in many plant species, including *Arabidopsis* (Arteca and Arteca, 2007), mustard (Masood et al., 2012), soybean (Chmielowska-Bąk et al., 2013b), and pea (Rodríguez-Serrano et al., 2009). Experiments with young soybean seedlings revealed that an increase in ET concentration was accompanied not only with the induction of the genes encoding the enzymes of the ethylene biosynthesis pathways, but also the genes related to the proteins involved in the polyamine metabolism, NO generation, and MAPK cascades (Chmielowska-Bąk et al., 2013b). In mustard plants, an increase in ethylene concentration was correlated with augmented 1-aminocyclopropane-1-carboxylic acid synthase activity (ACS), a key enzyme in the ethylene biosynthesis pathway (Masood et al., 2012). Furthermore, experiments performed on bean and onion plants with the use of an inhibitor of ethylene synthesis (Maksymiec, 2011), and tomato mutants with the antisense ACS gene (Liu et al., 2008) pointed to ethylene as a link in Cd-induced accumulation of H_2O_2. Ethylene together

Table 3 | Exemplary studies of growth regulators level (endogenous) under different experimental background.

Growth regulator	Species/organ	Cadmium concentration	Time of treatment	Changes in level of growth regulator	References
Ethylene	Soybean/roots	10 mgL^{-1} (89 μM) 25 mgL^{-1} (223 μM)	6–24 h	↑	Chmielowska-Bąk et al., 2013b
	Pea/leaves	50 μM	14 days	↑	Rodríguez-Serrano et al., 2009
	Arabidopsis/leaves	400 μM	24 h	↑ (youngest leaves) ↑ (oldest leaves)	Arteca and Arteca, 2007
Salicylic acid	Pea/roots	50 μM	14 days	↑	Rodríguez-Serrano et al., 2006
	Maize/leaves	10–25 μM	14 days	↑	Krantev et al., 2008
	Kosteletzkya virginica/leaves	5 μM	1–3 weeks	↓ (1 week) ↑ (2 and 3 weeks)	Han et al., 2013
Jasmonic acid	Runner bean/leaves/young plants	100 μM	0–120 h	↑ (14 h); ↓ (120 h)	Maksymiec et al., 2005
	Runner bean/leaves/oldest plants	100 μM	0–120 h	↓ (14 h); ↑ (120 h)	
	Pea/leaves	50 μM	14 days	↑	Rodríguez-Serrano et al., 2009
Abscisic acid	Potato/roots	0.1 mM	5–48 h	↑	Stroiński et al., 2013
	Rice (Cd-tolerant)/leaves	0.5 mM	0–3 days	↑	Hsu and Kao, 2003
	Wheat	100–1000 μM	30 days	↓ (400 μM, 1000 μM)	Moussa and El-Gamal, 2010a
Auxin	Arabidopsis/root	50 μM	7 days	↓	Zhu et al., 2013
	Poplar/stem	50 μM	24 days	↓	Elobeid et al., 2012
	Kosteletzkya virginica/leaves	5 μM	1–3 weeks	↑	Han et al., 2013
Polyamines	Sunflower/shoots	0.1–1 mM	0–16 days	↑ (1 mM, Put, Spm)	Groppa et al., 2007
	Soybean/roots	50 μM, 200 μM	0–6 days	↑ (Put) ↓ (Spd)	Balestrasse et al., 2005
	Tobacco cell suspension	0.05 mM, 1 mM	12–72 h	↑ (0.05 mM)	Kuthanová et al., 2004

↑, *Increase in level;* ↓, *Decrease in level. Put, putrescine; Spd, spermidine; Spm, spermine. For more details, see References.*

with increased H_2O_2 production and the activation of the PLC and PLD signaling pathways seems to be involved in the induction of apoptosis in tomato suspension cultures treated with Cd (Yakimova et al., 2006). On the other hand, experiments on mustard plants treated with ethylene biosynthesis inhibitor strongly suggest that ET plays an important role in the alleviation of Cd stress on photosynthesis *via* modulation of the sulfur metabolism and GSH synthesis (Masood et al., 2012). In turn, a comparison of ethylene-insensitive mutant and control tomato plants revealed a very similar pattern of Cd-induced response in terms of growth parameters, metal accumulation, lipid peroxidation, H_2O_2 production, and in the activity of most antioxidant enzymes (Monteiro et al., 2011). However, the mutant showed augmented H_2O_2 production and enhanced ascorbate peroxidase activity in its fruit, and reduced leaf chlorophyll degradation, indicating that ethylene signaling can modulate the biochemical pathways of oxidative stress in a tissue-dependent manner.

In addition to ethylene, SA and JA also seem to play a role in cadmium signal transduction. The accumulation of endogenous SA under cadmium stress has been noted in pea (Rodríguez-Serrano et al., 2006), maize (Krantev et al., 2008), *Arabidopsis* (Zawoznik et al., 2007), and halophyte *Kosteletzkya virginica* (Han et al., 2013). The significance of endogenous SA as a signaling molecule necessary to modulate Cd-induced oxidative stress has been well-demonstrated on SA-accumulating and SA-deficient lines of *Arabidopsis* (Zawoznik et al., 2007; Tao et al., 2013). Mutants exhibited varying levels of H_2O_2, lipid peroxidation, and antioxidant enzyme activity compared to wild plants. High endogenous SA significantly increased Cd-induced plant growth retardation, whereas SA deficiency decreased the growth inhibition. However, the majority of reports concern the effect of exogenous application of SA. In most studies, SA displays a protective effect *via* alleviation of Cd-induced oxidative stress. Modulation of the ROS level (mainly H_2O_2) and the activity of the antioxidant system after SA pretreatment was observed in many different species, including maize (Krantev et al., 2008), rice (Panda and Patra, 2007), mung bean and common vetch (Zhang et al., 2011), flax (Belkadhi et al., 2013), Kentucky bluegrass (Guo et al., 2013), and mustards plants (Ahmad et al., 2011). It has also been suggested that SA-induced protection against Cd oxidative stress is mediated through H_2O_2 accumulation produced by NADPH oxidase (Chao et al., 2010). Moreover, experiments on ryegrass plants (Wang et al., 2013) and lupine seedlings (Arasimowicz-Jelonek et al., 2012) imply intensive cross-talk

among SA, H_2O_2 and NO in long-distance signaling pathways under cadmium treatment. SA also seems to play a protective role in photosynthesis. Plants pretreated with SA and subjected to Cd challenge showed a diminished reduction in chlorophyll content and/or photosynthetic enzyme activity (Krantev et al., 2008; Popova et al., 2009; Moussa and El-Gamal, 2010a). On the other hand, castor beans pretreated with SA and exposed to Cd displayed potentiated symptoms of Cd toxicity in terms of plant growth and photosynthetic parameters (Liu et al., 2011). All these reports suggest that the mode of SA action depends on the concentration of SA and the plant's susceptibility to this hormone. The lack of a clear tendency following cadmium treatment can also be observed in another stress hormone, namely JA. Elevated levels of JA have been noted in several plant species treated with cadmium, including pea (Rodríguez-Serrano et al., 2006), runner bean, and *Arabidopsis* plants (Maksymiec et al., 2005). It has been suggested that JA has a protective effect against Cd action at lower concentrations (Maksymiec and Krupa, 2002; Noriega et al., 2012). However, at higher concentrations (10^{-4} mol/L), it may induce changes usually observed under heavy metal stress, such as growth reduction, chlorophyll degradation, and inhibition of various photosynthetic parameters (Maksymiec and Krupa, 2002). JA might also interact with ROS signaling—it has been shown to mediate the generation of ROS in *Arabidopsis* plants exposed to cadmium (Maksymiec and Krupa, 2006).

The response of ABA to cadmium is also ambiguous and depends on the experimental background. An increase in hormone concentrations has been reported in potato plants (Stroiński et al., 2013), halophyte *Kosteletzkya virginica* (Han et al., 2013), and two rice cultivars, however a Cd-tolerant rice cultivar showed much greater ABA accumulation (Hsu and Kao, 2003). Moreover, exogenous application of ABA resulted in enhanced tolerance to cadmium stress and a decrease in uptake of this heavy metal in a sensitive rice cultivar. Experiments on potato plants treated with Cd and an inhibitor of ABA biosynthesis implicated the participation of ABA in the transduction of the Cd signal to the cells of potato roots and phytochelatin synthesis *via* increased phytochelatin synthase activity (Stroiński et al., 2013). The protective role of ABA against cadmium stress has also been demonstrated by experiments comparing wild type *Arabidopsis* plants and ABA-deficient plants, in which the mutants proved to be more sensitive to the metal (Sharma and Kumar, 2002). These findings strongly suggest that ABA may be involved in signal pathways during Cd stress. Moreover, ABA might also initiate the production of metal detoxification compounds (i.e., phytochalatins) and influence the metabolic regulation of other hormones, such as cytokinins (Hayward et al., 2013). Bioinformatic analysis of the promoter sequences of Cd-inducible genes in soybean seedlings revealed that their promoters possess several regulative motifs associated with plant response to stress factors, ABA, and ethylene signaling (Chmielowska-Bąk et al., 2013b). On the other hand, studies of ABA-deficient and ABA-insensitive mutants of *Arabidopsis* excluded a direct mediatory role for ABA in Cd-imposed phytotoxic effects on germination and growth assays (Sharma and Kumar, 2002). A decrease in ABA content has also been observed in wheat plants treated with 400 and 1000 μM of $CdCl_2$ (Moussa and El-Gamal, 2010b).

Auxin (IAA) is crucial plant growth hormone controlling physiological and developmental processes, but its involvement in cadmium response is still poorly recognized. An increasing body of evidence indicates that cadmium disturbs auxin homeostasis by affecting its level, distribution, metabolism, transport, and balance with other phytohormones (Elobeid et al., 2012; Hu et al., 2013). The modulation of endogenous auxin concentrations after Cd treatment has been observed in *Arabidopsis* (Zhu et al., 2013), halophyte *Kosteletzkya virginica* (Han et al., 2013), and poplar (Elobeid et al., 2012), but, similar to other phytohormones, there is no clear tendency. Experiments with auxin inhibitor have demonstrated the involvement of the hormone in the effective alleviation of Cd-induced root growth inhibition, H_2O_2 production, and root swelling, but only at a low concentration (10 μM) of the metal (Tamas et al., 2012). Additionally, the application of exogenous auxin might alleviate Cd toxicity in plants by inhibiting heavy metal biosorption, reducing Cd translocation, or stimulating antioxidant enzymes (Piotrowska-Niczyporuk et al., 2012; Zhu et al., 2013). Moreover, auxin signaling might also be involved in defense response to Cd-stress by activation of a detoxification enzyme (Bočová et al., 2013). In general, the concentration and distribution of auxin under cadmium stress seems to be modulated by the regulation of auxin metabolism gene expression through, e.g., the MAPKs cascade and ROS signaling pathways (Zhao et al., 2011, 2013; Hu et al., 2013). Another possibility is increased activity of enzymes involved in the inactivation and/or degradation of the hormone (Chaoui et al., 2004; Elobeid et al., 2012).

Apart from phytohormones, polyamines (PA) such as putrescine (Put), spermidine (Spd), and spermine (Spm) have been proven to play a crucial role in the signaling network and plant defense to cadmium. The pathways of the PA metabolism may crosstalk with other signaling molecules, such as phytohormones, ROS, and NO (Groppa et al., 2008a; Yang et al., 2013). The modulation effect of cadmium on polyamine concentration and the activity of their biosynthesis enzymes was observed in a variety of plant species, including frogbit (Yang et al., 2013), sunflower, wheat (Groppa et al., 2003, 2008b), *Potamogeton crispus* (Yang et al., 2010), mungbean (Choudhary and Singh, 2000), carnation (Serrano-Martínez and Casas, 2011), soybean (Balestrasse et al., 2005), and tobacco cells (Kuthanová et al., 2004). Most of the data indicate the protective role of polyamines in Cd stress response. Experiments with exogenous PA application have provided evidence for the important role of polyamines (Spd and Spm) in cadmium stress by influencing the expression and function of the antioxidant system (Groppa et al., 2001; Wen et al., 2011; Kumar et al., 2012; Piotrowska-Niczyporuk et al., 2012), a reduction in ROS generation, and the prevention of lipid peroxidation (Yang et al., 2013). In addition, an experimental approach with antisense inhibition in a Spd synthase gene revealed increased lipid peroxidation and ineffective induction of the antioxidant system in a transgenic pear plant as compared to the wild type (Wen et al., 2011). All of these findings indicate that polyamines are key biological compounds in the signaling network, but like other growth regulators, the specific response under cadmium stress seems to depend on the species, applied concentrations, and time of exposure to the metal.

MITOGEN-ACTIVATED PROTEIN KINASE CASCADES

An increasing body of evidence suggests that in plants, MAPKs cascades may function in the Cd-signaling pathways and play an essential role in plant defense or stress responses against metal. Differentiated levels of MAP kinases gene expression have been observed in Cd-exposed seedlings and cell suspensions of rice (Agrawal et al., 2003; Kim et al., 2003; Yeh et al., 2004), *Arabidopsis* plants (Opdenakker et al., 2012), and soybean seedlings (Chmielowska-Bąk et al., 2013b). In addition, exposure to cadmium ions activated four distinct MAPKs (SIMK, MKK2, MKK3, SAMK) in alfalfa seedlings (Jonak et al., 2004) and two (MPK3, MPK6) in *Arabidopsis* (Liu et al., 2010). Activation of the plant MAPK cascade by Cd is achieved within minutes and is probably mediated through distinct signaling pathways, including ROS (Yeh et al., 2007; Liu et al., 2010), Ca^{2+}-dependent protein kinase, and phosphatidylinositol 3-kinase (Yeh et al., 2007). Furthermore, experiments with a mitochondrial permeability transition pore opening blocker indicated that Cd-induced MAP kinase activities are dependent on the functional state of mitochondria (Yeh et al., 2007). Moreover, the NO signaling pathway followed by MAP kinase activation is probably involved in Cd-induced PCD. In *Arabidopsis* plants, the metal-induced activity of caspase-3-like protease was promoted by increased NO production *via* up-regulation of MPK6 activity (Ye et al., 2013).

REGULATION OF GENES EXPRESSION

Exposure to cadmium leads to the changes in expression of numerous genes. The microarray analysis revealed that this metal modulates expression of nearly 400 genes in *Arabidopsis* and more than 1700 in rice (Kovalchuk et al., 2005; Ogawa et al., 2009). Cadmium has been shown to up-regulate genes encoding pathogen related proteins, antioxidant enzymes, transporters, TFs, and proteins associated with glutathione metabolism. In turn genes encoding proteins connected with photosynthesis were down-regulated in response to short-term cadmium stress (Fusco et al., 2005; Ogawa et al., 2009). The extensive impact of this heavy metal on gene activity requires engagement of various gene regulating mechanisms. Data from the literature imply that Cd-dependent regulation of genes expression is mediated by changes in the activity of TFs, the modulation of micro RNA levels, and modifications in chromatin.

Plants possess an average of 590 TFs grouped in various families usually named after their DNA-binding motifs (Charoensawan et al., 2010). The response to cadmium stress involves TFs belonging to the MYB, HSF, bZIP, WRKY, and DREB families. Analysis of the expression levels of over 160 genes encoding the TFs belonging to the MYB family in *Arabidopsis* showed that 20% of them were affected by cadmium and salt stress (Yanhui et al., 2006). Also, in soybean roots short-term cadmium stress caused induction in the gene encoding MYBZ2 (Chmielowska-Bąk et al., 2013b). In turn, wheat and rice plants treated with cadmium were characterized by an elevated expression of the *HsfA4* gene. Moreover, plants over-expressing this TF were more tolerant to cadmium stress, while plants with hampered HsfA4 expression exhibited reduced resistance to this metal (Shim et al., 2009). Among the TFs belonging to the bZIP family, bZIP62, ThbZIP1, and BjCdR15 were shown to be involved in plant response to cadmium stress. The gene encoding bZIP62 was induced by cadmium in soybean roots, while *ThbZIP1* showed increased expression in *Tamarix hispida* (Wang et al., 2010; Chmielowska-Bąk et al., 2013b). Transgenic *Arabidopsis* plants over-expressing BjCdR15 exhibited higher tolerance to cadmium accompanied by a higher accumulation of this metal in leaves. It is suggested that BjCdR15 confers resistance to cadmium through regulation of its root-to-shoot translocation and induced phytochelatin synthesis (Farinati et al., 2010). Analysis of microarray expression profiles demonstrated that exposure to cadmium leads to an elevated expression of *OsDREB1A*, *OsDREB1B,* and *WKRY09* in rice and TFs belonging to the ATAF, DREB2A, bZIP, and WRKY families in *Arabidopsis* (Suzuki et al., 2001; Ogawa et al., 2009). Cd-dependent induction of WKRY25 and WKRY29 genes in *Arabidopsis* has also been proven by the real-time PCR technique (Opdenakker et al., 2012). In opposition to the described results, it has been demonstrated in *Solanum torvum* plants that exposure to this metal leads to inhibited expression of TFs belonging to the DREB family (Yamaguchi et al., 2010). The described examples show that cadmium affects the mRNA levels of various TFs. This metal might also influence TF activity through changes in their structure. Experiments performed with the use of the NMR technique revealed that Cd^{2+} replaces Zn^{2+} in a basic leucine zipper motif in the SUPERMAN (SUP37) TF isolated from *Arabidopsis*. The described substitution leads to changes in SUP37 conformation which can alter its DNA binding ability (Malgieri et al., 2011). Cadmium stress might also influence TF structure indirectly through the induction of NO production. It has been demonstrated that NO-dependent nitrosylation of cysteine residues in the AtMYB2 transcription factor leads to hampered DNA binding (Serpa et al., 2007).

TFs bind to defined DNA sequences called *cis*-acting elements. In bean plants, a *cis*-acting sequence called PvSr2 was shown to be associated with heavy metal stress. Transgenic tobacco plants containing the PvSr2 sequence exhibited an increased expression of the reporter gene in response to Cu^{2+}, Zn^{2+}, Hg^{2+}, and Cd^{2+} (Qi et al., 2007). Analysis of *cis*-acting elements is useful not only in the search for metal-responsive sequences, but also in uncovering the signaling molecules involved in Cd-dependent gene regulation. In soybean, Cd-responsive genes contained in their promoter region elements associated with ethylene and ABA signaling suggesting that these plant hormones are involved in the response to cadmium stress (Chmielowska-Bąk et al., 2013b). In fact, as it is described in the section concerning plant hormones, the induction of ethylene synthesis by cadmium was noted in various plant species (Rodríguez-Serrano et al., 2006; Arteca and Arteca, 2007; Masood et al., 2012; Chmielowska-Bąk et al., 2013b).

The levels of transcribed mRNA can be regulated by micro RNAs. Recent research shows that cadmium stress affects the levels of miRNAs in rice, soybean, and rape plants (Ding et al., 2011; Zhou et al., 2012; Fang et al., 2013; Zhang et al., 2013). In rice, 19 miRNAs were sensitive to this heavy metal. Interestingly, only one of them, miR528, was induced by cadmium, while the other 18 exhibited diminished expression. The affected miRNAs were involved in the regulation of the genes encoding signaling elements, including the TFs and proteins involved in miRNA

processing. Inhibited expression of miR168, miR166, and miR390 was correlated with elevated levels of the target mRNAs encoding the AGO protein, HD-Zip TF, and protein kinase, similar to RLK (Ding et al., 2011). Extensive microarray analysis of 953 soybean miRNAs showed that 14 of them were affected by cadmium stress in Cd-tolerant Huaxia3 cultivar and 21 in Cd-sensitive cultivar Zhonghuang24. Their target transcripts were involved in various processes, including development, reproduction, metabolism, and response to stimuli (Fang et al., 2013). Cadmium also caused changes in the levels of several miRNAs in the roots and shoots of rape. The affected miRNAs included miR395. More detailed research concerning the involvement of miR395 in plant response to cadmium demonstrated that this molecule is involved in cadmium uptake, root-to-shoot translocation, the alleviation of oxidative stress, and regulation of the expression of the genes encoding phytochelatines and one of the sulfur transporters, Sultr1;1 (Zhou et al., 2012; Zhang et al., 2013).

The expression of genes can be influenced by changes in chromatin, including histone modifications and DNA methylation. Increased levels of methylated DNA is associated with the repression of genes activity and vice versa—hypomethylation loosens the chromatin structure and facilitates genes activation (Chinnusamy and Zhu, 2009). Several studies report that cadmium modulates the levels of DNA methylation in animals and plants; however, there is no clear pattern in the observed changes. In the case of plants, hypermethylation has been observed in radish plants and *Posidonia oceanic*. In the latter case, increased methylation was correlated with elevated expression of methylotrasferase, indicating the *de novo* methylation process (Yang et al., 2007; Greco et al., 2012). Hypermethylation was also noted in garden cress in response to lower cadmium concentrations; however, more intense cadmium stress caused a decrease in the levels of DNA methylation (Yanez Barrientos et al., 2013). In turn, in *Gracilaria dura* exposure to cadmium resulted in a decrease in methylated DNA (Kumar et al., 2012). Significantly, DNA methylation exhibits epigenetic effects. Therefore, at least some of Cd-dependent changes in the pattern of genes expression might be "memorized" and passed on to plant offset.

POST-TRANSCRIPTIONAL MODIFICATION OF PROTEINS

Protein activity can be influenced not only by changes in the expression levels of encoding mRNAs, but also by post-translation modifications. Heavy metals, including cadmium, can bind to the functional groups of biological molecules, leading to changes in their structure and activity (Latowski et al., 2005; Sharma et al., 2008). Cadmium might also affect protein functions by replacing other divalent ions, such as Ca^{2+} or Zn^{2+} (Chmielowska-Bąk et al., 2013a). This type of process, called molecular mimicry, has been observed in radish. Substitution of Ca^{2+} by Cd^{2+} in calmodulin resulted in inhibited activity of this sensor protein (Rivetta et al., 1997). Cadmium may also modify proteins indirectly through the induction of ROS and NO accumulation. Over-production of ROS leads to oxidative damage in proteins manifested by protein carbonylation (Braconi et al., 2011). In fact, an increase in the levels of carbonylated proteins has been observed in maize, pea, alfalfa, cucumber, sunflower, and potato plants treated with cadmium (Romero-Puertas et al., 2002; Pena et al., 2006, 2007; Gonçalves et al., 2009). Some of the oxidized proteins in pea plants were identified as Rubisco and antioxidant enzymes namely glutathione reductase, manganese superoxide dismutase and catalase (Romero-Puertas et al., 2002). Interestingly, as it has been described in the section concerning ROS, peptides derived from oxidatively modified proteins might serve as organelle specific signaling molecules (Møller and Sweetlove, 2010).

As has also been mentioned, NO can modify proteins by binding to critical Cys residues, leading to their S-nitrosylation (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). The use of the Biotin Switch method has indicated numerous putative protein targets for S-nitrosylation in plants, including various signaling/regulating proteins associated with plant stress responses (Kovacs and Lindermayr, 2013). It has been also shown that under cadmium stress S-nitrosylation affect the activity of catalase and glycolate oxidase (Ortega-Galisteo et al., 2012; Romero-Puertas et al., 2013). S-nitrosylation of the elements involved in signal transduction pathways may lead to alterations in their functioning, as has been observed in the case of the AtMYB2 TF (Serpa et al., 2007).

CONCLUSIONS

In summary it can be concluded that plants' response to cadmium involves various signaling elements, such as plant hormones, polyamines, calcium ions, ROS, NO, MAPK cascades, TFs, and microRNAs. The mentioned elements are often interrelated with one another and form a complex signaling network. Although significant progress has been made in recent years in the uncovering of the role of compounds participating in this network, there are still many ambiguities:

- It is postulated that peptides derived from oxidatively damaged proteins may act as secondary ROS messengers and regulate specific genes; however, it has not been proven that such an ROS signaling pathway is involved in the response to cadmium stress.
- The role of endogenous NO and other RNS during Cd stress is still very puzzling; therefore, the recognition of the molecular targets of RNS will be an exciting challenge for future research.
- Plant growth regulators are substantially involved in the signaling pathways of plant response to cadmium, but their mutual interaction and exact crosstalk with the overall signaling network is still not fully recognized.
- Recent research implies that cadmium stress leads to the induction of various TFs; however, information concerning their role in plant response to this heavy metal is still scarce.
- There is a lack of research examining whether Cd-dependent changes in the levels of DNA methylation are associated with acquiring long-term resistance to this stress factor that can be memorized and passed to the offset.

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Disruption of the ammonium transporter *AMT1.1* alters basal defenses generating resistance against *Pseudomonas syringae* and *Plectosphaerella cucumerina*

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Disruption of the high-affinity nitrate transporter *NRT2.1* activates the priming defense against *Pseudomonas syringae*, resulting in enhanced resistance. In this study, it is demonstrated that the high-affinity ammonium transporter *AMT1.1* is a negative regulator of *Arabidopsis* defense responses. The T-DNA knockout mutant *amt1.1* displays enhanced resistance against *Plectosphaerella cucumerina* and reduced susceptibility to *P. syringae*. The impairment of *AMT1.1* induces significant metabolic changes in the absence of challenge, suggesting that *amt1.1* retains constitutive defense responses. Interestingly, *amt1.1* combats pathogens differently depending on the lifestyle of the pathogen. In addition, N starvation enhances the susceptibility of wild type plants and the mutant *amt1.1* to *P. syringae* whereas it has no effect on *P. cucumerina* resistance. The metabolic changes of *amt1.1* against *P. syringae* are subtler and are restricted to the phenylpropanoid pathway, which correlates with its reduced susceptibility. By contrast, the *amt1.1* mutant responds by activating higher levels of camalexin and callose against *P. cucumerina*. In addition, *amt1.1* shows altered levels of aliphatic and indolic glucosinolates and other Trp-related compounds following infection by the necrotroph. These observations indicate that *AMT1.1* may play additional roles that affect N uptake and plant immune responses.

Keywords: *AMT1.1*, basal resistance, metabolomics, transceptor, *NRT2.1*

INTRODUCTION

Arabidopsis defense responses against *P. syringae* and *P. cucumerina* have been widely studied. These pathogens have very different life styles, in that *P. syringae* is a hemibiotrophic bacterial pathogen that penetrates through natural openings, whereas *P. cucumerina* is a necrotrophic fungus that can overcome cell barriers and penetrate into the cytoplasm (Berrocal-Lobo et al., 2002). Salicylic acid-dependent defenses have emerged as the most effective resistance mechanism against the bacterium (Brooks et al., 2005; Glazebrook, 2005). The effector secretion machinery of *P. syringae* is currently under study because of its effectiveness in suppressing *Arabidopsis* defenses. Although the direct link remains unknown, Camañes et al. (2012b) demonstrated that coronatine, a *P. syringae* effector, targets *NRT2.1* and subsequently causes a H_2O_2 burst. The *nrt2* mutant remains insensitive to this manipulation and the increased JA-signaling that is responsive to coronatine (Melotto et al., 2006). *P. cucumerina* is more complex. *Arabidopsis* defenses against this pathogen are horizontal and involve several defense signaling pathways, such as SA, JA, IAA, and ABA-mediated callose deposition (Lorenzo and Solano, 2005; García-Andrade et al., 2011; Sánchez-Vallet et al., 2012; Gamir et al., 2014). N fertilization influences plant-pathogen interactions; therefore, the following two factors affect plant resistance: the total amount of N and the source of N. Over-fertilization increases the severity of mildew in several grapevine cultivars and many crop plants (Keller et al., 2003; Marschner, 2012). The level of N in fertilization programs also affects plant resistance based on the pathogen lifestyle. For example, elevated levels of N promote susceptibility against biotrophs, such as powdery mildew, whereas it results in reduced disease development of *Botrytis cinerea* (Walters and Bingham, 2007). However, the source of N appears to have a greater influence in plant resistance (Gupta et al., 2013). Fertilizing tobacco plants with NO_3^- accelerates the hypersensitive response following *P. syringae* pv. *Phaseolicola* infections and provides enhanced resistance. This resistance is related to NO_3^- reduction and NO production that triggers SA synthesis (Durner and Klessig, 1999). Plants fed NO_3^- display increased *PR1* expression and higher levels of SA. By contrast, tobacco plants fed with NH_4^+ are more susceptible to the bacterium. The bypass of nitrate reduction avoids NO production and triggers 4-aminobutyric accumulation, which is a nutrient for the pathogen (Solomon and Oliver, 2001), increasing the susceptibility of the plant.

Previously, first in animal sciences and then in plant sciences, the term transceptor is applied to membrane proteins that perform a dual transport and signaling function. Gojon et al. (2011) revised the transceptor role of the nitrate transporter family member NRT1. In addition to binding to low affinity nitrate, this protein can sense nitrate in the root environment. Additional studies demonstrated that both NRT1.1 and the high-affinity transporter NRT2.1 are transceptors. The sensing network regulated by NRT1.1 has not yet been elucidated, although several

of its components have been recently described. One target of NRT1.1 is the transcription of *NRT2.1*. The dual role of the induction of *NRT2.1* during early time-points of nitrate supply and repression during later time-points both directly and indirectly involve NRT1.1. Of the N metabolism components, mutants impaired in *NRT1.1*, such as *chl1* are also affected in *NIA1*, *NiR* (encoding nitrate reductase and nitrite reductase, respectively) (Wang et al., 2009) and *CIPK8*, which has a regulatory role on *NRT2.1* (Hu et al., 2009). Glutamic acid (Glu) is important in N flow and also regulates root architecture by promoting root branching. This effect is antagonized by excess NO_3^-, and this antagonistic action is also regulated by *NRT1.1*-dependent signaling (Walch-Liu and Forde, 2008). Root development and branching is also conditioned to auxin signaling, and it appears that *NRT1.1* couples NO_3^- sensing and auxin signaling.

There is recent evidence for other NO_3^- sensors coordinating signaling events in *Arabidopsis*. *NRT2.1* displays *NRT1.1*-independent roles and is also unrelated to NO_3^- uptake activity. This gene is a repressor of lateral root initiation under high sucrose and NO_3^- supply, but surprisingly, this gene seems to sense abiotic stress and concomitantly suppresses biotic stress responses. We have demonstrated that the mutant *nrt2* is affected in bacterial effector manipulation (Camañes et al., 2012b). Mutation of *NRT2.1* has significant consequences in the transcriptome of *Arabidopsis* upon *P. syringae* infection, suggesting a complex regulatory function for the *NRT2.1* gene and also the NRT2.1 protein (Camañes et al., 2012b). Therefore, we hypothesize that *NRT2.1* should be designated a transceptor as previously proposed by Gojon et al. (2011). In *Arabidopsis*, the link between the immune system and nitrate transport is not fully understood. However, recent advances indicate two different relevant events. First, low nitrate treatment induces a rapid burst of ethylene production and upregulates *CTR1, EIN3*, and *EIL1* (Zheng et al., 2013). Second, we demonstrated that a low N treatment enhances *P. syringae* susceptibility (Camañes et al., 2012b), which may be related to the increase of ET-dependent signaling that is antagonistic to the SA-signaling that is recognized as an efficient defense against biotrophic pathogens (Pieterse et al., 2009).

Interactions with the plant immune system are not restricted to *NRT2.1*. Other NRT family members, such as *NRT2.6* show enhanced expression upon *Erwinia amylovora* infection (Dechorgnat et al., 2012). Despite high NO_3^- levels that trigger *NRT2.6* expression, no nitrate-related phenotype has been associated with the *nrt2.6* mutant. Furthermore, *nrt2.6* is less tolerant to *E. amylovora* because of the reduced ability to produce H_2O_2 upon infection.

Some members of the *AMT1* family are high-affinity ammonium transporters. Of these, *AMT1.1* has the highest affinity for NH_4^+ (Shelden et al., 2001). The removal of N increases *AMT1.1* expression, and this correlates with an increase in NH_4^+ uptake. This function is affected by 30–40% in the insertional T-DNA mutant *amt1.1*. Interestingly, *amt1.1* plants are indistinguishable from wild type under optimal fertilization and growth conditions. Furthermore, *amt1.1* displays wild type levels of total N in the presence of N or after 4 days of N starvation (Kaiser et al., 2002). Although the ammonium supply may affect the plant responses to pathogens, to the best of our knowledge, no NH_4^+ transporter is involved in the plant immune system. *NRT2.1* and *AMT1.1* are the primary high-affinity transport systems (HATS) involved in NO_3^- and NH_4^+ uptake, respectively (Cerezo et al., 2001; Kaiser et al., 2002), and reciprocal regulation between *AtNRT2.1* and *AtAMT1.1* expression has been observed (Camañes et al., 2012a). High-affinity ammonium and nitrate transporters act as sensors of low NH_4^+ and NO_3^- in the root environment; therefore, thus the gene expression of *AMT1.1* and *NRT2.1* is enhanced upon low fertilization. Interestingly, its role in HATS under normal N supply conditions (>1 mM NO_3^- or NH_4^+) is negligible (Gansel et al., 2001). Using an experimental system with optimal fertilization, we focused on the role of the high-affinity ammonium transporter gene in the *Arabidopsis* immune responses against the hemibiotrophic bacterium *P. syringae* and the necrotrophic fungus *P. cucumerina*. Metabolomic and genetic studies revealed an interplay between *AMT1.1* and plant resistance.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of the Arabidopsis accession Col-0 were obtained from the SALK institute (Alonso et al., 2003), the EMS *lin1* line (in Col-0 background) was generously supplied by Jocelyn Malamy (University of Chicago, EEUU, Little et al., 2005) and the line *nrt3.1* (salk_043672) in the Col-0 background was obtained from SALK institute (Alonso et al., 2003). The background *Col3-gl1* and the mutant *amt1.1* (in *Col3-gl1* background) were provided by Alain Gojon (INRA, Montpelier, France).

For the bioassays of bacterial resistance, 1 week after germination, the seedlings were transferred to 33-ml soil pots. The plants were cultivated at 20°C day/18°C night with 8.5 h of light (105 μE m^{-2} s^{-1}) per 24 h and 60% relative humidity.

All plant genotypes were germinated in soil, and, 1 week after germination, seedlings were individually transferred to 33 ml pots containing commercial potting soil (TKS1, Floragard GmbH; http://www.floragard.de). Plants were cultivated at 20°C day/18°C night temperatures with 8.5 h of light (105 μE m^{-2} s^{-1}) per 24 h and 60% relative humidity.

For fungal resistance assays. About 50 seeds were germinated in 33-ml soil pots. Plants were grown in the same conditions as described for bacterial experiments.

PATHOGEN STRAINS AND RESISTANCE ASSAYS

The bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 was grown overnight at room temperature in King's B solid medium with appropriate antibiotics and diluted to the desired concentration with 10 mM $MgSO_4$ for plant inoculation. The bacteria were used to infect 5-week-old (or otherwise mentioned) *Arabidopsis* plants by dipping in a suspension of 2.5×10^5 colony-forming units (cfu)/ml using 0.02% Silwet L-77 (Tornero and Dangl, 2001), control plants were treated with Silwet accordingly. Three days after challenge inoculation, the disease level was determined by harvesting the plants a plating a range of dilutions in agar-KB medium containing rifampicin. After incubation at 28°C for 3 days, the number of rifampicin-resistant colony

forming units per gram of infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

The adapted strain of *P. cucumerina* BMM was kindly provided by Brigitte Mauch-Mani (Universitè de Neuchâtel, Switzerland). Two week old plants were sprayed with 1×10^3 spores/mL and maintained at 100% relative humidity. Two days after inoculation leaves were sampled by freezing in liquid N_2 and stored at −80°C until analysis. Disease rate was determined by microscopical analysis after tripan blue stainings of infected leaves (Flors et al., 2008). Leaves were classified in a disease ranking as explained in the figure legend.

GROWTH CONDITIONS FOR STARVATION EXPERIMENTS

Arabidopsis thaliana was grown hydroponically as described in Lejay et al. (1999). The seeds were germinated directly on top of modified Eppendorf tubes filled with pre-wet sand. Plants were grown until the age of 5 weeks on a 1 mM NH_4NO_3 nutrient solution (repressed plants), which prevented any growth difference between the two genotypes (Lejay et al., 1999). Before inoculation experiments, plants were transferred for 48 h to N-free solution (de-repressed plants).

HPLC-ESI FULL SCAN MASS SPECTROMETRY (Q-TOF INSTRUMENT)

Metabolome analysis was performed using an Acquity UPLC system (Waters, Mildford, MA, USA) interfaced to hybrid quadrupole time-of-flight (QTOF Premier). The LC separation was performed by HPLC SunFire C18 analytical column, 5 μm particle size, 2.1 × 100 mm (Waters). Analytes were eluted with a gradient of methanol and water containing 0.01% HCOOH. Chromatographic conditions and QTOF MS parameters were followed as described in Gamir et al. (2014).

HPLC-ESI TANDEM MASS SPECTROMETRY (TRIPLE QUADRUPOLE INSTRUMENT). HORMONAL ANALYSIS

An Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Mildford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The chromatographic separation conditions were closely related to those described previously. Chromatograpic conditions and TQD parameters were followed as described in Flors et al. (2008) and Gamir et al. (2012). Masslynx v 4.1 (Waters, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and samples.

FULL SCAN DATA ANALYSIS

Raw data obtained from Masslynx software was transformed to.CDF using Databrigde provide by Masslynx package. The.CDF data was process with R for statistical computing using XCMS package for relative quantification (Smith et al., 2006). Principal Component Analysis (PCA) were used as explained in http://www.numericaldynamics.com/ as a tool to define major changes in the metabolome of the plant under priming condition during fungal infections.

For heatmap construction and clustering of metabolites it was used the software MarVis Filter and MarVis cluster (http://marvis.gobics.de/; Kaever et al., 2012).

KINETICS OF $^{15}NH_4^+$ INFLUX

The kinetics of $^{15}NH_4^+$ influx as a function of external $^{15}NH_4^+$ concentrations ($[^{15}NH_4^+]_0$) was measured with $[^{15}NH_4^+]_0$ ranging from 0.02 to 0.8 mM. To kinetics studies control plants and de-repressed plants of three genotypes (Col-0 and *nrt3.1*) were used. Influx of $^{15}NH_4^+$ into the roots were assayed as described by Gazzarrini et al. (1999). Col-0 and *nrt3.1* plants with a normal fertilization or N depletion were sequentially transferred to 0.1 mM $CaSO_4$ for 1 min and to complete nutrient solution (pH 6.0) containing $^{15}NH_4^+$ (98% atom excess ^{15}N) for 5 min, at the concentrations indicated in figures. At the end of the ^{15}N labeling, roots were washed for 1 min in 0.1 mM $CaSO_4$ and were separated from shoots. The roots were dried at 70°C for 48 h, weighed, crushed in a hammer-mill and analyzed for total ^{15}N content using an integrated system for continuous flow isotope ratio mass spectrometry (Euro-EA elemental analyser, EuroVector S.P.A.; and Isoprime mass spectrometer; GV Instruments). Root influx is expressed in μmol $^{15}NH_4^+$ (g root DW)$^{-1}$ h^{-1}. To kinetics studies data-transformation method based on the Michaelis-Menten formalism was used. The experiment was repeated three times.

RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

Gene expression by quantitative real-time RT-PCR was performed using RNA samples extracted from root tissue using the RNA kit (Omega Bio-Tek Inc, Doraville, GA, USA) according to the manufacturer instructions. To avoid contaminating DNA, the samples were treated with DNAse I. A total of 1 μg of total RNA was annealed to oligo-dT and reverse transcribed using Omniscript Reverse Transcription kit (QIAGEN) to obtain cDNA. The sequences of the gene-specific oligonucleotides designed and used for real-time PCR are the following: *AMT1.1* forward: 5′acactgtggccagttaggcg3′ and reverse: 5′ccgtg gggatgtctttgaga3′, *Tubuline (TUB)* forward: 5′cgattccgttctcgatgt tgt3′ and reverse: 5′aatgagtgacacacttggaatcctt3′ and EF1α forward: 5′gtcgattctggaaagtcgacc3′ and reverse: 5′aatgtcaatggtgataccacgc3′. Real-time PCR was conducted using the QuantiTect™ SYBR Green PCR Kit (QIAGEN) and the SmartCycler II instrument (Cepheid). The experiment was repeated three times.

RESULTS

ALTERATION IN NITRATE AND AMMONIUM TRANSPORTERS ENHANCES BASAL RESISTANCE

It has been reported that deletions in *NRT2.1* and *NRT2.2* cause the reduced susceptibility of *Arabidopsis* to *P. syringae* (Camañes et al., 2012b). *NRT2.1* has been proposed as a transceptor that delivers and coordinates distal signaling that affects primary metabolism and defense signaling (Gojon et al., 2011; Camañes et al., 2012b). The function of *NRT2.1* in transporting nitrate is also influenced by the membrane protein *NRT3.1*, which is not a transporter itself, but its mutation significantly affects the HATS of nitrate (Okamoto et al., 2006; Orsel et al., 2006). To determine whether deficiencies in *NRT3.1* and the ammonium transporter gene *AMT1.1* have consequences in the basal resistance, we characterized the responses of the mutant lines *nrt3.1* and *amt1.1* toward *P. syringae* and *P. cucumerina*. The enhanced resistance mutant *lin1* (blocked in *NRT2.1*) was used as a control. All three mutants showed enhanced resistance against both

P. syringae and *P. cucumerina* compared to their respective control wild type plants (**Figures 1A,B**). The mutant *lin1* was more resistant to the bacterium, whereas *nrt3.1* and *amt1.1* showed significantly reduced susceptibility, although lower than *lin1*. Because *NRT3.1* influences nitrate transport, we tested whether it also affects ammonium transport. The mutant *nrt3.1* showed wild type $^{15}NH_4^+$ uptake kinetics both with N and after 48 h of N depletion (**Figure 2A**). In addition, we also determined

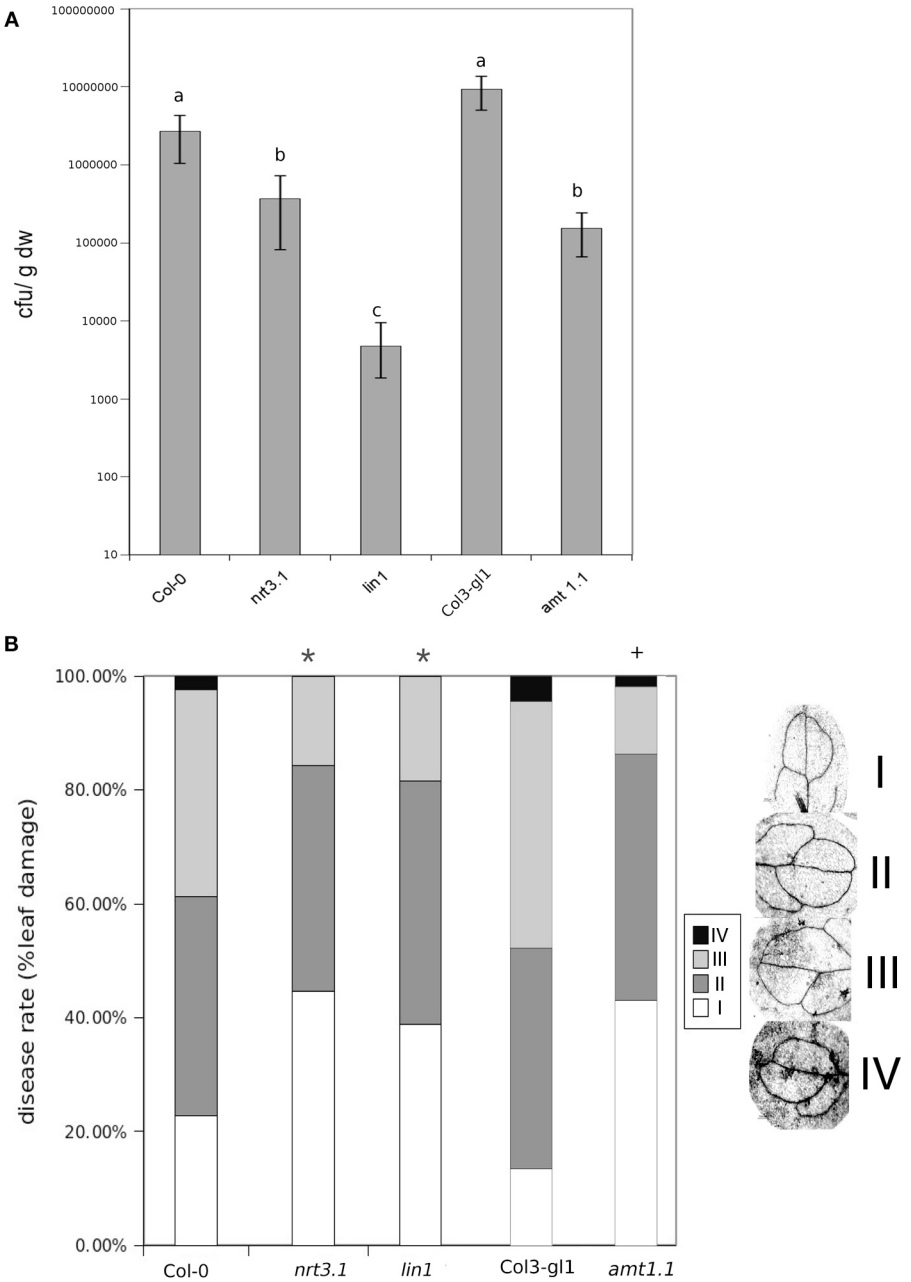

FIGURE 1 | Bacterial proliferation and disease rate in Col-0, *lin1, nrt3.1,* and *Col3-gl/1* and *amt1.1* plants infected with *P. syringae* and *P. cucumerina* respectivelly. (A) Five week old plants were challenge-inoculated by dipping in a bacterial suspension of *P. syringae* at 2×10^5 c.f.u. mL^{-1}. The values presented are means (±SD) of the log of the proliferation values. Data represent the average of three independent experiments ($n = 3$). Different letters mean significant statistical differences (ANOVA, LSD test; $p < 0.05$). **(B)** Two week old plants were inoculated by spraying with 1×10^3 spores × mL^{-1} with PcBMM. Disease symptoms were recorded by trypan-blue staining at 5 days post inoculation. Disease rate was ranked according to the infected leaf surface: level I no infection, level II less than 25% of infected leaf surface, level III between 25 and 50% of infected leaf surface, level IV more than 50% of infected leaf surface. The figure shows a representative experiment that was repeated three times with the same results. Data presented are the means of the percentage of diseased leaves per plant. Asterisk indicate statistically significant differences compared with non-induced control plants (*t*-test; $^*p < 0.05$; $^+p < 0.05$ with their respective controls; n∼50 leaves).

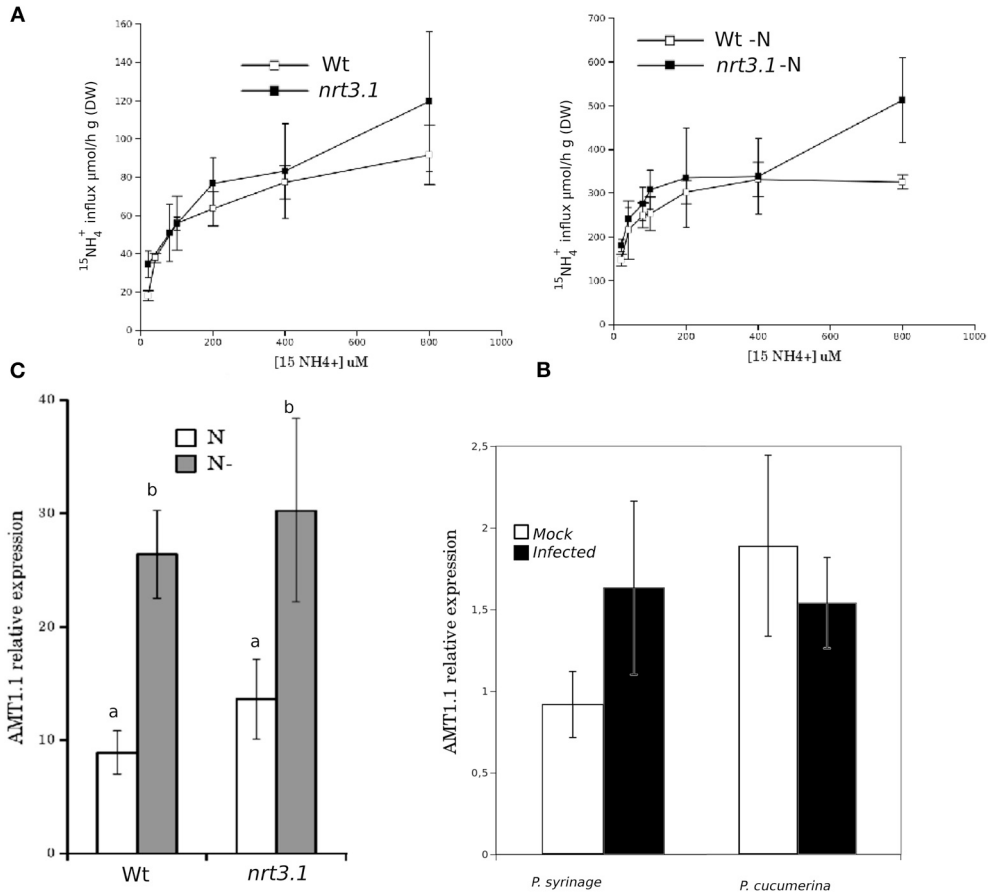

FIGURE 2 | (A) Kinetics of $^{15}NH_4^+$ influx in Col-0 and *nrt3.1* roots in the low $^{15}NH_4^+$ concentration range. Plants were grown hydroponically with N supplied as 1 mM NH_4NO_3 during 6 weeks. After that one group of plants were transferred to 1 mM NH_4NO_3, other group of plants were transferred to N-free nutrient solution (-N) during 48 h. $^{15}NH_4^+$ influx was measured at different concentrations of external $^{15}NH_4^+$. Each data is the mean of 30 replicates ±SE. **(B)** Real-time PCR analysis of the expression of *AMT1.1* in Col-0 and *nrt3.1* plants fertilized normally along 5 weeks and exposed to N starvation 2 days (-N). The *AMT1.1* transcript levels were normalized to the expression of *TUB* measured in the same samples. The experiment was repeated using *EF1α* with similar results. Each bar represents average data with standard error bars from two technical replicates three independent experiments ($n = 6$). **(C)** Real-time PCR analysis of the expression of *AMT1.1* in mock and *P. syringae* or *P. cucumerina* wild type infected plants. The *AMT1.1* transcript levels were normalized to the expression of *TUB* measured in the same samples. The experiment was repeated using *EF1α* with similar results. Each bar represents average data with standard error bars from two technical replicates three independent experiments ($n = 6$).

whether the *AMT1.1* gene is upregulated in the absence of N under the same experimental conditions. N depletion induces (de-represses) *AMT1.1* expression in wild type plants (Gazzarrini et al., 1999), whereas *nrt3.1* shows wild type *AMT1.1* expression (**Figure 2B**). Both results suggest that *NRT3.1* does not modulate ammonium kinetics uptake and that it does not alters *AMT1.1* de-repression.

Finally, we tested whether infection with *P. syringae* or *P. cucumerina* affects *AMT1.1* expression in a manner that may act as targets for bacterial or fungal effectors. Following infection, *AMT1.1* expression was not significantly modified by any of the pathogens (**Figure 2C**).

NITROGEN STARVATION ENHANCES *P. syringae* SUSCEPTIBILITY BUT DOES NOT AFFECT *P. cucumerina* RESISTANCE

Previously, we determined that nitrate starvation for 48 h increased the susceptibility of *Arabidopsis* toward *P. syringae* and that *nrt2.1* was also impaired (Camañes et al., 2012b). To determine whether N depletion affects the basal resistance against both pathogens and to determine the contribution of the ammonium transporter *AMT1.1* to this interaction, we performed a hydroponic experiment by inducing N depletion for 48 h. According to our previous study (Camañes et al., 2012b), wild type starved plants were more susceptible to *P. syringae* (**Figure 3**), whereas they were not affected in their resistance to the fungus (**Figure 3**). Notably, *amt1.1* starved plants also showed increased susceptibility against *P. syringae*, whereas their resistance to the fungus was not affected.

amt1.1 DISPLAYS ALTERED HORMONAL DEFENSE RESPONSES AGAINST *P. syringae* AND *P. cucumerina*

Because *amt1.1* showed reduced susceptibility and enhanced resistance toward the bacterium and the fungus, respectively, compared to the wild type plants, we analyzed the main

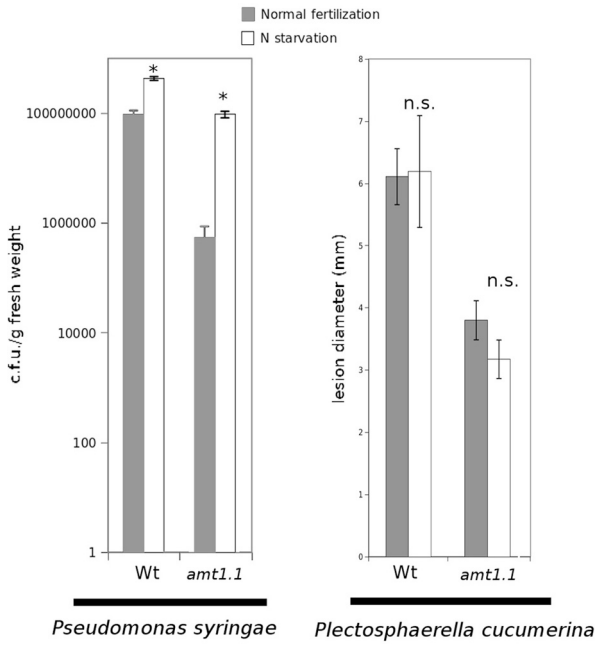

FIGURE 3 | *P. syringae* proliferation and lesion diameter cause by *P. cucumerina* in *Col3-gl1* and *amt1.1* plants fertilized normally (Normal fertilization) along 5 weeks and exposed to N starvation 2 days before inoculation (N starvation). Hydroponically growing plants were challenge-inoculated either with a bacterial suspension of *P. syringae* at 2.5×10^5 c.f.u./ml or with a drop of 1×10^5 spores $\times$ mL^{-1} of PcBMM. Data represent the average of three independent experiments ($n = 3$). The values presented are means of infected plants (±SD). Asterisk indicates statistically significant differences (LSD test; $p < 0.05$).

hormones involved in defense signaling. Surprisingly, the SA, JA, and JA-Ile levels remained lower in the *amt1.1* plants infected with *P. syringae* for the entire experiment compared to the wild type plants (**Figure 4A**). Indoleacetic acid (IAA), however, did not significantly change in the *amt1.1* plants. A reduction of JA and JA-Ile in *amt1.1* plants suggests that this mutation may counteract bacterial effectors by enhancing resistance. However, this hypothesis is unlikely because the SA levels remained lower in the mutant. Therefore, the hormonal analysis did not clearly explain the reduced susceptibility of *amt1.1* to the bacterium. By contrast, the hormonal responses of the mutant against the fungus may contribute to resistance. JA-Ile and IAA following infection are increased compared to the wild type plants; however, SA at 48 and 72 hpi is reduced in the mutant infected with *P. cucumerina* (**Figure 4B**). We also tested other common defense responses that effective against *P. cucumerina*, such as the camalexin levels and callose accumulation (Ton and Mauch-Mani, 2004; Gamir et al., 2014). Both defensive responses were strongly enhanced in the mutant response to the pathogen. Camalexin and callose in *amt1.1* remained significantly higher at 48 hpi (**Figure 5**).

METABOLOMIC PROFILING OF *amt1.1* IN RESPONSE TO PATHOGEN ATTACK

The hormonal profile of *amt1.1* in response to bacterial infection did not explain its enhanced resistance; therefore, we performed full metabolomic profiling to understand the metabolic changes that may contribute to the resistance of this mutant. For *P. cucumerina,* the classical defenses against necrotrophs, such as JA-Ile, callose, and camalexin, over-accumulated in *amt1.1* plants infected with the fungus. We also performed metabolic profiling following infection with the fungus to determine the extent to which *amt1.1* affects the responses to fungal infection. We used untargeted reverse-phase liquid chromatography-mass spectrometry (HPLC-QTOF-MS) for the profiling. *Col3-gl1* and *amt1.1* plants were either mock or *P. syringae* or *P. cucumerina* infected. Metabolomic analysis and data reporting were performed as described by Pitzschke and Hirt (2010), Fernie et al. (2011), and Kaever et al. (2012).

The acquired raw data were transformed into.cdf files using Databridge from the Masslynx 4.1 software (Masslynx 4.1, Waters). These data were subsequently subjected to analysis using the free software R for statistical purposes. The signals from the positive and negative electro-spray analysis (ESI+; ESI−) were processed separately, these ionization modes assure that all compounds that tend to form either cations or anions will be detected by the mass spectrometer. We performed a PCA of all signals obtained in ESI+ and ESI−. The infection with *P. syringae* and *P. cucumerina* induced strong changes in the metabolome of the wild type plants and *amt1.1* plants that differed for the positively and negatively ionized compounds (**Figure 6**). The impact of the infection in ESI+ appeared stronger in *amt1.1* upon *P. cucumerina* infection compared with *P. syringae*; however the changes in ESI− are more subtle because there is an overlap in the variability of *amt1.1* with or without infection independently of the pathogen.

Notably, in the absence of infection, the basal metabolomes of *Col3-gl1* and *amt1.1* do not overlap in any of the PCA with the exception of ESI− in the seedlings. This suggests that many metabolites are already altered in *amt1.1* in the absence of infection, and these changes may contribute to its enhanced resistance, discarding a general priming phenomenon to explain the phenotypes observed against both pathogens. Additionally, the wild type plants and *amt1.1* plants show differences in the basal responses between both experiments without inoculation, which may be due to a difference in the age of the plants (5 and 2 week-old plants for the *P. syringae* and *P. cucumerina* experiments, respectively) but also to the different mock treatments (dip inoculated Silwet for *P. syringae* and sprayed 10 mM $MgSO_4$ for *P. cucumerina*). A heatmap analysis was performed to identify metabolites involved in the resistance phenotypes of *amt1.1*, and the clusters of metabolites that over-accumulated in *amt1.1* either in the absence or presence of the infection were selected (**Figure S1**). Clusters from the heatmaps that showed overaccumulated compounds in the mutant (red color) were selected for a detailed metabolic study and pathway analysis.

Prior to identifying the signals in the selected clusters, we performed a detailed analysis of the amino acids in the metabolome. For such purpose, we constructed a library of amino acid standards for the identity assignation using the exact mass provided by the Q-TOF analyser and the retention time parameter (Gamir et al., 2014).

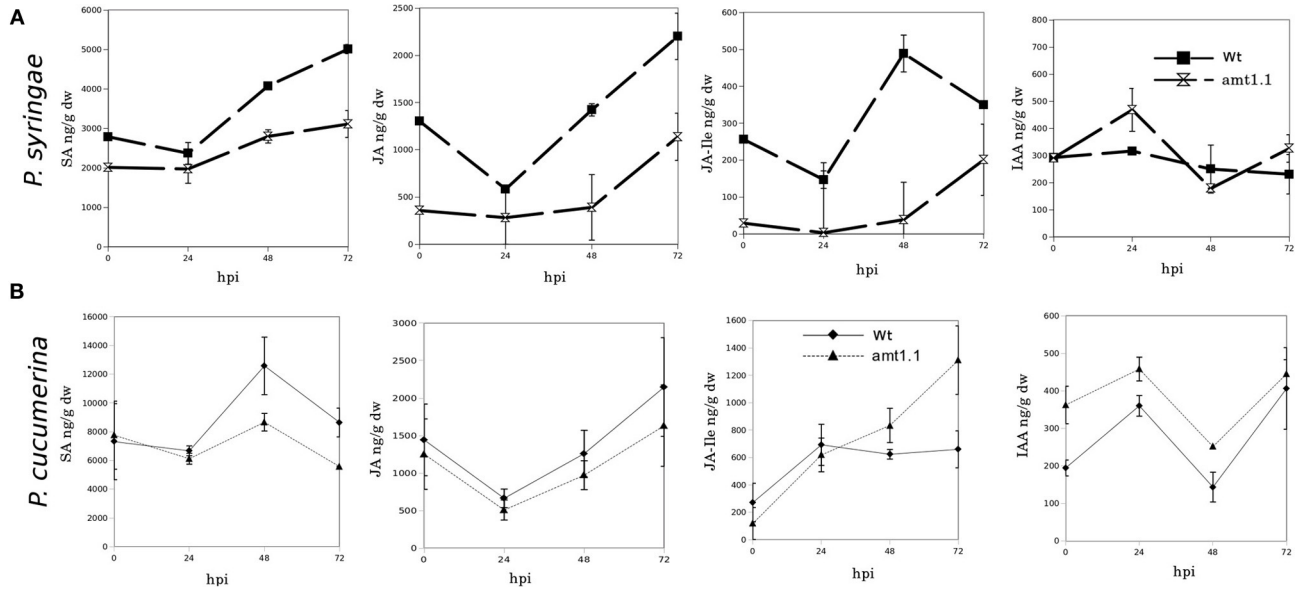

FIGURE 4 | SA, JA, JA-Ile, and IAA profiling upon *P. syringae* and *P. cucumerina* infection. Plants were challenged as described in **Figure 1**. Both mock and pathogen infected plants were harvested at different time-points. Freeze dried material was processed for a targeted quantification analysis by TQD-MS. The concentration of the hormones was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Leaf material from 15 individual plants for *P. syringae* **(A)** resistance assays and 150 plants for *P. cucumerina* **(B)** resistance assays were pooled together for each treatment × genotype combination. Data represent average three independent experiments ±SD; $n = 3$.

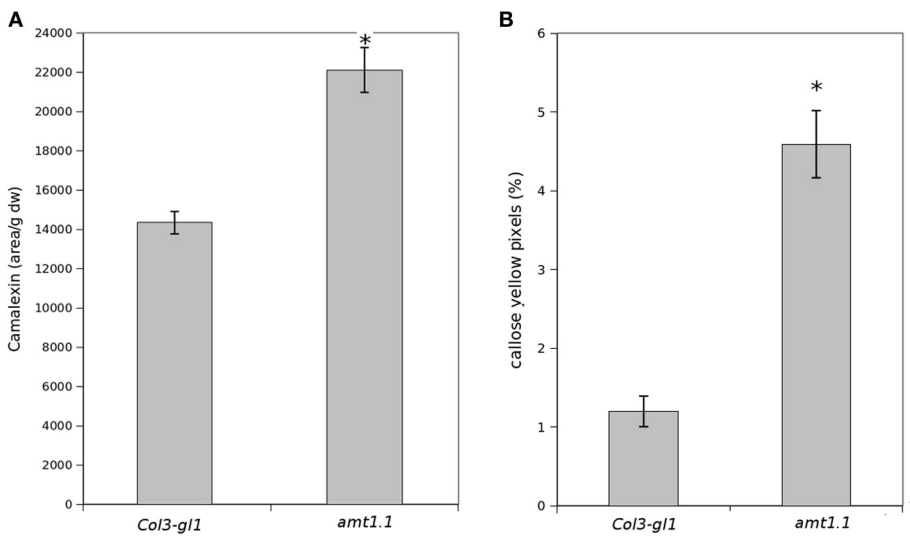

FIGURE 5 | Camalexin and callose levels upon *P. syringae* and *P. cucumerina* infection. Plants were challenged as described in **Figure 1**. Either mock or pathogen infected plants were harvested at 48 hpi. **(A)** Freeze dried material was processed for a targeted quantification analysis of camalexin by TQD-MS. The relative concentration was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Leaf material from 150 individual plants were pooled together for each treatment × genotype combination. Data represent average three independent experiments. **(B)** Callose was visualized by aniline blue staining and epifluorescence microscopy (UV). Quantification was performed by determining the number of yellow pixels per million pixels corresponding to pathogen-induced callose on digital photographs of infected leaf areas. Asterisk indicates statistically significant differences (LSD test; $p < 0.05$). Data shown are means (±SD; $n = 20$) of the relative number of yellow pixels per photograph.

In response to *P. syringae,* all the amino acids, together with azelaic acid and pipecolic, were not increased in the *amt1.1* mutant in response to the bacterium (data not shown). However, a different profile was observed following *P. cucumerina* infection. The majority of amino acids were increased in *amt1.1* plants with or without infection (**Figure 7**). Pro, Thr, His, and Met were previously elevated in the absence of infection and this increase occurred until 48 hpi. Ala, Arg, Asn, Ile+Leu (not separated in

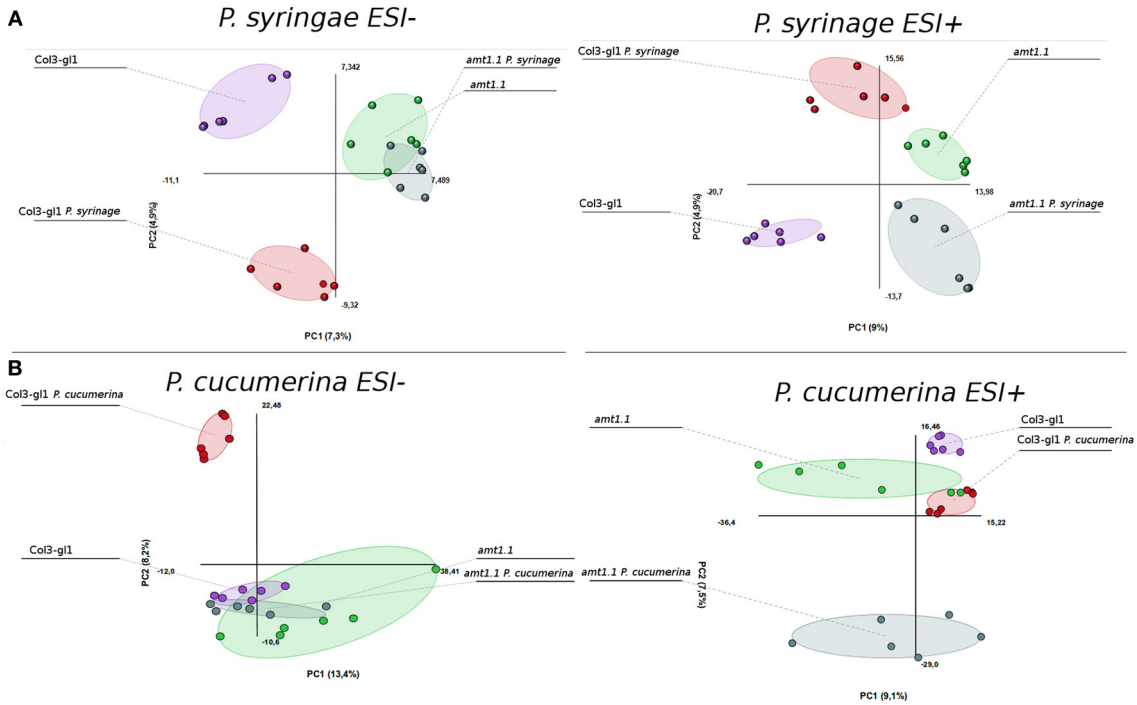

FIGURE 6 | Non-supervised Principal Component Analysis. (PCA) analysis representation of major sources of variability of ESI+ and ESI− signals obtained from a non-targeted analysis by HPLC-QTOFMS to monitor metabolomic changes during bacterial **(A)** and fungal invasion **(B)**. **(A)** Five week old plants were dip inoculated with *P. syringae* with $2.5 \times 10E^5$ c.f.u/ml. 48 hpi Leaf material from 15 individual plants were pooled together for each treatment × genotype combination. **(B)** Two week old plants were sprayed inoculated with $10E^3$ spores/ml of *P. cucumerina* and samples for analysis were collected 48 hpi. Leaf material from 150 individual plants were pooled together for each treatment × genotype combination. Data points represent two technical replicates from three independent experiments (biological replicates; $n = 6$) injected randomly into the HPLC-QTOFMS. The signals corresponding to different treatments were compared using the non-parametric Kruskal-Wallis test, and only data with a *P*-value lower than 0.01 between groups was used for subsequent processing.

our chromatographic analysis), Cys, Gln, Lys, and Tyr showed a primed profile because they remained at the same levels in the wild-type plants before the inoculation; however, after infection, *amt1.1* displayed elevated levels compared to *Col3-gl1*. Only Asp, Glu, Phe, Trp, and Val in the mutant remained at the same levels as wild type infected plants (data not shown). Next, we performed a full comparative analysis of the metabolome. To identify the compounds, we used a library of standards with both the exact mass provided by the Q-TOF detector and the retention time. For the compounds with no available standards, we used the exact mass and fragmentation spectrum, when available, from the Massbank and Metlin databases. For such compounds, we assigned a tentative identification. After the signals were either exactly or tentatively identified, the compounds were searched against the Aracyc (http://pathway.gramene.org/gramene/aracyc.shtml) and Kegg (http://www.genome.jp/kegg/) databases to assign a putative metabolic pathway and a biological function. The analysis of the hormones or amino acids in *amt1.1* yielded a plausible explanation for its reduced susceptibility to *P. syringae*. However, the selection of the metabolite clusters from the heatmap that over-accumulated in *amt1.1* compared to the wild type plants following infection provides valuable information. Following the tentative identification of metabolites and their classification into metabolic pathways of *Arabidopsis*, we observed that fatty acid-CoA conjugates were increased in *amt1.1* either in the absence or presence of *P. syringae* (**Figure 8**). Nucleotides, such as ATP or UTP were also increased in *amt1.1*. Interestingly, three chemical species derived from TDP were also found for the *amt1.1* over-accumulated compounds. Dipeptides and tripeptides were another group of interest, which was surprising because the free amino acids in *amt1.1* are not different from the wild type plants. Although SA was not increased in the mutant, several benzoyl compounds over-accumulated in *amt1.1*, both with and without infection. The metabolic pathways with a higher number of hits in the cluster of over-accumulated compounds in *amt1.1* were the flavonoid and phenylpropanoid phytoalexins (**Table 1**). Although infection induced a strong increase in these compounds in *Col3-gl1*, *amt1.1* showed higher levels in the absence of infection. Finally, an aliphatic glucosinolate and vitamins B1 and B6, all of which are involved in defense, were also increased in the mutant (**Figure 8**). For the interaction with *P. cucumerina*, the identified compounds matched four secondary metabolism pathways of *Arabidopsis*, indolic glucosinolates, aliphatic glucosinolates, the Trp pathway and phenylpropanoids (with a total of 19 compounds) (**Figure 9**). Notably, many secondary metabolites in these groups accumulated in *amt1.1* without infection, and all were then either increased or maintained in response to *P. cucumerina* in *amt1.1*

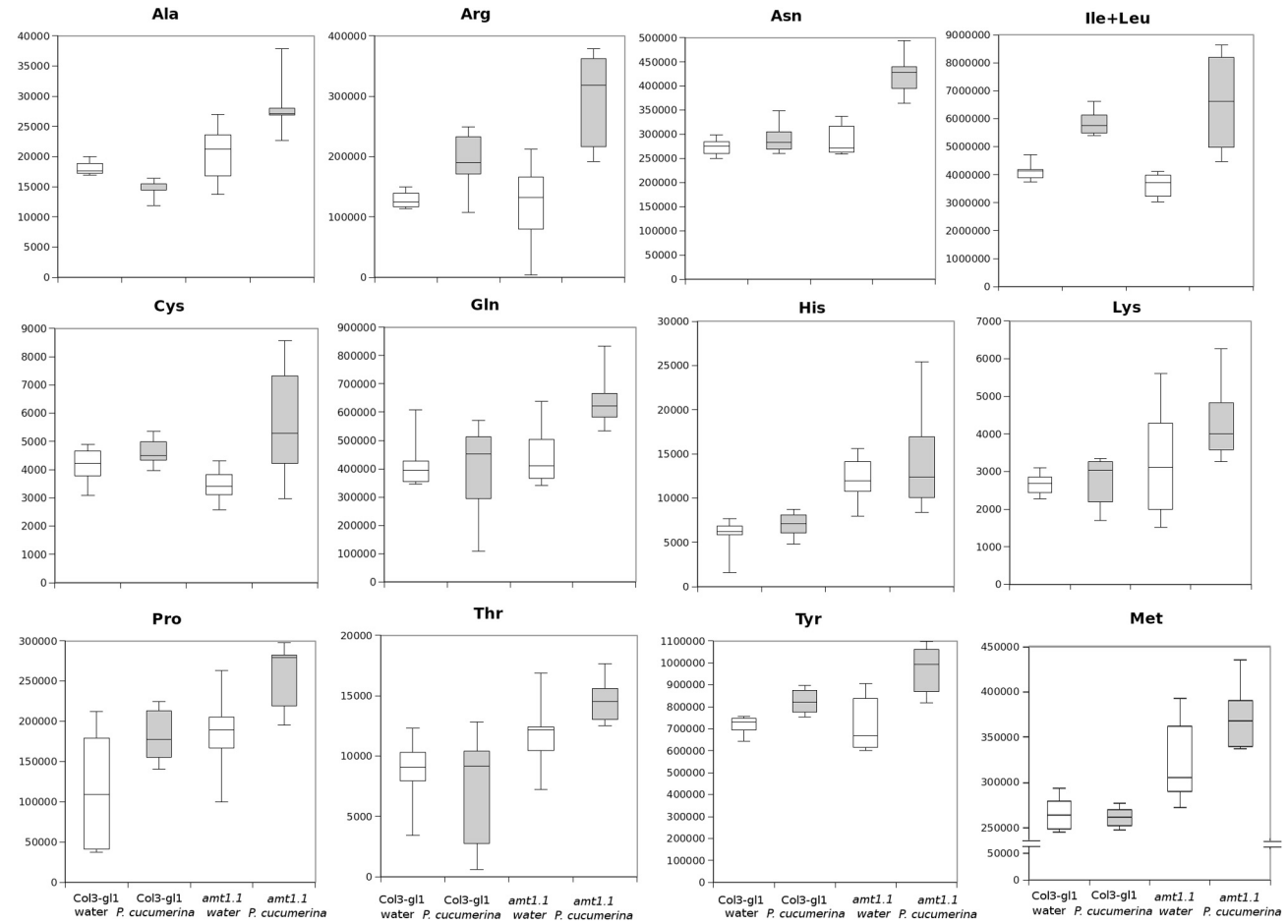

FIGURE 7 | Aminoacid profiling upon *P. cucumerina* infection. Two week old *Col3-gl/1* and *amt1.1* plants either mock or *P. cucumerina* inoculated were processed for relative quantification analysis by HPLC-QTOFMS data. The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. White bars are mock inoculated and filled bars are *P. cucumerina* infected plants. Leaf material from 150 individual plants were pooled together for each treatment × genotype combination. Boxplots represent average three independent experiments with two technical replicates ($n = 6$).

plants compared to wild type plants. In addition to these pathways, although there were fewer hits, tentative identification of the metabolites showed that *amt1.1* responded to infection by increasing three mevalonic acid derivatives, four nucleotide intermediaries, three shikimic acid metabolites, two polyamines, and several hormones, such as GA3, GA-A58, and ABA (**Figure S2**).

DISCUSSION

We previously reported (Camañes et al., 2012b) that under optimal nutritional conditions, the total contents of N in *nrt2.1* and *amt1.1* plants are identical, which indicates that the N content is not responsible for altering the basal resistance in the mutants. Based on our previous study, we performed all resistance experiments, except otherwise noted, under the optimal fertilization conditions. First, we demonstrated that mutations in several genes related to N transport resulted in an increased resistance against two pathogens with different lifestyles. The HATS of nitrate uses a membrane complex in which the NRT3.1 protein modulates the function of *NRT2.1*, and mutation of *NRT3.1* results in the most significant losses of HATS function compared to *NRT2.1* mutations (Orsel et al., 2006). Therefore, we tested two different hypotheses. First, we tested whether this gene may also regulate ammonium transport, and second, we tested whether *NRT3.1* is involved in the plant defense response. The uptake experiments with $^{15}NH_4^+$ showed that the absorption of ammonium in *nrt3.1* does not differ from Col-0 plants, which suggests that it is not involved in ammonium HATS. We also demonstrated that *AMT1.1* gene expression with or without N was similar for Col-0 and *nrt3.1* plants. These observations confirm that this gene is not involved in *AMT1.1* modulation. Because *nrt3.1* plants show resistance against *P. cucumerina* and *P. syringae*, this suggests that it may have overlapping mechanisms with *NRT2.1* to stimulate defense; however, this hypothesis must be confirmed.

We ascertained the influence of *AMT1.1* on plant resistance because our experiments suggest that *AMT1.1* acts as a transceptor to coordinate plant tolerance against N depletion and defense against pathogens as suggested previously for NRT1 and NRT2.1 (Gojon et al., 2011; Camañes et al., 2012b). The

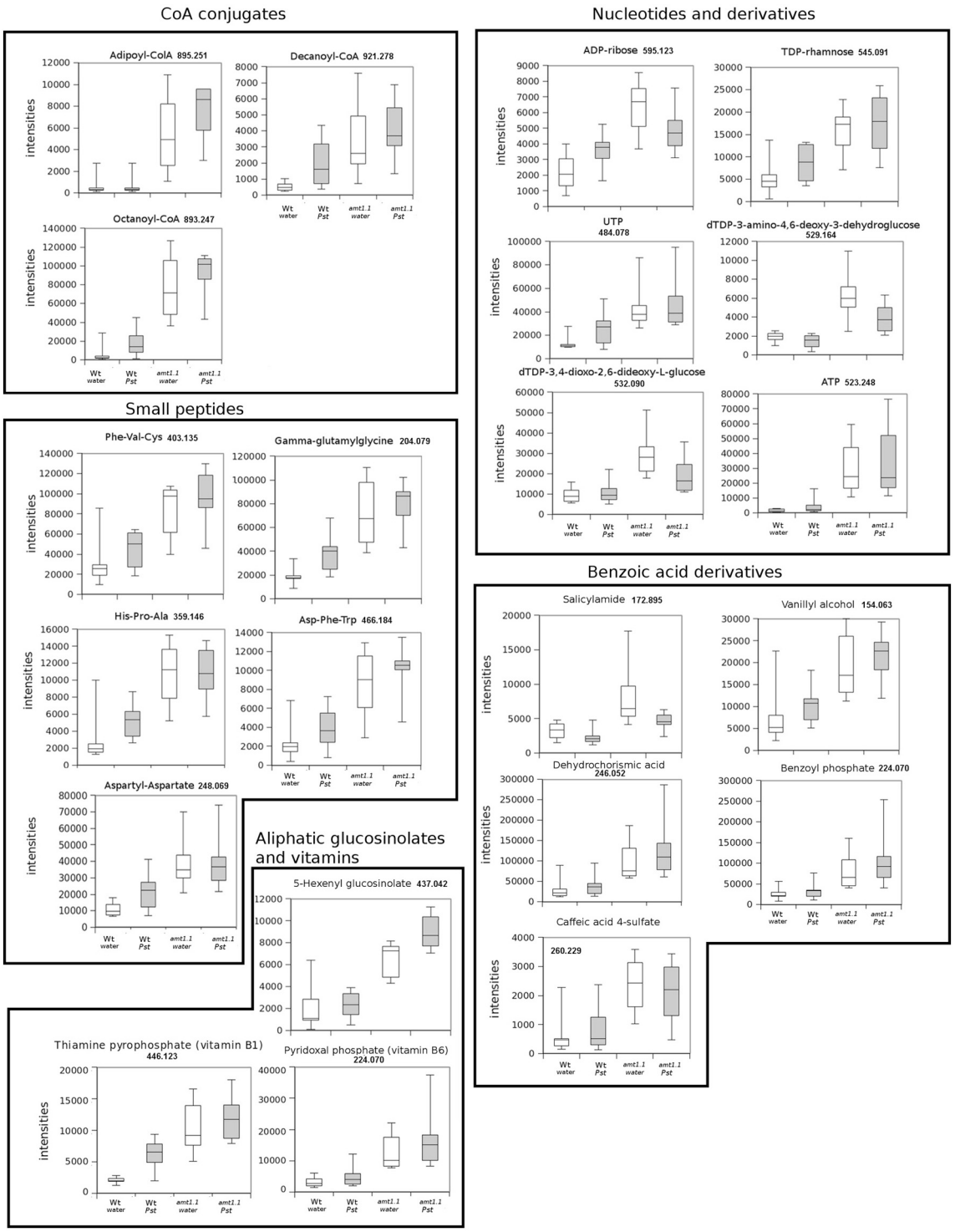

FIGURE 8 | Profiling of the main hits in the overaccumulated compounds upon *P. syringae (Pst)* infection in *amt1.1* compared with *Col3-gl1*. Five week old *Col3-gl1* and *amt1.1* were infected as described in **Figure 1**. After 48 hpi plants were processed for relative quantification analysis by HPLC-QTOFMS data. The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. The compounds were tentatively identified using the exact mass criteria using the METLIN and Massbank databases. The compounds were grouped by metabolic pathways according to KEGG and AraCyc databases. White bars are mock inoculated and filled bars are *P. syringae* infected plants. Leaf material from 15 individual plants were pooled together for each treatment × genotype combination. Boxplots represent average three independent experiments with two technical replicates. Only data showing a *p*-value below 0.05 after a Kuskal Wallys test were used for pathway assignation ($n = 6$).

Table 1 | Phenylpropanoid profiling upon *P. syringae* infection.

Neutral mass	*Col3-gl1*-Mock	*Col3-gl1-P. syringae*	amt1.1-Mock	amt1.1-*P. syringae*	Tentative identification
ESI−					
868.141	5717.574 ± 1339.009	7903.077 ± 1171.697	15069.108 ± 3900.508	10608.243 ± 1332.910	Quercetagetin 7-methyl ether 3-(2‴-caffeoylglucosyl)-(1→2)-glucuronide
784.183	1993.974 ± 416.230	5584.543 ± 1443.404	6600.123 ± 1439.933	5310.700 ± 908.973	6-Hydroxyluteolin 7-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-3-glucuronide
920.272	1741.940 ± 548.621	4257.727 ± 1356.214	9965.213 ± 1679.580	8535.086 ± 1714.162	Quercetin 3-(2‴-caffeylsambubioside)-7-glucoside
546.072	2872.295 ± 527.433	5379.807 ± 1033.293	10884.914 ± 2827.895	20974.750 ± 3776.673	4,6-Dideoxy-4-oxo-dTDP-D-glucose
982.276	1986.003 ± 537.055	6063.620 ± 1171.669	11885.748 ± 1351.898	15605.830 ± 897.168	Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside
356.221	3751.581 ± 770.196	2451.929 ± 1357.613	11677.959 ± 1955.930	11193.410 ± 1678.297	4,8,11,14-Eicosatetraenoic acid, 6-hydroxy-, (E,Z,Z,Z)-
402.132	97604.298 ± 15455.088	190757.113 ± 33780.888	343773.018 ± 49529.885	404922.286 ± 53046.318	7-Hydroxyflavanone beta-D-glucopyranoside
892.242	16249.517 ± 9694.477	39546.429 ± 13717.218	164163.569 ± 34206.807	186117.251 ± 20320.571	Palargonidin 3-(6″-ferulylglucoside)-5-(6‴-malonylglucoside)
528.126	834.255 ± 209.720	1438.870 ± 373.901	6122.663 ± 1052.023	6546.308 ± 1472.074	4,2′-Dihydroxy-3,4′,6′-trimethoxychalcone 4-glucoside
344.123	6814.904 ± 2501.614	9237.129 ± 1791.413	17129.393 ± 2994.036	18527.512 ± 1827.166	5,6,7,4′-Tetramethoxyflavanone
460.129	25299.855 ± 13806.666	40022.020 ± 15639.629	158482.998 ± 18759.482	169688.892 ± 18730.315	7,8,3′,4′-Tetramethoxy-6″,6″-dimethylpyrano[2″,3″:5,6]flavone
894.252	2544.518 ± 1377.062	5227.236 ± 2004.211	25842.316 ± 5738.193	29016.835 ± 3447.355	Genistein 7,4′-bis(O-glucosylapioside)
622.151	2809.060 ± 709.473	7451.498 ± 1212.428	12048.485 ± 1529.533	12526.350 ± 1257.233	Kaempferol 3-[2‴-acetyl-alpha-L-arabinopyranosyl-(1→6)-galactoside]
ESI+					
292.042	1186.017 ± 392.336	1807.786 ± 476.655	4104.516 ± 661.600	4953.756 ± 1425.747	5,7,3′-Trihydroxyisoflavone
360.181	853.215 ± 287.879	1370.833 ± 540.537	6396.268 ± 2111.103	4320.185 ± 1595.289	5,2′,3′-Trihydroxy-3,7,8-trimethoxyflavone
306.072	5958.788 ± 1705.843	7086.008 ± 2216.577	23547.293 ± 5398.893	19686.483 ± 5614.565	Pelargonidin
400.116	30044.197 ± 4429.060	48455.063 ± 8051.467	89620.850 ± 16202.253	69018.310 ± 10018.920	Flavonol 3-O-D-galactoside
544.609	1029.246 ± 505.676	2338.742 ± 704.251	5440.121 ± 1619.737	5301.904 ± 2113.984	Quercetin 3-glucoside-3′-sulfate
498.147	65764.676 ± 21821.696	45436.512 ± 10350.166	202317.111 ± 40047.133	192242.002 ± 55136.009	Quercetin 7-methyl ether 3,3′-disulfate
460.138	12122.235 ± 2423.017	16230.111 ± 3295.099	27447.715 ± 4518.375	24785.374 ± 4133.228	Luteolin 4′-methyl ether 7,3′-disulfate
636.387	137818.486 ± 17769.746	86637.426 ± 7725.291	71514.777 ± 4284.190	63127.380 ± 9084.052	flavonoid Isoscutellarein 4′-methyl ether 8-(2″,4″-disulfatoglucuronide)

Five week old plants either mock Col3-gl/1 and amt1.1 or P. syringae inoculated (Col3-gl/1 Pc and amt1.1 Pc) plants were processed for relative quantification analysis by HPLC-QTOFMS data. Tentative identification of signals was performed by contrasting the exact mass in the METLIN and Massbank databases. The concentration of selected signals within the phenylpropanoid metabolism was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Data show average values of three independent biological replicates containing pools of 15 plants in each replicate are shown with their standard deviation.

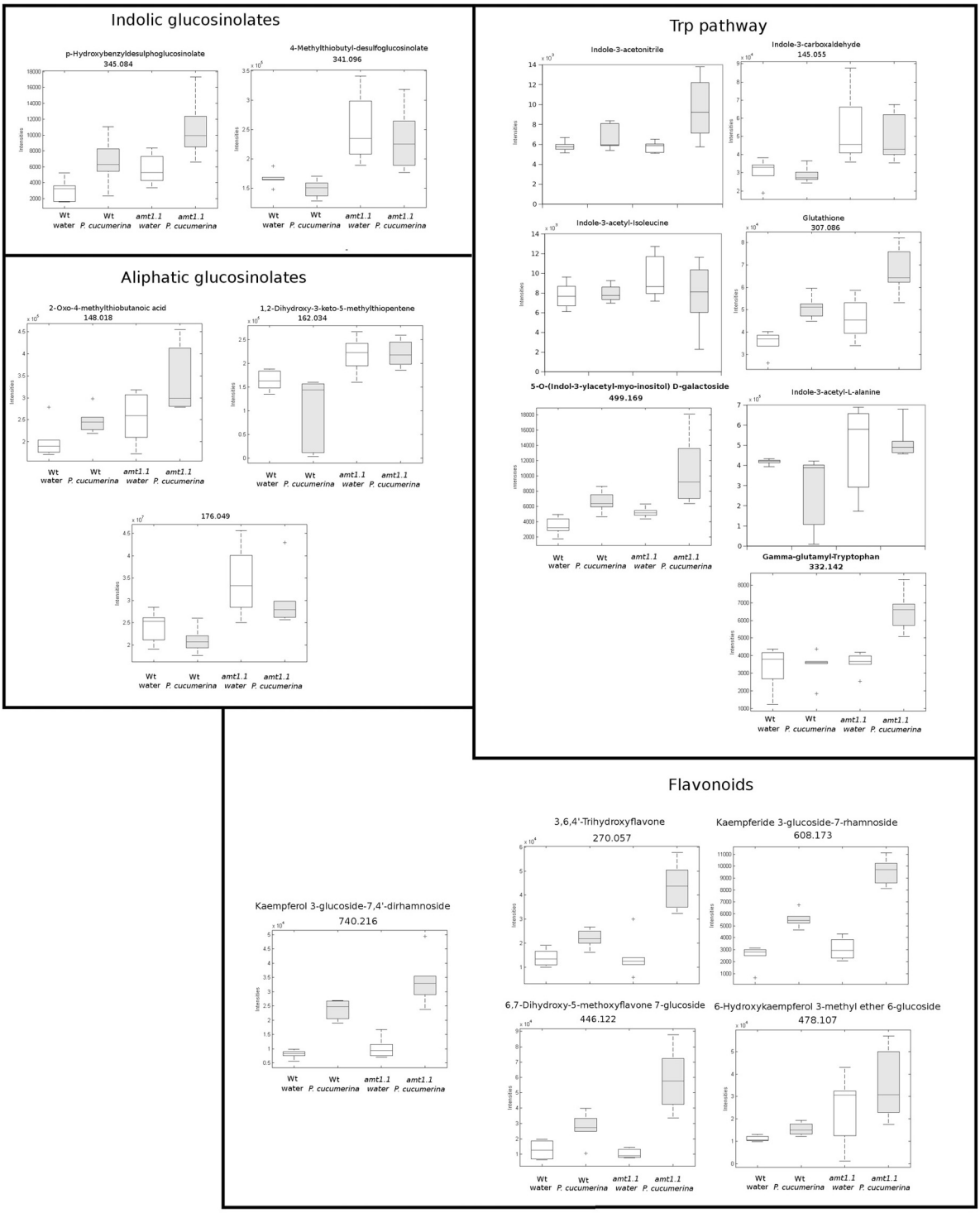

FIGURE 9 | Profile of the main hits in the overaccumulated compounds upon *P. cucumerina* infection in *amt1.1* compared with *Col3-gl1.* Two week old *Col3-gl1* and *amt1.1* were infected as described in **Figure 1**. After 48 hpi plants were processed for relative quantification analysis by HPLC-QTOFMS data. The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. The compounds were tentatively identified using the exact mass criteria using the METLIN and Massbank databases. Those compounds tentatively identified have the exact mass indicated. Te compounds fully identified have no reference to the mass. The compounds were grouped by metabolic pathways according to KEGG and AraCyc databases. White bars are mock inoculated and filled bars are *P. cucmerina* infected plants. Leaf material from 150 individual plants were pooled together for each treatment × genotype combination. Boxplots represent average three independent experiments with two technical replicates. + indicates outliers. Only data showing a *p*-value below 0.01 after a Kuskal wallys test were used for pathway assignation ($n = 6$).

mutant *amt1.1* showed reduced susceptibility against *P. syringae*, and surprisingly, *amt1.1* showed strong resistance toward *P. cucumerina* infection compared to wild type plants. Previously, we demonstrated that environmental factors contribute to the enhanced expression of *NRT2.1*. For example, N depletion affects the resistance of *Arabidopsis* to *P. syringae*. Although the depletion of N induces *AMT1.1* gene expression (**Figure 2**), it also increased the susceptibility in *amt1.1* plants, suggesting that it is the absence and not the overexpression of *AMT1.1* that alters resistance mechanisms. Notably, neither infection with *P. syringae* or *P. cucumerina* significantly altered the *AMT1.1* transcripts. We tested whether this also applies to resistance against necrotrophs. The enhanced resistance of *amt1.1* against *P. cucumerina* was not altered by N depletion; therefore, the absence of these genes impacts *Arabidopsis* defense against this pathogen but not their overexpression, suggesting posttranscriptional or post-translational regulation. This is not so surprising because NRT1.1 and NRT2.1 have a complex protein regulation mechanism mediated by proteins, such as CIPK23 and NAR2.1 (Okamoto et al., 2006; Ho et al., 2009).

To identify the molecular mechanisms for the *amt1.1* phenotypes, we performed several targeted and non-targeted metabolomic studies. For the targeted studies, hormones regulate the plant immune system (Pieterse et al., 2009). Active SA-dependent responses against *P. syringae* promote resistance in *Arabidopsis*. Additionally, because of the existing negative crosstalk between the SA and JA signaling pathways, resistance against biotrophs is associated with the concomitant downregulation of JA and JA-Ile levels (Pieterse et al., 2009). This has been observed in *amt1.1* plants upon *P. syringae* infection. In fact, SA, JA, and JA-Ile remain lower compared to wild type plants during a time-course experiment. Therefore, the reduced susceptibility of *amt1.1* to the bacterium cannot be caused by changes in the hormonal balance. Except for IAA, the hormones tested remained at lower levels in the absence of infection. This suggests that disruption of *AMT1.1* affects the basal levels of SA, JA, and JA-Ile, producing several constitutive changes. Interestingly, these basal changes are not visible in 2-week old plants; therefore, some alterations affected by the mutation are age dependent. For non-host resistance, such as for *P. cucumerina*, the defense responses are horizontal, multigenic and not controlled by single hormonal pathway, because other hormones in addition to SA, JA, ABA, and ET influence *Arabidopsis* responses against this necrotroph (Sanchez-Vallet et al., 2010). These compounds include the β-subunit of the heterotrimeric G-protein (Delgado-Cerezo et al., 2012) and glucosinolates and other Trp derivatives (Sánchez-Vallet et al., 2012; Gamir et al., 2014). In our first approach, we showed that *amt1.1* influences the SA, JA-Ile, and IAA profile during infection. Both JA-Ile and IAA over-accumulate, whereas SA is at lower levels compared to *Col3-gl1*. This may partially explain the observed phenotype because *P. cucumerina* has a necrotrophic lifestyle and is sensitive to JA-dependent signaling (Thomma et al., 2000). Recent research has suggested complex interplay between hormones in addition to the negative crosstalk between SA and JA (Pieterse et al., 2009), IAA also has antagonistic effects with SA, and both may explain the lower amounts of SA observed in *amt1.1,* although the final link between the ammonium transporter and hormonal signaling remains to be elucidated. *Arabidopsis* can also resist *P. cucumerina* through two other major mechanisms, callose deposition and camalexin (Ton and Mauch-Mani, 2004; García-Andrade et al., 2011; Sánchez-Vallet et al., 2012). Both mechanisms are directly or indirectly influenced by *AMT1.1*. Early callose deposition is enhanced in the mutant, and the camalexin levels remain higher at 48 hpi compared to *Col3-gl1*.

In our *Arabidopsis-P. syringae* system, we observed that *amt1.1* does not influence the basal amino acid accumulation in response to the bacterium. By contrast, there is a significant change in the amino acid profile for *P. cucumerina*. The mutant *amt1.1* over-accumulates several amino acids in the absence of infection, but these changes are even more robust after infection. Up to 11 amino acids over-accumulate in *amt1.1* infected plants compared to *Col3-gl1.* Surprisingly, Trp remains at the same level as the wild type plants with or without *P. cucumerina*. This may be explained by the over-accumulation of other Trp derivatives, such as indolic glucosinolates, IAA, camalexin, and other indole conjugates (**Figure 9**). These compounds are metabolic sinks that may trap the putative overproduction of Trp.

According to the PCA, the distance between *Col3-gl1* and *amt1.1* upon infection by both pathogens is rather marked; however, the diversity of the metabolic responses against *P. cucumerina* is much wider compared with the bacterium. In fact, of all of the compounds analyzed by targeted and untargeted chromatography that were found in clusters of over-accumulated metabolites in *amt1.1* upon *P. syringae* infection, only five pathways were identified, and of 45 signals, 24 were tentatively assigned to the phenylpropanoids (**Table 1**) and 5 to benzoic acid derivatives. This suggests that shikimic acid derivatives may compete with SA accumulation, which is a likely explanation for the reduced susceptibility of *amt1.1* and the reduced levels of free SA. Phenylpropanoids have been associated with plant resistance against a wide range of pathogens (Shadle et al., 2003). Two other compounds over-accumulated in amt1.1 plants are vitamins B1 and B6. Both have been linked to disease resistance (Denslow et al., 2005; Ahn et al., 2007). Exogenous treatment with vitamin B1 can induce defense priming in *Arabidopsis* against *P. syringae* mediated by H_2O_2 accumulation, callose deposition, and *NPR1.* Vitamin B6 is involved in superoxide quenching and stress responses, and during *P. syringae* infection in *Nicotiana*, vitamin B6 acts as an antioxidant and modulator of active oxygen species (Denslow et al., 2005). The relevance of the other metabolites, such as small peptides, nucleotides and CoA conjugates in the defense against pathogens must be clarified. Of the signals over-accumulated in *amt1.1* infected with *P. cucumerina,* 51 were tentatively or fully identified. These compounds were mainly involved in the indolic and aliphatic glucosinolates pathways, Trp pathway, flavonoids, nucleotides, and fatty acids. Notably, glucosinolates have been directly linked to resistance against *P. cucumerina* Sánchez-Vallet et al., 2012; Gamir et al., 2014, and also callose deposition in response to PAMPs (Clay et al., 2009). Although no direct evidence for *AMT1.1* and glucosinolates has been previously reported, this may be worth investigating. The NRT/PTR transporters have been associated with glucosinolate translocation in *Arabidopsis*, mainly

aliphatic (Nour-Eldin et al., 2012). Additionally, Met is a precursor of aliphatic glucosinolates (Kraker and Gershenzon, 2011). This amino acid highly accumulates in amt1.1 plants infected with *P. cucumerina*. Furthermore, three precursors of aliphatic glucosinolates are also elevated in *amt1.1* (**Figure 9**). These findings suggest a repressive influence of AMT1.1 on plant defenses against necrotrophic infection that result in changes in amino acid metabolism and/or peptide transport that causes increased defenses, most likely mediated by aliphatic and indolic glucosinolates. Other relevant metabolic contributions from different pathways may also be involved, such as chlorogenic acid, ABA, spermidine, several fatty acids, and other unclassified compounds that are also over-accumulated in *amt1.1* plants upon infection.

We previously demonstrated that the member of the high-affinity transport system of nitrate, the *NRT2.1* gene, in addition to transporting nitrate, is involved in the coordination of plant defense responses (Camañes et al., 2012b). This gene is de-regulated upon low nitrate concentrations in the roots, subsequently, the HATS is activated, providing tolerance mechanism against abiotic stresses (Gansel et al., 2001). Despite these functions, *NRT2.1* represses plant responses against bacterial and fungal pathogens. The metabolic interplay between nitrate uptake and resistance against pathogens is being elucidated. Because this resistance mechanism is multicomponent and non-pathogen-specific, the disruption of *NRT2.1* results in enhanced resistance against *P. syringae* and *P. cucumerina* through different defense mechanisms (Camañes et al., 2012b; Gamir et al., 2014).

Here, we demonstrate that *AMT1.1* acts as a negative regulator of *Arabidopsis* defense. Its disruption has a moderate impact on metabolomic changes upon *P. syringae* infection, which is correlated with a reduced susceptibility to the bacterium. By contrast, the metabolomic changes of *amt1.1* upon *P. cucumerina* challenge are significant, targeting Trp-derivatives and indolic glucosinolates. These changes may be responsible for the enhanced resistance of *amt1.1* against the fungus. This study and previous findings from our laboratory indicate that N transporters are transceptors that impact the transcriptome (Camañes et al., 2012b) and metabolome of *Arabidopsis* (Gamir et al., 2012, 2014) to coordinate abiotic stress tolerance and the negative regulation of biotic stress responses. The manner in which *NRT2.1* and *AMT1.1* regulate stress signaling remains to be identified; however, it is clear that the plant has mechanisms to distinguish and regulate different sources of stress. Transceptors appear to play a key role in integrating the environmental signals and plant defense responses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00231/abstract

Figure S1 | Heat map analysis performed with Marvis (Filter and Cluster packages). Five and two week old plants either mock (*Col3-gl/1* and *amt1.1*) or either *P. syringae Col3-gl/1 P. syringae* and *amt1.1 P. syringae* or *P. cucumerina* inoculated (*Col3-gl/1 Pc* and *amt1.1 Pc)* plants were processed for relative quantification analysis by HPLC-QTOFMS data. The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. Heatmaps are generated by using Mar-Vis Filter and Cluster following a Kruskal-Wallys test ($p < 0.01$). Clusters overepresented *amt1.1* mock or infected (intense red colors) compared with wild type plants were selected for subsequent data analysis.

Figure S2 | Profile of additional hits in the overaccumulated compounds upon *P. cucumerina* infection in *amt1.1* compared with *Col3-gl1*. For experimental details see **Figures 1**, **9**. The concentration of the metabolites was determined in all the samples by normalizing the chromatographic area for each compound with the dry weight of the corresponding sample. The compounds were tentatively identified using the exact mass criteria using the METLIN and Massbank databases. Those compounds tentatively identified have the exact mass indicated. Te compounds fully identified have no reference to the mass. The compounds were grouped by metabolic pathways according to KEGG and AraCyc databases. White bars are mock inoculated and filled bars are *P. cucmerina* infected plants. Leaf material from 150 individual plants were pooled together for each treatment × genotype combination. Boxplots represent average three independent experiments with two technical replicates. Only data showing a *p*-value below 0.01 after a Kuskal wallys test were used for pathway assignation ($n = 6$).

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Enhancing crop innate immunity: new promising trends

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Plants are constantly exposed to potentially pathogenic microbes present in their surrounding environment. Due to the activation of the pattern-triggered immunity (PTI) response that largely relies on accurate detection of pathogen- or microbe-associated molecular patterns by pattern-recognition receptors (PRRs), plants are resistant to the majority of potential pathogens. However, adapted pathogens may avoid recognition or repress plant PTI and resulting diseases significantly affect crop yield worldwide. PTI provides protection against a wide range of pathogens. Reinforcement of PTI through genetic engineering may thus generate crops with broad-spectrum field resistance. In this review, new approaches based on fundamental discoveries in PTI to improve crop immunity are discussed. Notably, we highlight recent studies describing the interfamily transfer of PRRs or key regulators of PTI signaling.

Keywords: innate immunity, pattern-triggered immunity, pattern-recognition receptor, leucine-rich repeat receptor kinase, lectin receptor kinase, pathogen, microbe

INTRODUCTION

Monocultures of highly fertilized crops that are typical of intensive agriculture practices are very sensitive to disease by adapted pathogens (Bruce, 2012). The development of resistant crops is thus critical for better yields. Although prone to disease, plants have evolved diverse defense mechanisms to cope with potential pathogens. To sense invaders, plants are equipped with surveillance machineries such as plasma membrane surface-localized proteins called pattern recognition receptors (PRRs), which detect foreign (non-self) pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs; Boller and Felix, 2009; Bohm et al., 2014; Zipfel, 2014), as well as self-derived damage-associated molecular patterns (DAMPs; Boller and Felix, 2009; Newman et al., 2013; Zipfel, 2014). MAMPs are evolutionarily conserved across a certain class of microbes and are essential for the microbial lifestyle. Some examples of bacterial MAMPs and their corresponding PRRs include flagellin/FLAGELLIN SENSING2 (FLS2; Gómez-Gómez and Boller, 2000), EF-Tu/EF-Tu RECEPTOR (EFR; Zipfel et al., 2006), *Xanthomonas* eMAX/RECEPTOR OF eMAX (ReMAX; Jehle et al., 2013) and peptidoglycan (PGN)/LYSIN-MOTIF1 (LYM1) and LYM3 (Willmann et al., 2011). Fungal MAMPs/PRRs pairs are exemplified by chitin/CHITIN ELICITOR RECEPTOR KINASE1 (CERK1; Miya et al., 2007; Wan et al., 2008), xylanase/ETHYLENE INDUCING XYLANASE2 (Eix2; Ron and Avni, 2004), and avirulence gene Ave1/VERTICILIUM1 (Ve1; de Jonge et al., 2012). DAMPs are endogenous molecules released upon cell damage or pathogen recognition. The known DAMPs/PRRs pairs include Pep peptides/PEP1 RECEPTOR 1 (PEPR1) and PEPR2 (Huffaker et al., 2006; Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010), cell wall fragment oligogalacturonides (OGs)/WALL-ASSOCIATED KINASE 1 (Brutus et al., 2010), and extracellular ATP (eATP)/DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1; Choi et al., 2014).

The recognition of MAMPs or DAMPs by PRRs activates the pattern-triggered immunity (PTI) response (Tsuda and Katagiri, 2010). Increased cellular Ca^{2+} concentration, production of reactive oxygen species (ROS), and activation of mitogen-activated protein kinase (MAPK) cascades are considered as early PTI responses, whereas callose deposition and marker gene up-regulation are observed later during PTI (Boller and Felix, 2009; Zipfel and Robatzek, 2010; Tena et al., 2011). These first layers of defense are believed to be sufficient to prevent the invasion of a wide range of pathogens. Functional PRRs are crucial for the success of PTI, as a defective recognition system makes plants more vulnerable to their surrounding environment. Notably, loss-of-function mutations in *FLS2* impair *Arabidopsis thaliana* resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 bacteria (Zipfel et al., 2004) and *Arabidopsis efr* mutants show increased susceptibility to *Agrobacterium tumefaciens* (Zipfel et al., 2006). Similarly, *Arabidopsis cerk1* mutants display enhanced sensitivity to fungal pathogens (Miya et al., 2007; Wan et al., 2008), and *Arabidopsis pepr1 pepr2* plants are more susceptible than wild-type plants to *Pst* DC3000, *Botrytis cinerea*, and *Colletotrichum higginsianum* (Ma et al., 2012; Liu et al., 2013; Ross et al., 2014). In addition to PRRs, other regulators are required for full activation of PTI (Macho and Zipfel, 2014). For example, BRI1-ASSOCIATED RECEPTOR-LIKE KINASE/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3) acts as a co-receptor for the conserved 22-amino acid epitope flg22 of the bacterial flagellin, and forms a complex with FLS2 upon flg22 perception (Chinchilla et al., 2007; Sun et al., 2013). BOTRYTIS-INDUCED KINASE1 (BIK1) is also critical for flg22-mediated signal transduction from the FLS2/BAK1 receptor complex (Lu et al., 2010; Zhang et al., 2010). Accordingly, loss-of-function mutants of *BAK1* or *BIK1* display impaired flg22-induced responses (Chinchilla et al., 2007; Heese et al., 2007; Lu et al., 2010; Zhang et al., 2010). Recent studies

showed that L-type lectin receptor kinases (LecRKs) modulate the PTI response (Singh and Zimmerli, 2013). LecRK-I.9, also known as DORN1, is necessary for eATP recognition, and is required for MAMP-induced callose deposition (Bouwmeester et al., 2011a; Choi et al., 2014). In addition, LecRK-V.5 negatively regulates MAMP-induced ROS burst in guard cells (Desclos-Theveniau et al., 2012), and LecRK-VI.2 associates with FLS2 and positively modulates PTI upstream of MAPK signaling (Singh et al., 2012a; Huang et al., 2014a).

Though PTI is sufficient to limit the proliferation of a wide variety of microbes, successful pathogens often break plant resistance via delivering virulence molecules called effectors into the apoplast or host cells to suppress PTI (Dou and Zhou, 2012). As a counter measure, plants deploy resistance (R) proteins that generally perceive directly or indirectly perturbations of effectors to mount another layer of defense called effector-triggered immunity (ETI; Jones and Dangl, 2006; Dodds and Rathjen, 2010). ETI is characterized by the induction of a strong and transient immune response often correlated with localized cell death to restrict pathogen spread (Jones and Dangl, 2006). However, rapidly evolving pathogens are able to overcome ETI via frequent mutations in effectors, escaping host R protein detection (Gassmann et al., 2000; Jones and Dangl, 2006; Huang et al., 2014b).

It is a slow process to generate disease resistant crop varieties via traditional breeding, which involves crossing between different varieties and multiple backcrossing to select progenies with the most positive and least negative traits. With the advances in genetic engineering, novel basic knowledge on plant immunity can be applied successfully toward the rapid development of disease resistant crops. To combat crop diseases, relevant defense-related genes can be transferred from one plant species to another. Interspecies or interfamily gene transfer has been shown to be feasible with heterologous genes remaining functional after transfer (Wulff et al., 2011; Dangl et al., 2013). Detailed molecular mechanisms are however not yet well understood. The compatibility of gene transfer across plant species/families suggests that components of defense signaling pathways are conserved between species. In agreement with this, MAPK cascades are crucial for various defense responses in *Arabidopsis*, tomato, *Nicotiana benthamiana*, and rice (Rodriguez et al., 2010; Meng and Zhang, 2013). Similarly, the plant ROS burst represents a general hallmark of pathogen recognition and defense activation (Torres, 2010).

With the increasing number of plant defense regulators identified, many examples have been established to test the efficacy of heterologous gene transfer on enhancing disease resistance. In this review, we discuss recent findings on improving plant immunity via transfer of defense-related genes, with a special focus on approaches exploiting PTI to confer broad-spectrum resistance.

RATIONALES FOR PTI-BASED BIOENGINEERING

Strategies to improve crop immunity via transfer of *R* genes were extensively used in the past decades (Wulff et al., 2011; Dangl et al., 2013). However, the durability of *R* gene-mediated resistance can be greatly hampered by the rapid evolution of pathogen effectors, which are only partially critical for pathogen fitness and virulence (Gassmann et al., 2000; Zhou et al., 2007; Ayliffe et al., 2008; Huang et al., 2014b). Moreover, effectors are species, race, or strain specific, making it difficult to combat diverse pathogens with a single *R* gene transfer (Chisholm et al., 2006; Jones and Dangl, 2006; Bent and Mackey, 2007; Thomma et al., 2011). By contrast, MAMPs, which are conserved within a class of microbes, are less likely to adopt mutations since they are crucial for the fitness and survival of microbes. For example, the MAMP flagellin from bacterial flagella is critical for bacterial motility, and peptidoglycans are inherent of the cell wall of Gram-positive bacteria (Felix et al., 1999; Nürnberger et al., 2004; Zipfel and Felix, 2005; Gust et al., 2007; Erbs et al., 2008). Similarly, DAMPs, which serve as common danger signals released from stressed-cells, induce plant immune responses against a large variety of pathogens (Huffaker et al., 2006; Yamaguchi et al., 2010; Liu et al., 2013). Accordingly, approaches exploiting PTI may stand a better chance in engineering crops with durable resistance against diverse pathogens.

GAIN OF NEW MAMP PERCEPTION CAPABILITIES

Recognition of MAMPs by PRRs is the first step in PTI activation and consequently, plants defective in MAMP recognition are more susceptible to pathogens. Conversely, the introduction of a new PRR to a given plant species may boost PTI responses via additional PTI activation signaling from the new MAMP/PRR recognition system. This experimental hypothesis was demonstrated to be feasible through the interfamily transfer of EFR, a bacterial EF-Tu receptor (Lacombe et al., 2010). EF-Tu is one of the most abundant, widely conserved, and slow-evolving protein in bacteria (Lathe and Bork, 2001; Kunze et al., 2004; Lacombe et al., 2010). Recognition of EF-Tu (or its eliciting epitope elf18) is *Brassicaceae* specific (Kunze et al., 2004; Zipfel et al., 2006) and *Solanaceous* plants such as *N. benthamiana* and tomato do not possess EFR (Kunze et al., 2004). Remarkably, *N. benthamiana* and tomato plants with stable expression of *EFR* gain responsiveness to elf18 as illustrated by the accumulation of ROS and PTI-responsive mRNAs (Lacombe et al., 2010). Moreover, heterologous expression of *EFR* in *N. benthamiana* and tomato greatly increases resistance toward a wide range of pathogens carrying the eliciting EF-Tu (Lacombe et al., 2010). Transgenic expression of *EFR* in *N. benthamiana* and tomato does not result in constitutive defense responses or defects in growth and development when assessed in laboratory conditions (Lacombe et al., 2010), fulfilling basic agronomical requirements. Theoretically, host co-evolved pathogens are unlikely to possess effectors that target the new PRR signaling originally absent from the host (Lacombe et al., 2010), making PRR genetic engineering a promising tool in agricultural biotechnology. Similarly, *N. benthamiana* plants lacking ReMAX, the PRR for perception of the MAMP eMAX respond to eMAX treatment when a chimeric receptor engineered from ReMAX and the tomato Eix2 is transiently expressed (Jehle et al., 2013). It would be interesting to test whether stable transformation of *N. benthamiana* with ReMAX could confer resistance to a wide range of *Xanthomonas* bacteria. *Xanthomonas* bacteria are indeed known to cause serious diseases in major crops, and effective strategies are required to control such diseases (Ryan et al., 2011). Bacterial blight of rice, caused by *Xanthomonas oryzae*

pv. *oryzae* (*Xoo*) is one of the most devastating disease in rice (Nino-Liu et al., 2006). Transfer of the potential PRR XA21 (Song et al., 1995) from wild rice *Oryza longistaminata* to the susceptible rice cultivar (*Oryza sativa* subsp. japonica var. Taipei 309) confers resistance to multiple isolates of *Xoo* (Wang et al., 1996), suggesting that XA21 can be used as a tool to control rice blight. Similarly, *Xanthomonas campestris* pv. *musacearum* (*Xcm*) causes banana *Xanthomonas* wilt (BXW) and has a huge impact on banana yield (Tripathi et al., 2009). Comparative genomic analysis between *Xoo* and *Xcm* revealed a conserved set of bacterial genes required for the activation of XA21-mediated immunity, suggesting that XA21 can be used for engineering resistance against *Xcm* (Tripathi et al., 2014). This hypothesis was confirmed by the evaluation of transgenic banana plants expressing *Xa21* for BXW resistance (Tripathi et al., 2014). After inoculation of *Xcm*, non-transgenic banana plants display typical BXW symptoms such as yellow ooze in pseudostem, spreading of *Xcm*, and complete wilting, whereas transgenic banana plants expressing *Xa21* show only few or no symptoms, indicating enhanced resistance (Tripathi et al., 2014). Like in tomato plants expressing *EFR*, growth is not altered in banana plants expressing *Xa21* (Tripathi et al., 2014). Interestingly, transfer of XA21 to dicot plants such as sweet orange (*Citrus sinensis*) or tomato also confers resistance against *Xanthomonas axonopodis* pv. *citri* and *Ralstonia solanacearum*, respectively (Mendes et al., 2010; Afroz et al., 2011). XA21 thus stands as a promising candidate for engineering resistance against diverse pathogens in different plant species.

BOOSTING THE PTI RESPONSE

LecRKs belong to a class of receptor kinases characterized by an extracellular lectin domain, and are involved in plant development and stress responses (Bouwmeester and Govers, 2009; Vaid et al., 2012; Singh and Zimmerli, 2013). Although the lectin motif is suggested to bind to oligosaccharides or small hydrophobic ligands (Barre et al., 2002; Andre et al., 2005; Bouwmeester and Govers, 2009), a recent study showed that *Arabidopsis* LecRK-I.9/DONR1 acts as a PRR for the DAMP eATP (Cao et al., 2014; Choi et al., 2014). In mammalian cells, abnormal or uncontrolled increase of eATP represents a danger signal from damaged or stressed cells, and is involved in activating the innate immune system (Hanley et al., 2004). Similarly, plant eATP is increased upon various stresses and is proposed to play a central role in regulating plant immunity (Tanaka et al., 2010; Cao et al., 2014; Choi et al., 2014). Importantly, *Arabidopsis lecrk-I.9/dorn1* displays impaired ATP-triggered PTI responses, such as Ca^{2+} influx, activation of MAPK, and up-regulation of stress-induced genes (Choi et al., 2014). LecRK-I.9/DONR1 was initially identified as a target of the *Phytophthora infestans* RXLR-dEER effector IPI-O (Gouget et al., 2006). LecRK-I.9/DONR1 also contributes to *Arabidopsis* resistance against *Phytophthora brassicae*, and is important for maintaining cell wall (CW)-plasma membrane (PM) continuum (Bouwmeester et al., 2011a). Ectopic expression of *LecRK-I.9/DONR1* in *Solanaceous* potato and *N. benthamiana* plants results in enhanced resistance against *Phytophthora infestans* (Bouwmeester et al., 2014). The CW-PM continuum is hypothesized to be critical for guarding pathogen invasion, and virulent pathogens destabilize through effector action the CW-PM continuum to facilitate colonization (Bouwmeester et al., 2011a,b). The enhanced resistance observed in transgenic potato and *N. benthamiana* may thus be the result of a strengthening of CW-PM adhesions by ectopic expression of *LecRK-I.9/DONR1* (Bouwmeester et al., 2011a,b, 2014). Alternatively, heterologous expression of *LecRK-I.9/DONR1* may trigger an enhanced PTI response via perception of eATP released from pathogen-stressed cells (Choi et al., 2014). When grown in greenhouse condition, stable transgenic potato lines expressing *LecRK-I.9/DONR1* display aberrant plant development including wrinkled leaves, decreased leaflet separation, and malformed tuber (Bouwmeester et al., 2014). The strengthening of CW-PM adhesion by heterologous expression of *LecRK-I.9/DONR1* may disrupt normal plant development (Bouwmeester et al., 2014).

Arabidopsis LecRK-VI.2 was first identified as being involved in ABA inhibition of seed germination (Xin et al., 2009), and was later shown to be a component of the FLS2 complex positively regulating PTI (Singh et al., 2012a; Huang et al., 2014a). *Arabidopsis* plants over-expressing *LecRK-VI.2* demonstrate a constitutively activated PTI, and display significant resistance against hemi-biotrophic *Pst* DC3000 and necrotrophic *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) SCC1 bacteria (Singh et al., 2012a). *Arabidopsis* plants over-expressing *LecRK-VI.2* demonstrate a dwarf phenotype (Singh et al., 2012a), as already observed in plants with constituve defense responses (Bowling et al., 1994; Li et al., 2001). LecRK-VI.2-mediated resistance in the *Brassicaceae* plant *Arabidopsis* can be extended to the *Solanaceous* family, as heterologous expression of *Arabidopsis LecRK-VI.2* in *N. benthamiana* enhances wild tobacco resistance against two strains of hemi-biotrophic *Pseudomonas* bacteria and to necrotrophic *Pcc* SCC1 bacteria (Huang et al., 2014a). Remarkably, even 2 weeks after inoculation with *Pseudomonas syringae* pv. *tabaci* 11528, *N. benthamiana* plants expressing *LecRK-VI.2* harbor only weak disease symptoms and normal development of flowers, whereas wild-type (WT) and empty Vector control plants are extremely stunted, and display severe necrotic symptoms with no flowering (Huang et al., 2014a). In line with what is observed in *Arabidopsis* (Singh et al., 2012b), *LecRK-VI.2* protective effect in *N. benthamiana* is bacteria specific (Huang et al., 2014a). However, heterologous expression of *LecRK-VI.2* in *N. benthamiana* does not directly activate, but only potentiates flg22-induced PTI responses. Priming of PTI may explain the observed enhanced resistance in transgenic *N. benthamiana* plants (Conrath et al., 2006; Conrath, 2011; Huang et al., 2014a). These emerging examples of heterologous expression of PRRs or of modulators of PRRs that can confer broad-spectrum resistance through a potentiated PTI response represent an interesting proof of concept approach that suggest feasibility for future applications to engineer resistant crops through primed PTI (**Figure 1**; Lacombe et al., 2010; Huang et al., 2014a). Similarly to transgenic expression of *EFR* (Lacombe et al., 2010), *N. benthamiana* plants expressing *Arabidopsis LecRK-VI.2* demonstrate a WT growth pattern under laboratory conditions (Huang et al., 2014a). The WT-like growth phenotype in *N. benthamiana* as opposed to the stunted phenotype observed in *Arabidopsis* may result from partial conservation of downstream PTI signaling in *N. benthamiana* (Huang et al., 2014a).

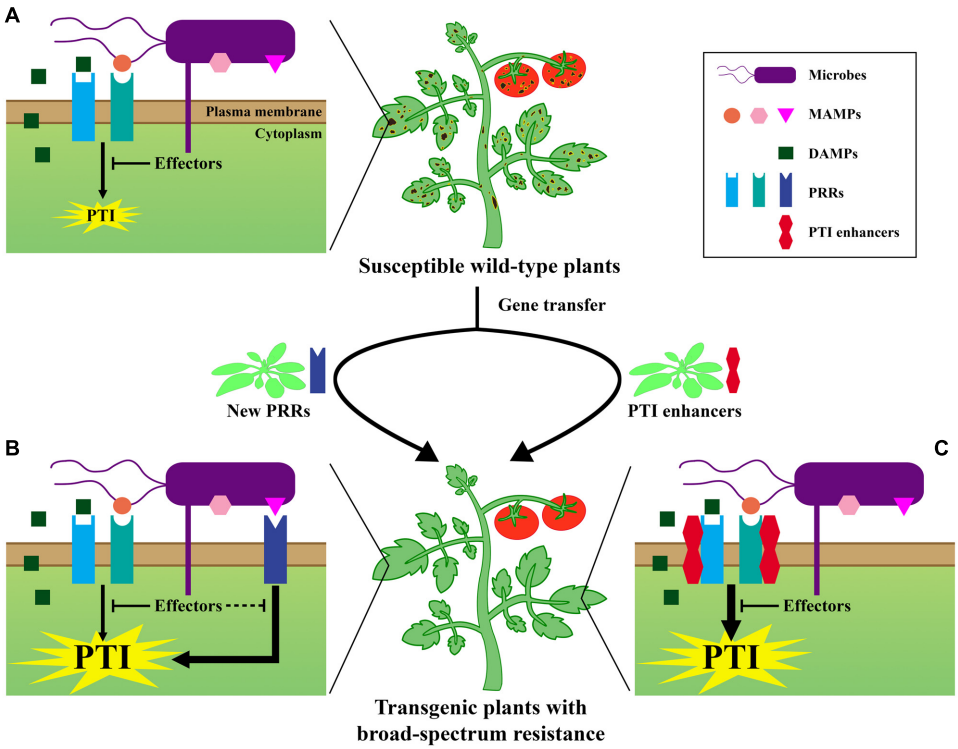

FIGURE 1 | Pattern-triggered immunity (PTI) mediated strategies to deploy broad-spectrum pathogen resistance in crops: a conceptual representation. (A) Perception of MAMPs and/or DAMPs by PRRs activates the PTI response. Adapted, virulent pathogens deliver effectors to suppress PTI, rendering wild-type plants susceptible to infection. **(B)** Interfamily or interspecies transfer of foreign PRRs improves MAMPs recognition and further enhances PTI. Host co-evolved pathogens are unlikely to possess effectors targeting PRRs from other plant species. **(C)** Heterologous expression of PTI enhancers/positive regulators boosts PTI signaling. Effectors may thus not be sufficient anymore to efficiently repress PTI.

In *Arabidopsis*, LecRK-VI.2 is crucial for the up-regulation of PTI marker genes responsive to numerous bacterial MAMPs such as flg22, elf18, PGN, and lipopolysaccharide (Singh et al., 2012a), that are recognized by different PRRs (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Willmann et al., 2011). In addition to associate with the PRR FLS2 (Huang et al., 2014a), LecRK-VI.2 may thus prime the PTI response through association and positive action at additional, different PRR complexes. Therefore, heterologous expression of *LecRK-VI.2* is likely to confer broad-spectrum resistance in other plant species via targeting of multiple PRRs. Therefore, *Arabidopsis* LecRK-VI.2 or LecRK-VI.2 orthologs and possibly other LecRKs stand as promising candidates in the development of crops with durable, wide-range resistance.

CONCLUSION AND PERSPECTIVES

Unlike R protein-mediated resistance that possesses narrow specificity, PTI is broad-spectrum and thus stands as a potential tool for engineering crops with enhanced immunity. Notably, interfamily transfer of genes encoding PRRs or key regulators of PTI enhances resistance of the recipient plant species against a broad range of virulent pathogens (**Figure 1**; Lacombe et al., 2010; Bouwmeester et al., 2014; Huang et al., 2014a; Tripathi et al., 2014). However, in some cases, such heterologous expression may lead to undesirable changes in growth and development (Bouwmeester et al., 2014). The emerging examples of interfamily transfer of PTI-related gene to confer broad-spectrum resistance is encouraging for the future development of resistant crops, but the durability and efficacy of this approach in the field is yet to be determined. In natural conditions, pathogens are constantly evolving to cope with host immunity (McDonald and Linde, 2002), and some pathogens acquire modified MAMPs to avoid recognition (Felix et al., 1999; Kunze et al., 2004; Lacombe et al., 2010). To achieve durable disease resistance, genetic-engineering should be used wisely, perhaps through stacking multiple PTI- and ETI-related genes, and proper field management should be deployed. The use of novel fundamental discoveries in PTI will definitively help the burgeoning of novel practical approaches to increase crop resistance to deleterious pathogens.

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Variation in plant responsiveness to defense elicitors caused by genotype and environment

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INTRODUCTION

The need to develop novel crop protection treatments that can be used in agriculture has driven much research into induced plant defense and is used as a justification for it. Plant defense elicitors could provide novel agrochemicals to protect crops from pests and diseases. However, in order to achieve this, treatments have to give consistent, reliable reductions in pest infestation or pathogen infection levels (Stephen Skillman, Syngenta, *personal communication*). When moving beyond controlled laboratory conditions one issue encountered has been the high variability of the induced defense approach—sometimes an effect is observed and sometimes it is not (Anderson et al., 2006; Wu and Baldwin, 2010). Inducing plant defenses is complex because the effect is via the plant, dependent on plant genetics and physiology and can be altered by the environmental context. This contrasts with conventional pesticides that have a direct toxic effect on target organisms and therefore more predictable effects.

This short opinion article will consider the role of plant genotype, environment and the interaction between genotype and environment in causing variation in induced plant defense responses.

GENOTYPE

Clearly not all plants are the same and some respond to defense elicitors better than others (Vallad and Goodman, 2004). The genotype of the plant can play a huge role in how well it responds to treatment. If particular inducible defense traits are absent in a given genotype nothing can be "switched on" by the treatment. Conversely, if a cultivar possesses inducible traits there is a "loaded gun" attached to the trigger (**Figure 1**). Many studies have revealed differences between plant genotypes in induced defense capacity and I will briefly review selected examples below.

There is natural variation between *Arabidopsis thaliana* accessions in resistance to *Botrytis cinerea* involving differences in *B. cinerea* induced camalexin accumulation and SA-dependent defenses (Denby et al., 2004; Rowe and Kliebenstein, 2008; Narusaka et al., 2013). Van Hulten et al. (2009) found natural variation in defense responsiveness amongst Arabidopsis accessions. Likewise, transcription profiling of wild Solanum species has revealed variation in induced defense responses between genotypes (Smith et al., 2014). Sharma et al. (2010) found that six tomato accessions varied significantly in inducibility of resistance against *Phytophthora infestans*. Natural variation in basal defense responsiveness to disease is reviewed by Ahmad et al. (2010).

Broekgaarden et al. (2007) found differences in transcriptional responses of two *Brassica oleracea* cultivars in response to induction by *Pieris rapae* attack. Of all the genes induced at any time point, only one third was induced in both cultivars tested. Similarly, Wu et al. (2008) found large differences between two *Nicotiana attenuata* accessions in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. Genotypic variation was observed in tomato when the cultivar "Carousel" responded to a seed treatment designed to induce defense whereas tomato cultivar "Moneymaker" did not (Smart et al., 2013). Some maize cultivars have a higher constitutive level of jasmonic acid (JA) based defenses as shown

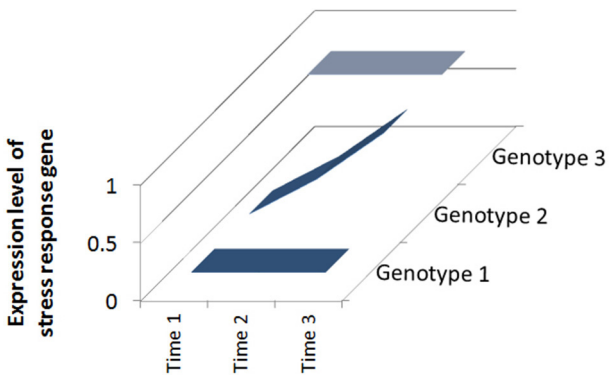

FIGURE 1 | Responsiveness of different plant genotypes to defense elicitors. Over a period of stress (Time 1–Time 3), Genotype 1 has low initial and final expression levels of a stress response gene and is susceptible to attack; Genotype 2 has increasing expression levels because the gene is inducible and Genotype 3 has high initial and final expression levels because it is constitutively expressed.

by Shivaji et al. (2010) which means there is less scope for further induction of them. The resistant inbred Mp708 had approximately 3-fold higher levels of jasmonic acid (JA) prior to herbivore feeding than the susceptible inbred Tx601. Han et al. (2009) found that wheat cultivars which were more resistant to aphids had greater constitutive levels of phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase activity than susceptible ones. Aphid infestation also induced activity of these enzymes in all cultivars, especially in susceptible ones. However, resistant varieties sometimes have higher levels of both constitutive and induced defenses.

Herbivore induced volatile (HIPV) emission plays an important role in indirect defense whereby natural enemies are attracted to plants after exposure to insect attack. Schuman et al. (2009) found variation between accessions of *Nicotiana attenuata* in HIPV emission. Variation in (HIPV) emission between Arabidopsis accessions has been demonstrated and this influenced the behavior of the parasitoid *Diadegma semiclausum* when offered headspace volatiles in two-choice experiments (Snoeren et al., 2010). Certain maize lines respond to elicitors in stemborer eggs that induce HIPV emission to attract natural enemies of the herbivore but most commercial hybrid maize cultivars have lost this trait (Tamiru et al., 2011). Furthermore, Degen et al. (2012) found big differences in HIPV emission between six different maize lines and in a field trial there were significant differences between the lines in the numbers of *Spodoptera frugiperda* recovered from the plants, their average weight gain and parasitism rates. There is also variation among genetic lines of *Datura wrightii* in herbivore and methyl jasmonate-induced volatiles (Hare, 2007): volatile emission from some lines after insect damage or MeJA treatment was lower than from other lines even before damage or MeJA treatment. Kappers et al. (2011) found variation in HIPV emission between cucumber varieties after infestation of the plants with herbivorous spider mites (*Tetranychus urticae*) and this influenced the attraction of carnivorous natural enemies. They suggested that the foraging success of natural enemies of pests can be enhanced by breeding for crop varieties that release specific volatiles.

ENVIRONMENT

The expression of induced plant defense responses is tightly regulated by the ecological context of the plant (Ballare, 2011). For a plant defense activator treatment to work well it must be well timed; timing is more critical than with a conventional pesticide. To induce or prime plant defenses, the treatment needs to be applied before the pest or disease attack as a preventative rather than curative treatment. However, a previous stress may have already switched on the defense and this may limit the magnitude of response to further elicitor treatment. Furthermore, plant responses to biotic stress are influenced by responses to abiotic stress (Suzuki et al., 2014).

The previous history of exposure can affect plant responsiveness. When *Pseudomonas putida* BTP1 infects roots of *Phaseolus vulgaris*, plants become more resistant to *Botrytis cinerea* on leaves (Ongena et al., 2005) and *Trichoderma asperellum* T203 root colonization of cucumber induces resistance to pathogens in above-ground parts of the plant (Shoresh et al., 2005). Infesting rice plants with the white-backed planthopper, *Sogatella furcifera*, dramatically increased the resistance of plants to rice blast, *Magnaporthe grisea* (Kanno and Fujita, 2003). Poelman et al. (2008) found that early season herbivory induces plant defense and differentially affects plant responses to subsequently colonizing herbivores. The specialist *Plutella xylostella* was more abundant on *Pieris rapae*-induced plants and preferred these plants over undamaged plants in oviposition experiments. This could perhaps be because the specialist is attracted to the HIPVs from its host plant. In contrast, the generalist *Mamestra brassicae* was more abundant on control plants and preferred undamaged plants for oviposition. The order of herbivore attack thus mediates the expression of plant defense phenotypes. There is negative crosstalk between plant defense pathways which means that attack by a different type of attacker could compromise responses to the defense elicitor (Bruce and Pickett, 2007). For example, attack that switches on the salicylic acid defense pathway would make a plant less responsive to a treatment designed to switch on the jasmonic acid defense pathway. For example, Zhang et al. (2009) showed that whiteflies interfere with indirect plant defense against spider mites in Lima bean.

INTERACTION BETWEEN GENOTYPE AND ENVIRONMENT

Plants are responsive to their environment and can adapt to stressful conditions. As described in the previous section, prior biotic or abiotic stress in the environment can alter how well a plant responds to subsequent treatment with a defense activator. Not only are these changes mediated by changes in metabolite levels and transcription factors but plants also have the capacity to reprogram expression levels of stress-response genes via epigenetic stress imprints (Bruce et al., 2007; Galis et al., 2009).

Evidence is accumulating that herbivore and pathogen attack can generate defense induction phenotypes across generations (Holeski et al., 2012; Kumar et al., 2013). Epigenetic changes can provide long lasting effects and even influence defense gene expression two generations later if the stress level is high enough (Luna et al., 2012). Dowen et al. (2012) profiled the DNA methylomes of Arabidopsis plants exposed to bacterial pathogen, avirulent bacteria, or salicylic acid (SA) and found numerous stress-induced differentially methylated regions, many of which were intimately associated with differentially expressed genes. The epigenomes of plants thus reflect the history of local genotype-environment interactions and much remains to be learnt about this. It is likely that epigenetic profiling can provide information about prior stress similar to how tree rings (Hughes and Brown, 1992) have been used to indicate previous drought stress. Although they would indicate more recent events, the level of detail about types of stress could be higher because of differential imprinting of different types of stress response genes.

CONCLUSIONS

Reliable and predictable treatment effects are required for practical use of plant defense activators by growers and for commercialization of such crop protection

products. However, the genotype of the plant, the environmental conditions and history of stress exposure, influence the magnitude of any boost in plant defenses obtained with an activator. Walters et al. (2013) have also highlighted host plant genotype and environmental considerations such as prior induction or trade offs between defense pathways as factors influence the field performance of induced resistance. It is not surprising that there is variation given that the defense activator treatment is only as good as the inducible plant defenses that it switches on.

Defense activators or elicitors need to be developed with the appropriate crop genotypes that can respond to the treatment. Variation between crop cultivars is a limitation if activators are developed ignoring this factor but it is also an opportunity to develop suitable packages of seeds and activator agrochemicals. Genetic variation in inducible defense traits complicates the use of plant defense activators but there is future potential to use particular plant activators in a package with selected crop cultivars that offer the best genetic potential for induced defense (Bruce, 2010; Kappers et al., 2011).

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Effects of light and the regulatory B-subunit composition of protein phosphatase 2A on the susceptibility of *Arabidopsis thaliana* to aphid (*Myzus persicae*) infestation

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The interactions between biotic and abiotic stress signaling pathways are complex and poorly understood but protein kinase/phosphatase cascades are potentially important components. Aphid fecundity and susceptibility to *Pseudomonas syringae* infection were determined in the low light-grown *Arabidopsis thaliana* wild type and in mutant lines defective in either the protein phosphatase (PP)2A regulatory subunit B′γ (*gamma*; *pp2a-b′γ*) or B′ζ (*zeta*; *pp2a-b′ζ 1-1* and *pp2a-b′ζ 1-2*) and in *gamma zeta* double mutants (*pp2a-b′γζ*) lacking both subunits. All the mutants except for *pp2a-b′ζ 1-1* had significantly lower leaf areas than the wild type. Susceptibility to *P. syringae* was similar in all genotypes. In contrast, aphid fecundity was significantly decreased in the *pp2a-b′γ* mutant relative to the wild type but not in the *pp2a-b′γζ* double mutant. A high light pre-treatment, which led to a significant increase in rosette growth in all mutant lines but not in the wild type, led to a significant decrease in aphid fecundity in all genotypes. The high light pre-treatment abolished the differences in aphid resistance observed in the *pp2a-b′γ* mutant relative to the wild type. The light and CO_2 response curves for photosynthesis were changed in response to the high light pre-treatment, but the high light effects were similar in all genotypes. These data demonstrate that a pre-exposure to high light and the composition of B-subunits on the trimeric PP2A holoenzymes are important in regulating plant resistance to aphids. The functional specificity for the individual regulatory B-subunits may therefore limit aphid colonization, depending on the prevailing abiotic stress environment.

Keywords: aphid fecundity, high light stress, *Myzus persicae*, redox signaling, protein phosphatase, photosynthesis, *Pseudomonas syringae*

INTRODUCTION

Plants respond to environmental stress through a complex signaling network involving stress receptors and hormones, plant growth regulators, calcium, and protein kinase cascades (Bostock, 2005; Pieterse et al., 2009; Verhage et al., 2010; Atkinson and Urwin, 2012). Identifying genes, processes, and regulators associated with plant stress responses will not only allow a deeper understanding of plant stress tolerance but also provide new opportunities for developing broad-spectrum stress tolerant crop plants. Cross-tolerance to environmental stresses is a common phenomenon that allows resistance to a range of different stresses upon exposure to only one type of stress (Pastori and Foyer, 2002; Mittler, 2006). Cross-tolerance occurs because of synergistic co-activation of non-specific stress-responsive pathways that cross biotic-abiotic stress boundaries (Bostock, 2005). In many cases, cross-tolerance has been linked to enhanced production of reactive oxygen species (ROS) and oxidative signaling (Foyer and Noctor, 2009). ROS-production and processing systems are intrinsically-linked to the plant response to infestation by insects, including phloem feeders such as aphids (Kerchev et al., 2012).

Aphids penetrate plant tissues to feed on photo-assimilates in the phloem by probing between the cells in the epidermal and mesophyll layers with their piercing-sucking mouthparts called stylets (Nam and Hardie, 2012). In addition to causing damage to tissues, aphid feeding transmits disease-causing viruses and fungi (Pimental, 2004). For example, the green peach aphid (*Myzus persicae*) is classed as "generalist" feeder because it can colonize more than 30 plant families. Moreover, *M. persicae* transmits over 100 viruses (Van Emden et al., 1969). A recent investigation of the responses of *A. thaliana* to *Myzus persicae* revealed that aphid attack resulted in rapid changes to the leaf transcriptome and metabolome signature, presenting evidence for the involvement of redox, salicylic acid (SA), and abscisic acid signaling pathways (Kerchev et al., 2013). The pattern recognition receptors in plants to induce the first stages of immunity to pathogens (sometimes called pathogen-associated molecular pattern-triggered immunity) are either RLKs or receptor-like proteins. BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KIN ASE3 (BAK1/SERK3), is a plasma membrane leucine-rich repeat

receptor like kinase that is required for ROS induction and callose deposition by *M. persicae* elicitors (Prince et al., 2014). Such studies suggest that innate immunity to aphids, as with resistance to other stresses, involves early perception of elicitors by cell surface-localized pattern recognition receptors leading to subsequent downstream immune signaling that involves redox signaling and protein kinase/phosphatase cascades (Kangasjärvi et al., 2012; Prince et al., 2014). The following studies were performed to analyse the role of trimeric protein phosphatase 2A (PP2A) in the plant response to aphid infestation because a cytoplasmic regulatory PP2A B'γ subunit was recently identified as a key component controlling pathogenesis responses in *A. thaliana* (Trotta et al., 2011; Li et al., 2014). Knockdown of the specific B'γ subunit resulted in a lesion mimic phenotype with constitutive activation of jasmonic acid and SA related defense pathways, premature disintegration of chloroplasts and cell death upon aging (Trotta et al., 2011). The constitutive immune responses of *pp2a-b'γ* were highly conditional, and became observable when the plants were grown in 50% relative humidity and moderate light intensity, but not when grown under high light (Trotta et al., 2011; Li et al., 2014). Proteomic studies suggested that the PP2A-B'γ dependent signaling effects involve antioxidant enzymes, such as copper/zinc superoxide dismutase 2 (CSD2) and monodehydroascorbate reductase 2 (MDAR2), with well-known roles in the maintenance of cellular ROS homeostasis (Trotta et al., 2011; Li et al., 2014). These observations also suggest that PP2A-B'γ is a focal point of cross-talk between plant responses to pathogens and light (Trotta et al., 2011; Li et al., 2014). In the following studies therefore we analyzed the role of PP2A in the responses to aphid infestation of *Arabidopsis thaliana* plants grown under either low or high light.

Trimeric PP2A phosphatases are composed of a catalytic subunit C, a scaffold subunit A, and a highly variable regulatory subunit B. In the *A. thaliana* genome these subunits are encoded by 5, 3, and 17 distinct genes, respectively, (Farkas et al., 2007; Sents et al., 2013). The catalytic C subunit of PP2A attains an active conformation only upon dimerization with a scaffold subunit A. Formation of a PP2A-A/C dimer in turn forms a platform for interaction with the regulatory B subunit, which is thought to determine the sub-cellular localization and target specificity of the PP2A holoenzyme (Farkas et al., 2007; Matre et al., 2009; Uhrig et al., 2013). It has also been proposed that the large number of isoforms for each subunit could provide extensive variability in subunit combinations, allowing versatile but highly specific functions for PP2A in the dephosphorylation of specific target proteins. Computational modeling predicted that both PP2A-B'γ and PP2A-B'ζ could interact with any of the PP2A A and C subunits, and therefore hold the potential to form similar PP2A trimers. However, the functional interactions between regulatory B-subunits remain poorly understood. We therefore examined the structural properties of PP2A in relation to the ability of *A. thaliana* to resist infestation by *M. persicae* in plants that had been grown under low light or had been given a pre-exposure to high light. The data presented here demonstrate that the B'γ subunit composition is an important determinant of the ability of the plants to limit aphid infestation at low but not at high light. Moreover, we show exposure to high light leads to cross tolerance responses that limit aphid fecundity in all genotypes.

MATERIALS AND METHODS

PLANT MATERIAL

Homozygote *pp2a-b'γ* (SALK_039172 for At4g15415), *pp2a-b'ζ1-1*, and *pp2a-b'ζ1-2* (SALK_107944C and SALK_150586 for At3g21650, respectively) mutant lines were identified from the SALK institute's collection by PCR analysis according to the institute's protocols (Alonso et al., 2003). A *pp2a-b'γζ* double mutant was constructed by crossing the SALK_039172 and SALK_107944C single mutants and selecting homozygotes from the F2 generation using the same set of PCR primers that were used to screen for the single *pp2a-b'γ* and *pp2a-b'ζ1-1* mutants. Insertion mutant information was obtained from the SIGnAL website at http://signal.salk.edu. To assess the expression of *PP2A-B'ζ* and *PP2A-B'γ* in the mutant lines, RNA was isolated with Agilent Plant RNA Isolation Mini Kit (product number 5185-5998) and thereafter DNAse-treated with Ambion DNA-*free* Kit (product number AM1906) according to the manufacturer's instructions for rigorous DNase treatment. One microgram of RNA was used for cDNA synthesis using the Invitrogen SuperScript III First-Strand Synthesis Super Mix for qRT-PCR (product number 11752-050). cDNAs of specific genes were amplified using Thermo Scientific Phire Hot Start II DNA Polymerase (product number F-122S). To confirm the absence of *PP2A-B'ζ* mRNA in *pp2a-b'ζ1-1*, *pp2a-b'ζ1-2*, and *pp2a-b'γζ* double mutant, the cDNA was used for RT-PCR with the primers FOR (5′-TGCCTATAGTCTTCCCAGCTCT-3′) and REV (5′GTGGACTCAGAGCTGCTTGT-3′). *pp2a-b'γ* is a knock-down mutant with 40% reduction in *PP2A-B'γ* transcript level (Trotta et al., 2011). To assess the abundance of *PP2A-B'γ* mRNA in *pp2a-b'γ*, *pp2a-b'ζ1-1*, and *pp2a-b'γζ* double mutant, PP2A-B′γ cDNA was specifically amplified with the primers FOR (5′-TGTGTTGCGTTGTGTTCGAC-3′) and REV (5′-GGTGCACCATGAATTTCCCG-3′) and normalized to *Actin 2* amplified with the primers FOR (5′-GTGAACGATTCCTGGACCTGCCTC-3′) and REV (5′-GAGAGGTTACATGTTCACCACAAC-3′). DNA bands stained with NIPPON Genetics Midori Green Advanced DNA stain (product number MG 04) in 1% agarose gels were detected with the PerkinElmer Geliance 1000 Imaging System. Band intensities were analyzed with ImageJ.

Unless otherwise stated *Arabidopsis thaliana* ecotype Columbia wild type and the mutant genotypes were grown in compost (SHL professional potting compost) in controlled environment chambers under an 8 h/16 h day/night regime, with an irradiance of 250 μmol m^{-2} s^{-1} (low light conditions). The relative humidity was 60% and day/night temperatures were 20°C.

AMINO ACID ALIGNMENT AND CONSTRUCTION OF PHYLOGENETIC TREE

Amino acid alignment was conducted using ClustalX program with gap penalty 10 and gap extension penalty set to 0.2. The amino acid sequence distances were inferred by using the Maximum Likelihood method based on the JTT matrix-based

model (Jones et al., 1992). The tree with the highest log likelihood (−8780.5476) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 17 amino acid sequences from Arabidopsis and the human ortholog B56γ. All positions containing gaps and missing data were eliminated. There were a total of 381 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

STRUCTURAL MODELING

Known X-ray structures of individual proteins were obtained from the Protein Data Bank (http://www.rcsb.org; Berman et al., 2000). Protein folds were assigned according to the SCOP database (http://scop.mrc-lmb.cam.ac.uk/scop; Murzin et al., 1995). Programs for protein structure modeling, Modeler (Sali and Blundell, 1993) and Homodge (in Bodil; Lehtonen et al., 2004) were used for homology modeling of domains based on known related structures. The Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information was used to search regions of sequence homology (http://www.ncbi.nlm.nih.gov/blast/). Discovery Studio (http://accelrys.com) and Sybyl (http://www.tripos.com) molecular modeling environments were used for additional modeling, structure superpositions, structure viewing and analysis. Identification of local atom environments, polar and non-polar interactions and contacts between amino acids were made using the CSU Analysis of Interatomic Contacts in Protein Entries software package (http://bip.weizmann.ac.il/oca-bin/lpccsu/), and the Automated Analysis of Interatomic Contacts software (Sobolev et al., 1999).

The PP2A holoenzyme (PDB code: 3FGA) composed of regulatory subunit α (Aα; *M. musculus*)/regulatory subunit γ (B56γ; *H. sapiens*)/catalytic subunit α (Cα; *H. sapiens*) bound to the synthetic construct microcystin LR *(Microcystis aeruginosa)* and to the 47 amino acid fragment of Shugoshin-like 1 protein (Sgo1, *H. sapiens*), at 2.7 Å was chosen as a template to model the Arabidopsis PP2A triple complex. Other available PP2A structures include (1) the PP2A holoenzyme (Aα/B56γ/Cα; *H. sapiens*) bound to the synthetic construct microcystin LR from Cyanobacteria at 3.3 Å (PDB codes: 2NPP, 2NYM, and 2NYL) and (2) the PP2A holoenzyme (Aα; *M. musculus*)/(B56γ; *H. sapiens*)/(Cα; *H. sapiens*) bound to the synthetic construct microcystin LR from *M. aeruginosa* at 3.5 Å (PDB codes: 2IAE). Amino acid sequences of Arabidopsis AT4G15415 and AT3G21650 (NCBI protein codes: NP_567464.1 and NP_188802.1) were taken to model the PP2A-B'γ and PP2A-B'ζ subunits, respectively. The AT1G10430 (NCBI protein code: NP_172514.1) sequence was taken to model PP2A-C2, and the AT1G25490 (NCBI protein code: NP_173920.1) sequence was taken to model PP2A-A1. The alignments used for modeling of PP2A-B'γ/B'ζ, PP2A-A, and PP2A-C are shown in **Figure 1A**, **Supplemental Figures 1**, **2**, respectively.

HIGH LIGHT TREATMENTS

Wild type and mutant plants were grown as described above under low light conditions (250 μmol m^{-2} s^{-1}) for 2 weeks in controlled environment chambers then half of the plants weregrown for a further 7 days under low light conditions in the controlled environment chambers and half were transferred to duplicate controlled environment cabinets in which the light intensity was 800 μmol m^{-2} s^{-1}(high light) and grown for a further 7 days. In both treatments, the day and night temperatures in the controlled environment chambers were carefully monitored and maintained at 20°C, with a relative humidity was 60%. In all cases, photosynthesis and aphid fecundity measurements were performed between 3 and 6 h into the photoperiod.

SHOOT GROWTH DETERMINATION

Leaf area was measured on 3 week-old plants. Photographs were taken with a Canon EOS 450 D (Canon Inc., Tokyo, Japan). Leaf area was measured and analyzed using Fiji ImageJ (http://fiji.sc/).

PHOTOSYNTHETIC GAS EXCHANGE MEASUREMENTS

Photosynthetic gas exchange measurements were performed on whole rosettes that had been grown for 2 weeks under low light conditions (250 μmol m^{-2} s^{-1}) and then grown for a further 7 days either under low light or under high light (800 μmol m^{-2} s^{-1}) conditions. Photosynthetic CO_2 assimilation rates and intracellular CO_2 (Ci) concentrations were measured using a portable Photosynthetic System (LI-6400XT) LI-COR at 20°C in the leaf chamber with a light intensity of (250 μmol m^{-2} s^{-1}) and an atmospheric CO_2 concentration of 400 μmol mol^{-1}. In all cases, rosettes were allowed to acclimatize to the chamber for 15 min prior to measurement to allow stabilization of parameters. Measurements were made on 5 plants per line per experiment.

CHLOROPHYLL *A* FLUORESCENCE QUENCHING PARAMETERS

The ratio of dark adapted variable chlorophyll *a* fluorescence (Fv) to the maximal value of chlorophyll a fluorescence (Fm) in the dark adapted state was measured in the leaves of 3 week-old plants following the transfer from LL growth conditions to HL conditions using a FluorPen, which is a portable, battery-powered fluorometer (FP 100-SN-FP-680 Equipements Scientifiques S.A., Nanterre, France).

PHOTOSYNTHETIC PIGMENTS

Leaves were harvested at the points indicated on the figures and weighed. Samples (100 mg fresh weight) were ground in liquid nitrogen. Photosynthetic pigments were extracted in 95% ethanol and determined according to the method of Lichtenthaler (1986).

APHID CULTURE CONDITIONS

Green peach aphids (*Myzus persicae* Sulzer) derived from stocks that had been collected in Scotland in the years 2002–2004 were obtained from Dr. Robert Hancock, James Hutton Institute, Invergowrie, UK. Aphid stocks were maintained on mature potato plants in transparent cages in an insectary under controlled environment conditions (16 h photoperiod and day/night temperatures of 20°C).

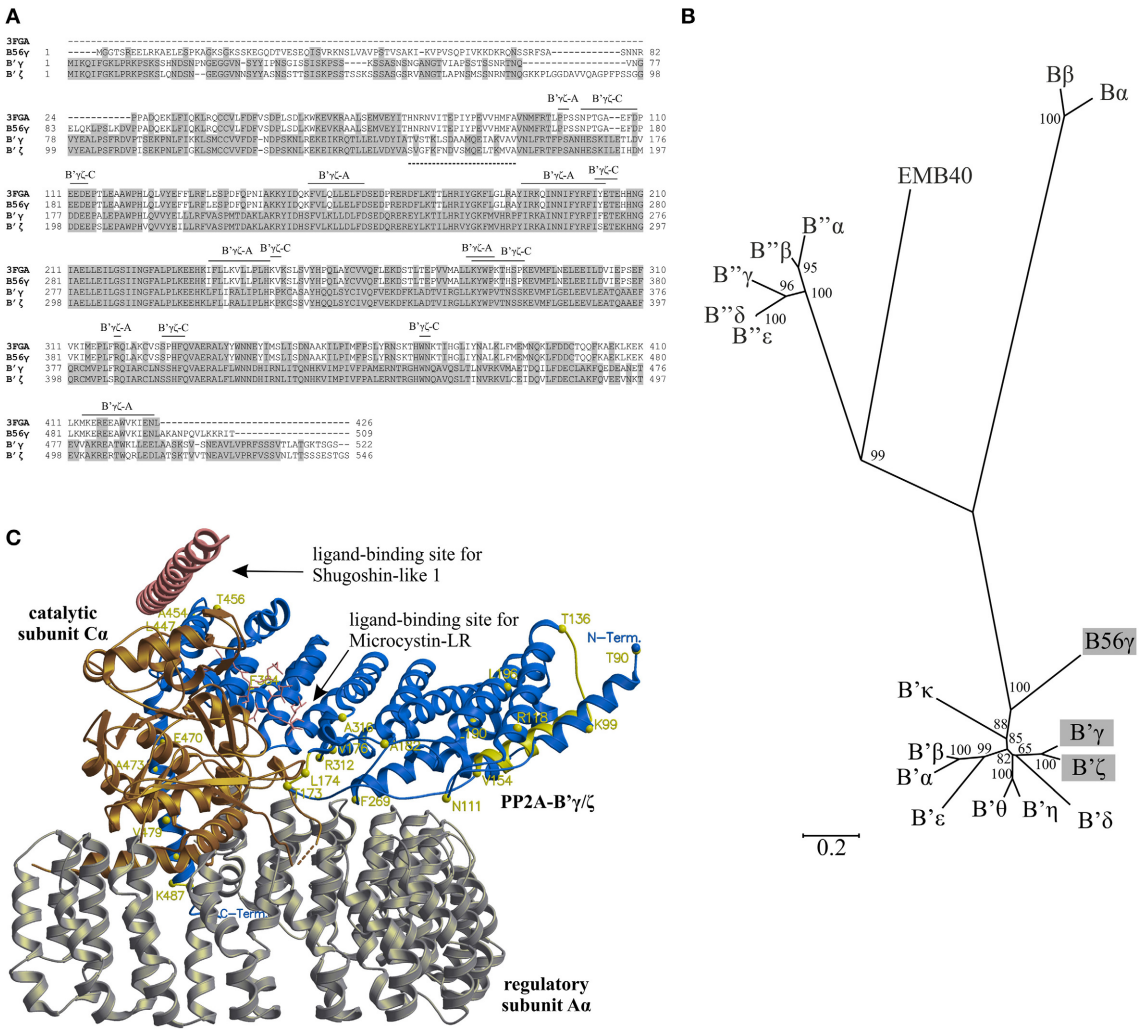

FIGURE 1 | Structural properties of PP2A-B′γ and PP2A-B′ζ. (A) Amino acid sequence alignment between *A. thaliana* PP2A-B′γ (B′γ) and PP2A-B′ζ (B′ζ), human PP2A-B56γ, and the sequence of PP2A-B56γ from the X-Ray structure (PDB Code: 3FGA). Gray areas indicate sequence identity and the lines indicate areas of interactions between PP2A-B′γ or PP2A-B′ζ and the PP2A-A and PP2A-C subunits, based on the structure of 3FGA. The dashed line indicates a variable segment between PP2A-B′γ and PP2A-B′ζ. Bα, AT1G51690; Bβ,AT1G17720; B′α, AT5G03470; B′β, AT3G09880; B′γ, AT4G15415; B′δ, AT3G26030; B′ε, AT3G54930; B′ζ, AT3G21650; B′η, AT3G26020; B′θ, AT1G13460; B′κ, AT5G25510; B″α, AT5G44090; B″β, AT1G03960; B″γ,AT1G54450; B″δ, AT5G28900; B″ε, AT5G28850; EMB40, AT5G18580; B56γ, Q13362. **(B)** Unrooted phylogeny tree showing evolutionary relationship between Arabidopsis PP2A regulatory B subunits and their human ortholog B56γ. The bar corresponds to substitutions per amino acid. The positions of Arabidopsis PP2A-B′γ and PP2A-B′ζ and human B56γ are highlighted with gray. **(C)** Structural model of a trimeric PP2A complex containing Arabidopsis PP2A-B′γ (blue), mouse PP2A-Aα (silver), and human PP2A-Cα (gold). Molecular modeling was based on the sequence alignment shown in **(A)**, using the structure 3FGA as a template. Sites of amino acid differences between PP2A-B′γ and PP2A-B′ζ are mapped in yellow. The N-terminal segment M1-V88 of PP2A-B′γ is not present in the structural model. All amino acid labels are as in PP2A-B′γ. Sites known to recognize the Shugoshin-like 1 and Microcystin-LR molecules in 3FGA are indicated.

APHID FECUNDITY

Aphid fecundity was determined on 3 week-old plants by the method of Fenton et al. (2010). A single 1-day-old nymph was placed in the center of a leaf (per plant) and was enclosed in a mesh perspex® cage (5 cm internal diameter) capped with a thin mesh (mesh size 200 μm). Plants with cages were then returned to the low light controlled environment chamber. After 15 days the total number of aphids was counted. Each fecundity experiment involved 15 plants per genotype per experiment and was repeated 3 times.

PSEUDOMONAS INOCULATION

For these experiments, the growth conditions for the wild type and the mutant genotypes were as described above except that the growth irradiance was 130 μmol m^{-2} s^{-1}. The virulent *Pseudomonas syringae* pv tomato strain DC3000 (Pst) was grown overnight in NYGA medium with 10 mg/mL tetracycline and 100 mg/mL rifampicin as in Trotta et al. (2011). Bacterial suspensions were washed twice in 10 mM $MgCl_2$, diluted to 10^5 colony forming units ml^{-1} and carefully infiltrated into fully expanded leaves using a needleless syringe on the abaxial surface. Four

days post-infection, bacterial growth cm^{-2} was determined in leaf material homogenized in 10 mM $MgCl_2$ to liberate the bacteria. Serial dilutions of the homogenates were plated (in duplicate) on NYGA medium supplemented with 10 mg/mL tetracycline and 100 mg/mL rifampicin. Colonies on the plates were counted after incubation at 28°C for 24 h.

STATISTICAL ANALYSIS

Data represent the mean ± standard error of the mean (SEM). Statistical analysis was performed by Student's *t*-test and a One-Way ANOVA (IBM SPSS Statistics—version 20). The values were considered statistically different when P was < 0.05.

RESULTS

PP2A-B'γ AND PP2A-B'ζ MAY FORM SIMILAR PP2A TRIMERS

Pairwise alignment of amino acid sequences revealed that Arabidopsis PP2A-B'γ and PP2A-B'ζ share 80% sequence identity (**Figure 1A**), and phylogenetic clustering of PP2A-B subunits illustrated a close evolutionary relationship of PP2A-B'γ and PP2A-B'ζ with their human (*Homo sapiens*) counterpart B56γ (**Figure 1B**, **Supplemental Figure 1**). The areas with greatest sequence variation between PP2A-B'γ and PP2A-B'ζ reside within the N and C terminal ends, with a unique 18 amino acid insertion present only in PP2A-B'ζ (**Figure 1A**). Additionally, the two proteins differ within a short segment between T136-V154 and S157-A175 in PP2A-B'γ and PP2A-B'ζ, respectively, (**Figure 1A**). In the middle parts of the proteins, only localized single amino acid differences occur between PP2A-B'γ and PP2A-B'ζ (**Figure 1A**), and these highly conserved areas incorporate all predicted sites of interaction with PP2A-A and PP2A-C, suggesting that PP2A-B'γ and PP2A-B'ζ may form similar PP2A trimers.

Next we analyzed structural differences and similarities between PP2A-B'γ and PP2A-B'ζ within putative PP2A complexes. The A and C subunits that associate with B'γ or B'ζ in PP2A heterotrimers have not yet been identified. Nevertheless, since PP2A-A and PP2A-C subunits are evolutionary highly conserved (**Supplemental Figures 2**, **3**), their mammalian counterparts present in a chimeric mouse (*Mus musculus*)/human PP2A (PDB code: 3FGA) could serve as a template for molecular modeling (**Figure 1C**). With these structural models, we aimed to dissect (1) whether the inter-subunit interacting areas between PP2A-B'γ and PP2A-B'ζ differ from each other and (2) whether these interacting areas would specifically interact with distinct *A. thaliana* PP2A-A and PP2A-C subunits only.

The very N-terminal regions of PP2A-B'γ and PP2A-B'ζ could not be modeled, since they were not present in any of the PP2A X-Ray structures available. However, it is likely that the N-terminal segment M1-V88 resides outside of the inter-subunit interface of PP2A, and interacts with the variable segment T136-V154, which forms an α-helix flanking

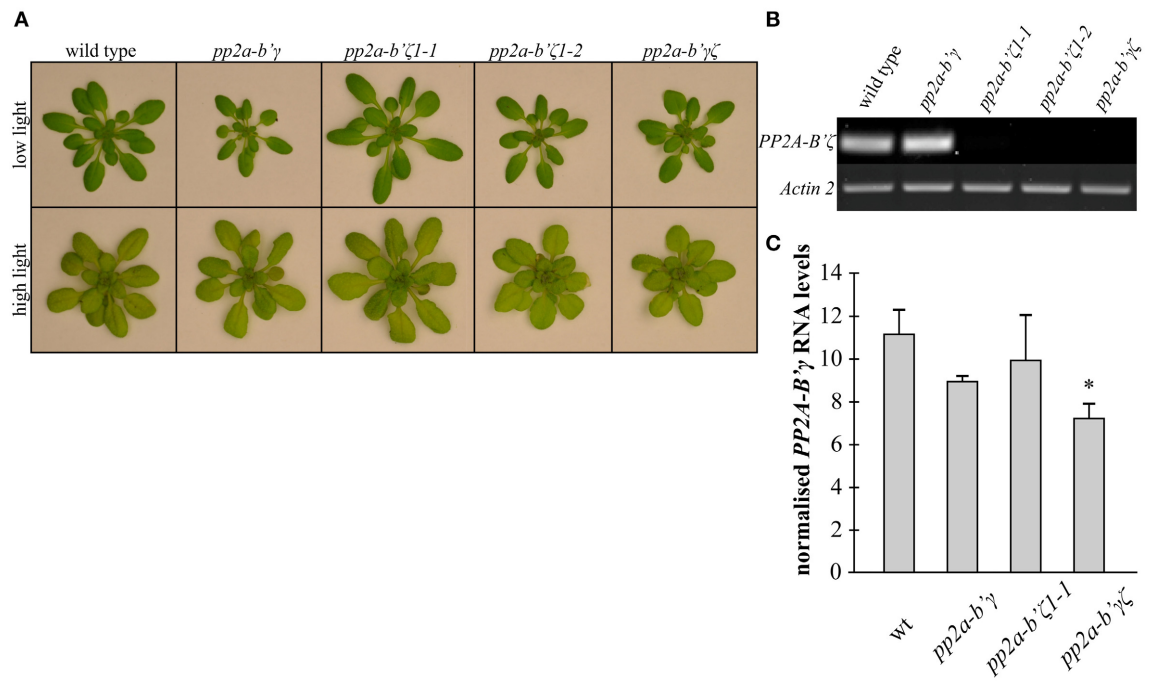

FIGURE 2 | A comparison of the rosette phenotypes of the *pp2a-b'γ*, *pp2a-b'ζ 1-1*, *pp2a-b'ζ 1-2*, and the *pp2a-b'γζ* double mutant grown under low and high light conditions. (A) Plants grown for 2 weeks under low light (250 μmol m^{-2} s^{-1}) and then either maintained for a further 7 days under low light growth conditions (top row) or transferred to high light (800 μmol m^{-2} s^{-1}) for 7 days (bottom row). A *pp2a-b'γζ* double mutant was constructed by crossing the SALK_039172 (*pp2a-b'γ*) and SALK_107944C (*pp2a-b'ζ 1-1*) single mutants. **(B)** RT-PCR analysis of *PP2A-B'ζ* mRNA in wild type and *pp2a-b'γ*, *pp2a-b'ζ 1-1*, *pp2a-b'ζ 1-2*, and the *pp2a-b'γ* ζ double mutant. Amplification of *Actin 2* is used to control for equal cDNA-levels. **(C)** RT-PCR analysis of *PP2A-B'γ* mRNA in wild type, *pp2a-b'γ*, *pp2a-b'ζ 1-1*, and the *pp2a-b'γ* ζ double mutant. Band intensity values of the *PP2AB'γ* -amplicon of each sample were normalized to *Actin 2 values*. Average values ± standard error values are shown ($n = 3$–4). The asterisk denotes a value that is significantly lower than the wild type-value ($P < 0.05$) as determined by a Student's *t*-test.

the N-terminal end of PP2A-B'γ and PP2A-B'ζ (**Figure 1C**). The N-terminal regions in either PP2A-B'γ and PP2A-B'ζ are therefore likely to form separate domains, which do not contribute to the inter-subunit communication within PP2A trimers.

Most single amino acid substitutions scattered across the PP2A-B'γ and PP2A-B'ζ sequences are either equivalent without major differences in chemical properties or reside outside of the PP2A inter-subunit interfaces (**Figures 1A,C**). Thus, the structural properties of the inter-domain interacting areas in PP2A-B'γ and PP2A-B'ζ would allow formation of similar PP2A trimers. The corresponding interacting areas of PP2A-A and PP2A-C lie within their regions of high identity, and are therefore unlikely to affect the formation of trimeric PP2A holoenzymes. Additionally, the variable N-terminal domains of PP2A-B'γ and PP2A-B'ζ may target PP2A holoenzymes to specific target proteins, thereby allowing functional specificity for the individual regulatory B-subunits.

EFFECTS OF LIGHT AND PP2A SUBUNIT COMPOSITION ON SHOOT GROWTH

When wild type and mutant plants were grown for 3 weeks under low light (250 μmol m^{-2} s^{-1}) the rosettes of all of the mutant genotypes except for *pp2a-b'ζ 1-1* were visibly smaller than the wild type at 3 weeks (**Figure 2A**). However, when the plants were grown for 2 weeks under low light (250 μmol m^{-2} s^{-1}) and then transferred for 7 days to high light (800 μmol m^{-2} s^{-1}), the rosette phenotypes were more similar in all genotypes than under low light, although the *pp2a-b'ζ 1-2* mutant and the *pp2a-b'γζ* double mutant were visibly smaller than the other lines under high light conditions (**Figure 2A**). Knockout of PP2A-B'ζ resulted in a strong depletion of PP2A-B'ζ transcripts in pp2a-b'ζ1-1, pp2a-b'ζ1-2, and the pp2a-b'γζ double mutants (**Figure 2B**). Knockdown *pp2a-b'γ* in turn harbors a T-DNA insertion in its promoter region (Trotta et al., 2011), and resulted in a 20% decline in the level of PP2A-B'γ mRNA in knockdown *pp2a-b'γ* and 35% in *pp2a-b'γζ* double mutants as compared to wild type plants (**Figure 2C**).

Leaf area measurements were performed on plants that had either been grown for 3 weeks under low light (250 μmol m^{-2} s^{-1}) or for 2 weeks under low light followed by 7 days under high light (800 μmol m^{-2} s^{-1}; **Figure 3**). The wild type plants had similar leaf areas under both high and low light growth conditions (**Figure 3**). In contrast, the leaf area was significantly increased in all of the mutant genotypes under high light relative to low light conditions (**Figure 3**). The high light-dependent increase in leaf area was most marked in the *gamma* (*pp2a-b'γ*) mutants (**Figure 3**).

The chlorophyll and carotenoid contents of the rosette leaves were similar in all genotypes under low light conditions (**Figure 4**). Growth under high light conditions for 7 days decreased leaf chlorophyll by about 30% in all genotypes relative to the leaves grown under low light conditions (**Figure 4**). The light-dependent decreases in leaf chlorophyll were similar in all genotypes (**Figure 4**).

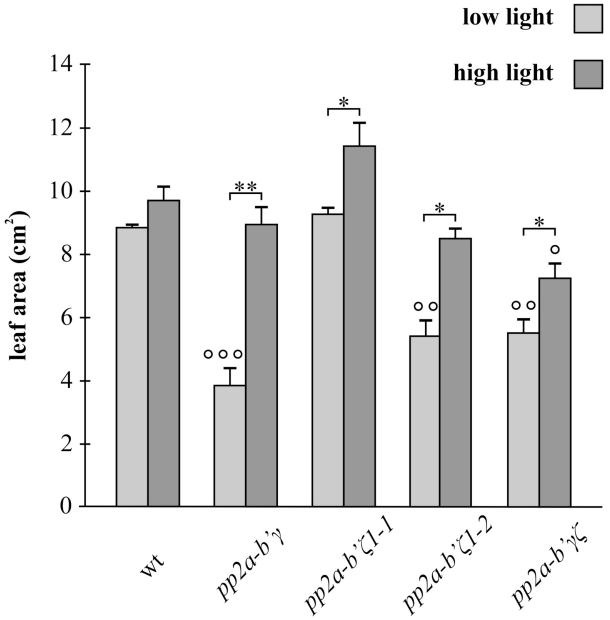

FIGURE 3 | A comparison of the rosette leaf areas of the *pp2a-b'γ*, *pp2a-b'ζ 1-1*, *pp2a-b'ζ 1-2*, and the pp2a-*b'γζ* double mutant grown under low and high light conditions. Leaf area measurements were performed on plants that had been grown for 2 weeks under low light (250 μmol m^{-2} s^{-1}) and then either maintained for a further 7 days under low light growth conditions or transferred to high light (800 μmol m^{-2} s^{-1}) for 7 days. The results are average values ± standard error values, $n = 5$. $^{*}p < 0.05$; $^{**}p < 0.01$ in Significance given from analysis by student's *t*-test and One-Way ANOVA analysis of LL and HL values for each genotype, as follows $^{\circ}p < 0.05$; $^{\circ\circ}p < 0.01$; $^{\circ\circ\circ}p < 0.001$ in Student's *t*-test and One-Way ANOVA comparisons between the mutant lines and wild type under LL or HL light conditions.

THE COMPOSITION OF PP2A SUBUNITS HAD NO EFFECT ON PHOTOSYNTHESIS

Photosynthetic CO_2 assimilation rates were similar in the leaves of all genotypes under low light growth conditions. Moreover, the ratio of dark-adapted variable chlorophyll a fluorescence (Fv) to maximal chlorophyll a fluorescence (Fm) was similar in all genotypes (**Supplemental Figure 4**). Growth under high light for 7 days decreased maximal rates of photosynthesis by about 40% relative to the leaves of plants that had been grown under low light and the Fv/Fm ratios were decreased by about 20% (**Supplemental Figure 4**). An analysis of the light response curves (**Figure 5A**) and the CO_2 response curves for photosynthesis (**Figure 5B**) showed that the initial slopes of both curves were decreased in the leaves of all genotypes that had been grown under high light for 7 days compared to those that had been maintained under low light growth conditions. Moreover, there was a significant highlight dependent decrease in the CO_2 saturated rates of photosynthesis measured in the CO_2 response curve analysis in all genotypes (**Figure 5B**). The highlight dependent decrease in the light-saturated rates of photosynthesis was less marked in light response curve analysis (**Figure 5A**). No significant differences in these parameters were observed between the wild type and mutant lines.

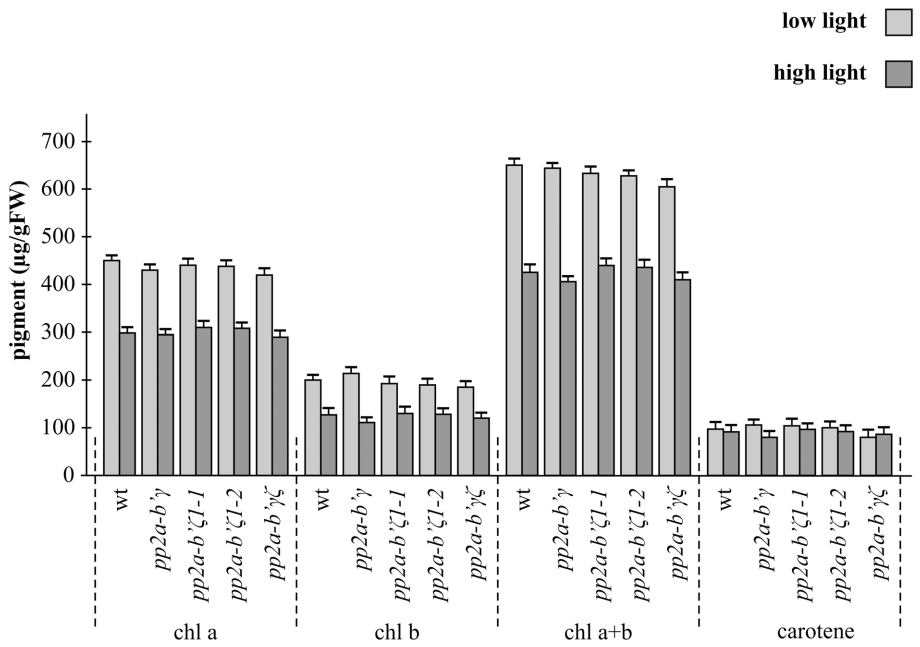

FIGURE 4 | A comparison of the leaf pigment contents in the *pp2a-b'γ*, *pp2a-b'ζ 1-1*, *pp2a-b'ζ 1-2*, and the pp2a-*b'γζ* double mutant grown under either low or high light conditions. Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (chl a+b) and total carotenoid pigments (carotene) analysis was performed on the whole rosettes of plants that had been grown for 2 weeks under low light (250 µmol m^{-2} s^{-1}) and then either maintained for a further 7 days under low light growth conditions or transferred to high light (800 µmol m^{-2} s^{-1}) for a further 7 days.

PRE-EXPOSURE TO HIGH LIGHT AND THE COMPOSITION OF PP2A SUBUNITS EFFECTS ON APHID FECUNDITY

Aphid fecundity measured in plants that had been grown only under low light growth conditions was similar in all genotypes, except for the mutants that lack the *gamma* (*pp2a-b'γ*) subunit of PP2A phosphatase (**Figure 6A**). The number of aphids was significantly lower (15%) on the leaves of the *pp2a-b'γ* mutant compared to the wild type, *zeta1* (*pp2a-b'ζ 1-1*), *zeta2* (*pp2a-b'ζ 1-2*), and *gamma zeta* double mutant (*pp2a-b'γζ*). Interestingly, the decrease in aphid fecundity observed in the *gamma* (*pp2a-b'γ*) mutant was not observed in the *gamma-zeta* (*pp2a-b'γζ*) double mutant (**Figure 6A**).

Growth under high light conditions for 7 days prior to the analysis of aphid fecundity led to a significant light-dependent decrease (up to 20%) in aphid numbers on all genotypes (**Figure 6A**). While aphid fecundity was similar in all genotypes that had been exposed to the high light pre-treatments, the light-dependent decrease in aphid fecundity was least marked (11%) in the *gamma* (*pp2a-b'γ*) mutant (**Figure 6A**).

THE GAMMA AND ZETA SUBUNITS OF PP2A HAVE NO EFFECT ON SUSCEPTIBILITY TO *PSEUDOMONAS SYRINGAE*

To evaluate whether the composition of PP2A subunits alters resistance to the hemibiotrophic bacterium, *Pseudomonas syringae* pv tomato DC3000 (Pst), we challenged leaf tissues of plants grown under an irradiance of 130 µmol m^{-2} s^{-1}, to this virulent pathogen, which proliferates in the intercellular spaces of leaf tissues of wild type plants causing disease with spreading, chlorotic lesions (Pavet et al., 2005). In a previous study, infection of plants with 10^8 colony forming units revealed no differences in bacterial growth at 2 days post-infection, and only a statistically insignificant eight-fold reduction in bacterial growth was observed at 5 days post-infection in *pp2a-b'γ* mutant compared to wild type (Trotta et al., 2011). Here we assessed bacterial growth by carefully infiltrating *Pseudomonas* in 10^5 CFU into the leaves and quantified bacterial growth *in planta* and hence the development of pathogen, 2 h after Pst inoculation, and 4 days after Pst inoculation, a point where typical disease symptoms were observed on the leaves. Pst proliferation was similar in all genotypes at both time points (**Figure 6B**).

DISCUSSION

Plants have co-evolved with an enormous variety of fungal pathogens and insect herbivores under conditions with very different types of abiotic stresses. They therefore harbor a large reservoir of natural adaptive mechanisms to maximize growth and survival while coping with different forms of stress simultaneously. In natural environments plants experience constantly changing light levels over each day and across the seasons. Exposures to high light can be stressful to plants, triggering the innate immune responses associated with pathogen-associated molecular patterns that enhance defenses against pathogen attack (Szechynska-Hebda et al., 2010; Karpinski et al., 2012). High light can therefore elicit cross-tolerance responses to different stresses. The data presented here show that a pre-exposure to high light induces adaptations in photosynthesis and changes in plant growth that may also give rise to a molecular memory of stress, leading to an enhanced resistance to aphid infestation (but

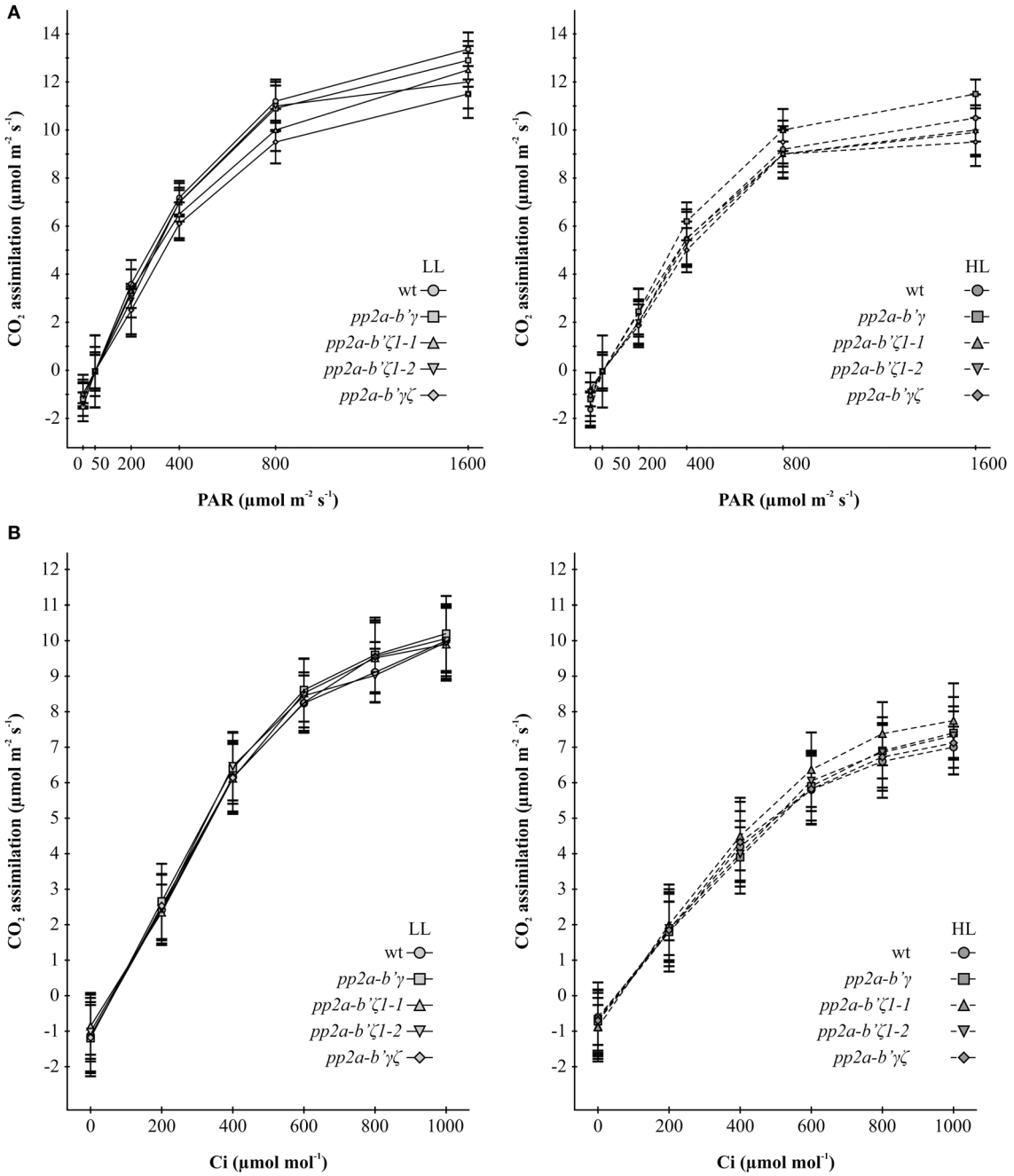

FIGURE 5 | A comparison of the light saturation curves for photosynthesis (A) and the CO_2 response curves for photosynthesis (B) in the *pp2a-b'γ*, *pp2a-b'ζ 1-1*, *pp2a-b'ζ 1-2*, and the pp2a-*b'γζ* double mutant grown under either low light (LL) or high light (HL) conditions. The light saturation curves for photosynthesis **(A)** and the CO_2 response curves for photosynthesis **(B)** were measured on rosette leaves of plants that had been grown for 2 weeks under low light (250 µmol m^{-2} s^{-1}) and then either maintained for a further 7 days under low light growth conditions or transferred to high light (800 µmol m^{-2} s^{-1}) for a further 7 days.

not to pathogen attack) in all the genotypes analyzed. The high light-dependent increase in growth in the mutants lacking the B'γ (*gamma*; *pp2a-b'γ*) or B'ζ (*zeta*; *pp2a-b'ζ 1-1* and *pp2a-b'ζ 1-2*) subunits or lacking both subunits is striking, and suggests that the restriction on growth imposed by the loss of the regulation of PP2A-dependent processes is overcome by highlight dependent pathways.

PP2A-B'γ regulates organellar ROS signaling and plays a key role in the negative control of SA-linked responses and associated metabolic alterations in *A. thaliana* (Trotta et al., 2011;

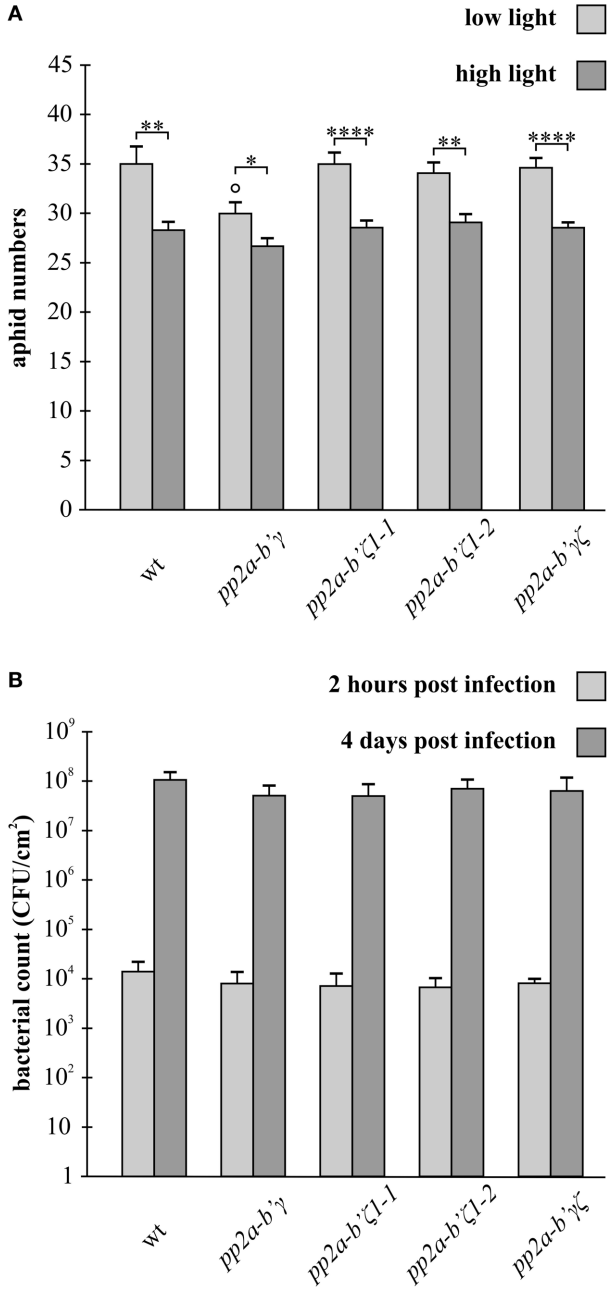

FIGURE 6 | A comparison of aphid fecundity in the *pp2a-b'γ*, *pp2a-b'ζ* 1-1, *pp2a-b'ζ 1-2,* and the pp2a-*b'γζ* double mutant plants grown under either low light or following a 7-day pre-treatment with high light (A), and the effects of these genotypes on infection by the hemibiotrophic pathogen *Pseudomonas syringae* (B). In **(A)**, the numbers of aphids present per plant were measured 2 weeks after the onset of infestation in plants that had either been grown for 5 weeks under low light (250 μmol m^{-2} s^{-1}) or on plants that had been grown for 2 weeks under low light followed by 7 days under high light (800 μmolm^{-2} s^{-1}) and then returned to the low light growth conditions prior to infestation and then grown with aphid infestation for a subsequent period of 2 weeks. The results are average values ± standard error values, $n = 14$. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ in Student's *t*-test and One-Way ANOVA comparisons between LL or HL light conditions. °$p < 0.05$

(Continued)

FIGURE 6 | Continued
in Student's *t*-test and One-Way ANOVA comparisons between the mutant lines and wild type in the LL or HL light conditions. In **(B)**, bacterial suspensions containing10^5 colony forming units ml^{-1} were carefully infiltrated into two fully expanded leaves of each plant and the abundance of the pathogen (CFU) was determined on low light-grown leaves 2 h and 4 days post inoculation. The results are averages ± standard error, $n = 5$.

Li et al., 2014). Metabolite profiling analysis has shown that the *pp2a-b'γ* mutation has only a minor impact on the contents of amino acids and sugars in non-stressed plants, with only slight increases in the levels of homoserine and tryptophan and reduced levels of malic acid and citric acid in *pp2a-b'γ* leaves (Li et al., 2014). However, when combined with a mutation in the photorespiratory form of *catalase 2* (*cat2*) in a double mutant, the *pp2a-b'γ* mutation increases the *cat2*-triggered accumulation of amino acids and camalexin, suggesting that PP2A-B'γ may influence pathways leading to secondary metabolism in response to oxidative signals (Li et al., 2014). This finding is consistent with observations demonstrating that PP2A-B'γ physically interacts with the cytoplasmic form of ACONITASE 3, a metabolic enzyme that is functionally associated with respiration, oxidative stress responses and cell death (Konert et al., authors own unpublished data). Further analysis of previously published microarray data revealed that SA signaling and cell death pathways are increased in *pp2a-b'γ* relative to the wild type (Trotta et al., 2011). Moreover, while no transcripts related to callose synthesis were differentially increased in the *gamma* (*pp2a-b'γ*) mutant (Trotta et al., 2011), the abundance of mRNAs encoding the beta-glucanase pathogenesis-related (PR) protein 2, which negatively regulates the deposition of the callose, was increased relative to the wild type, together with other PR transcripts.

The data presented here provides the first evidence that PP2A-B' negatively controls plant resistance to aphids in low light-grown *A. thaliana* plants, and that this regulatory pathway is functionally connected with PP2A-B'ζ, which in turn seems to have a positive impact on defense signaling (**Figure 6A**). While knock-down *pp2a-b'γ* mutants show decreased aphid fecundity under low light growth conditions, the *pp2a-b'γζ* double mutant supports aphid propagation in a similar manner to that observed in the wild type plants (**Figure 6A**). It should be noted that to date these observations have been made only on one mutant line because there are no other suitable mutant alleles available for analysis. Similarly, there are no transformed plants with appropriate RNAi constructs available as yet for these subunits. However, it is well known that PP2A phosphatases with different subunit compositions may regulate signaling networks at multiple nodes in both animals and plants (Hardie, 1990; Tang et al., 2011; Wu et al., 2011). Such multi-level action is possible because PP2A may assemble in a large number of different heterotrimeric holoenzymes with different functional properties and therefore high specificity toward specific target phospho-proteins.

The computational models of heterotrimeric PP2A complexes described here suggest that PP2A-B'γ and PP2A-B'ζ may bind

similar PP2A-A/C dimers (**Figure 1C**). Hence, when PP2A-B'γ is absent, PP2A-B'ζ can act as a positive mediator and may take over to promote defensive processes. Indeed, the perturbations in both regulatory subunits that occurs in the *pp2a-b'γζ* double mutant, appear to revert the situation back to the wild type (**Figure 6A**). Since the PP2A A and C subunits share high amino acid sequence identity (**Supplemental Figures 1**, **2**), the variable regulatory B subunits mediate essential roles in determining the substrate specificity and subcellular targeting of PP2A (Matre et al., 2009; Uhrig et al., 2013). Fluorescence-tagged versions of both B'γ and B'ζ have been observed in the cytoplasm. Moreover, B'ζ was localized to mitochondria (Matre et al., 2009; Trotta et al., 2011). Distinct functions of the GAMMA and ZETA subunits could also be mediated by the variable N-terminal domains that extrude from the core of the PP2A holoenzyme. The N-terminal domain may therefore determine interactions with other proteins, which because of the unique characteristics of the amino acid sequences are likely to differ between PP2A-B'γ and PP2A-B'ζ. Trimeric PP2A holoenzymes with B'γ or B'ζ may therefore regulate cellular functions in seemingly opposite ways. Furthermore, it is probable that both the GAMMA and ZETA subunits interact with a number of target phospho-proteins and therefore serve multiple functions, which might be changed in the mutants analyzed here. It is also possible that other B' subunits could replace the B' subunits in the *gamma zeta* double knockout because a number of different B' subunits will be available. However, higher order mutant combinations would be required to investigate this point.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00405/abstract

Supplemental Figure 1 | Amino acid sequence alignments of PP2A-B subunits. Seventeen known Arabidopsis PP2A-B subunits are aligned with human PP2A-B56γ.

Supplemental Figure 2 | Amino acid sequence alignment of Arabidopsis PP2A-A subunits. The mouse α subunit from the X-Ray structure of the known PP2A trimer (PDB code: 3FGA, chain A) is shown in parallel. Sequence similarity, based on the three Arabidopsis sequences, is highlighted.

Supplemental Figure 3 | Amino acid sequence alignment of Arabidopsis PP2A-C subunits. The human catalytic subunit from the X-Ray structure of the known PP2A trimer (PDB code: 3FGA, chain C) is shown in parallel. Sequence similarity is highlighted.

Supplemental Figure 4 | The change in the ratio of dark-adapted variable chlorophyll a fluorescence (Fv) to maximal chlorophyll a fluorescence (Fm) observed following the transfer of plants grown under low light to high light for 7 days.

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