Leaf stage-associated resistance is correlated with phytohormones in a pathosystem-dependent manner

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Abstract

It has been reported in several pathosystems that disease resistance can vary in leaves at different stages. However, how general this leaf stage-associated resistance is, and the molecular mechanism(s) underlying it, remain largely unknown. Here, we investigated the effect of leaf stage on basal resistance, effector-triggered immunity (ETI) and nonhost resistance, using eight pathosystems involving the hosts *Arabidopsis thaliana*, *Nicotiana tabacum*, and *N. benthamiana* and the pathogens *Sclerotinia sclerotiorum*, *Pseudomonas syringae* pv. *tabaci*, *P. syringae* pv. *tomato* DC3000, and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We found evidence that leaf stage-associated resistance exists ubiquitously in plants, but with varying intensity at different stages in diverse pathosystems. Microarray expression profiling assays demonstrated that hundreds of genes involved in defense responses, phytohormone biosynthesis and signaling, and calcium signaling, were differentially expressed between leaves at different stages. The *Arabidopsis* mutants *sid1*, *sid2-3*, *ein2*, *jar1-1*, *aba1* and *aao3* lost leaf stage-associated resistance to *S. sclerotiorum*, and the mutants *aba1* and *sid2-3* were affected in leaf stage-associated *RPS2/AvrRpt2*'-conferred ETI, while only the mutant *sid2-3* influenced leaf stage-associated nonhost resistance to *Xoo*. Our results reveal that the phytohormones salicylic acid, ethylene, jasmonic acid and abscisic acid likely play an essential but pathosystem-dependent role in leaf stage-associated resistance.
INTRODUCTION

In higher plants, there are usually three discrete temporal stages of postembryonic development: juvenile, adult, and reproductive. Plants at different stages are distinguished by a variety of developmental features, such as leaf shape and size, patterns of epidermal differentiation and the capacity to produce flowers or flower-bearing branches (Poethig 2003). The level of pathogen resistance also varies in leaves at different stages. Plants are generally more susceptible to pathogens in early stages than in late stages. Stage-associated resistance correlates with developmental stage and thus is a type of developmental resistance. This is different from age-related resistance (ARR), which does not relate to any particular physiological process or developmental stage (Develey-Riviere and Galiana 2007).

Leaf stage-associated disease resistance has been reported in several pathosystems. For instance, leaf stage was found to affect basal resistance, which is shown in host plants susceptible to pathogens. Compared with the lower leaves, the upper and/or apical leaves are more resistant to downy mildew (Hyaloperonospora parasitica) in cabbage (Coelho et al. 2009) and late blight (Phytophthora infestans) in potato (Solanum tuberosum) (Visker et al. 2003). By contrast, lower, older leaves show greater resistance to powdery mildew (Uncinula necator) (Doster and Schnathorst 1985) and downy mildew (Plasmopara viticola) (Reuveni 1998) in grapevine than the upper, younger leaves. In addition, leaf stage also influences induced/acquired resistance, which is activated in plants by an inducer that could be a pathogen or a component of pathogen and plant or even a chemical. The level of sulfated laminarin (PS3)-induced resistance to downy mildew (P. viticola) in grapevine was higher in adult leaves than in younger leaves (Steimetz et al. 2012). However, beta-aminobutyric acid (BABA)-induced resistance to late blight (P. infestans) in tomato was the opposite (Sharma et al. 2010). Overall, the previous results indicate that leaf stage can affect basal and induced/acquired resistance in a pathosystem-dependent manner. However, the effect of leaf stage on other types of resistance, such as nonhost resistance, which occurs in nonhost plants against nonadapted pathogens, and effector-triggered immunity (ETI), which is achieved in R gene-containing plants by recognizing pathogen effectors, remains unclear.

The molecular basis of leaf stage-associated resistance is largely unknown, although it seems
to be related to the ability to constitute a physical and chemical barrier to pathogens, such as accumulation of pathogenesis-related proteins, proteins modifying or strengthening cell walls, peroxide, and phenols (Hugot et al. 2004). Notably, the phytohormone salicylic acid (SA) plays a key role in ARR, a phenomenon whereby many plants exhibit enhanced resistance to disease at later developmental stages (Develey-Riviere and Galiana 2007). In *Arabidopsis*, SA is required for ARR against the bacterium *P. syringae* and the oomycete *H. parasitica* Emco5 (Kus et al. 2002; Cameron and Zaton 2004; McDowell et al. 2005), exhibiting direct antibacterial and antibiofilm activity to *P. syringae* (Wilson et al. 2017). Whether SA and other phytohormones similarly affect leaf stage-associated resistance remains to be elucidated.

In this study, using numerous pathosystems, we systematically investigated the effect of leaf stage on a variety of disease resistance types, including basal resistance, nonhost resistance and ETI, to various important pathogens. We performed microarray expression profiling assays to reveal the gene expression profiles at different stages, and used ten mutants deficient in SA, ethylene, jasmonic acid (JA) and abscisic acid (ABA) biosynthesis or signaling to unravel the roles of these phytohormones in leaf stage-associated resistance. Thus, we provide insights into the molecular basis of leaf stage-associated resistance.

**RESULTS**

**Leaf stage affects antifungal basal resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana* and *Nicotiana tabacum***

Both *Arabidopsis thaliana* and *Nicotiana tabacum* are hosts of *Sclerotinia sclerotiorum*, one of the most devastating fungal pathogens (Boland and Hall 1994; Cao et al. 2016), and thus exhibit basal resistance to *S. sclerotiorum*. *Arabidopsis* leaves at different stages exhibited different morphological characteristics. Juvenile leaves were rounder and less serrated than adult leaves, and reproductive leaves had very little petiole (Figure 1A). To examine the effect of leaf stage on basal resistance to *S. sclerotiorum*, leaves at different stages taken from 8-week-old *Arabidopsis* plants were inoculated with mycelial plugs of *S. sclerotiorum*, and disease severity
was investigated. To avoid induced resistance in systemic leaves, only one leaf per plant was inoculated (the same is true for other inoculation assays described hereafter).

The size of the diseased necrotic lesions in inoculated plants differed in leaves of different stages. Juvenile leaves developed the largest lesions, of 7.0 mm in diameter, while adult leaves formed medium-sized lesions of 5.2 mm in diameter, and reproductive leaves had the smallest lesions of 3.2 mm in diameter at 24 hours post-inoculation (hpi) (Figure 1A). This reveals that leaf stage significantly affects resistance to *S. sclerotiorum* in *Arabidopsis*, and that this resistance increases from juvenile to adult and from adult to reproductive leaves.

To further explore this result, a similar investigation was conducted in *Nicotiana tabacum*, a solanaceous host of *S. sclerotiorum*. As in *Arabidopsis*, *N. tabacum* leaves at different stages showed different morphological characteristics. Leaves of *N. tabacum* plants exhibited two distinguishable types of morphological characteristics. Juvenile leaves were smaller, rounder and thicker than adult leaves in upper positions of the plant (Figure 1B). Leaves that had fully expanded were selected for inoculation with *S. sclerotiorum*. Comparative analysis reveals that the disease severity caused by *S. sclerotiorum* varied significantly between leaves at different stages (Figure 1B). Juvenile leaves formed larger lesions with an average diameter of 10.6 mm, while adult leaves in upper leaf positions developed smaller lesions of 4.1 mm in diameter at 36 hpi (Figure 1B). This result supports the conclusion that leaf stage significantly affects resistance to *S. sclerotiorum* in *N. tabacum*, as observed in *Arabidopsis*.

Leaf stage influences antibacterial basal resistance to *Pseudomonas syringae* pv. *tabaci* in *Nicotiana benthamiana*, and to *P. syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana*

*Pseudomonas syringae* pv. *tabaci* (*Pstab*) is an important bacterial pathogen of *Nicotiana* species, and *N. benthamiana* plants have basal resistance to this pathogen. To deduce whether leaf stage influences basal resistance to *Pstab*, bacterial suspensions were inoculated into *N. benthamiana* leaves, and the severity of resulting necrotic symptoms in leaves at various positions was examined. At 18 hpi, water-soaked necrosis was apparent in inoculated areas of lower and lower-middle leaves; however, no or only very weak necrosis was seen in inoculated areas of middle-upper and upper leaves (Figure 2A). Assays of bacterial levels revealed that the
level of \textit{Pst} in leaves was significantly reduced in lower-middle, middle-upper and upper leaves compared to lower leaves by 0.4, 1.1 and 1.2 orders of magnitude, respectively (Figure 2A). This result coincides well with the severity of resulting necrosis symptoms (Figure 2A). However, at later stages of disease development, strong necrosis was observed to have expanded beyond the inoculated areas in all leaves. Neither necrotic symptoms nor bacterial levels differed significantly between leaves at different positions at 48 hpi (Figure 2A). Taken together, these results demonstrate that leaf stage affects basal resistance to early infection with the bacterial pathogen \textit{Pst} in \textit{N. benthamiana}.

To further characterize the effect of leaf stage on basal resistance to bacterial pathogens, another pathosystem, \textit{A. thaliana–P. syringae pv. tomato (Pst)} DC3000, was similarly examined. At 48 hpi, severe necrosis formed in inoculated areas of juvenile leaves; however, no clear necrosis was visible in inoculated areas of adult or reproductive leaves (Figure 2B). Bacterial enumeration assays showed that, compared with juvenile leaves, the quantity of \textit{Pst} DC3000 bacteria in adult and reproductive leaves was reduced by 0.8 and 1.2 orders of magnitude, respectively (Figure 2B). The obvious, less severe symptoms, and fewer bacterial cells in inoculated areas of adult and reproductive leaves compared with juvenile leaves remained clear at 72 hpi (Figure 2B). This result reveals that leaf stage affects basal resistance to the bacterial pathogen \textit{Pst} DC3000 in \textit{Arabidopsis}.

Taken together, these results demonstrate that, as with resistance to the fungal necrotrophic pathogen \textit{S. sclerotiorum} in \textit{Arabidopsis} and \textit{N. tabacum}, leaf stage affects basal resistance to the bacterial pathogens \textit{Pst} DC3000 and \textit{Pst} in \textit{Arabidopsis} and \textit{N. benthamiana}, respectively, and this resistance increases from lower to upper leaves.

\section*{Nonhost resistance to bacterial pathogens in \textit{N. benthamiana} and \textit{Arabidopsis} is strikingly affected by leaf stage}

Our finding that leaf stage affects basal host resistance to a variety of pathogens prompted us to investigate whether leaf stage also influences nonhost resistance in plants. To this aim, we tested three nonhost resistance pathosystems: \textit{N. benthamiana} and the bacterial pathogens
Xanthomonas oryzae pv. oryzae (Xoo) and Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) (Li et al. 2012, 2015; Du et al. 2013), and Arabidopsis and Xoo (Rahman et al. 2016). The hypersensitive response (HR) and hydrogen peroxide (H$_2$O$_2$) accumulation are hallmarks of nonhost resistance in these pathosystems (Li et al. 2012, 2015; Du et al. 2013; Rahman et al. 2016). In the Xoo-inoculated N. benthamiana plants, at 18 hpi, a strong HR was elicited in inoculated areas of lower and lower-middle leaves. No or only very weak HR-related necrosis was observed in inoculated areas of middle-upper and upper leaves (Figure 3A), although diaminobenzidine (DAB) staining assays revealed strong accumulation of H$_2$O$_2$ in these leaves, as in lower and lower-middle leaves (Figure 3A). Bacterial level assays showed that the quantity of Xoo bacteria in the middle-upper and upper leaves increased by nearly 0.7 orders of magnitude compared with lower and lower-middle leaves (Figure 3A). However, later on, a strong HR was observed in inoculated areas of all leaves. HR symptoms, H$_2$O$_2$ accumulation and bacterial levels were similar in all leaves at 24 hpi (Figure 3A). The development dynamics of the HR and resistance reveals that the development of nonhost resistance in middle-upper and upper leaves is delayed compared with lower and lower-middle leaves. Together, these results indicate that leaf stage significantly affects nonhost resistance to early Xoo infection in N. benthamiana. The level of this nonhost resistance is reduced from lower to upper leaves, in contrast to host resistance to S. sclerotiorum and Pstab.

To check the effect of leaf stage on nonhost resistance in other plant species, we performed similar experiments in Arabidopsis. The Xoo-induced HR in Arabidopsis appeared much later than in N. benthamiana. Nevertheless, the level of this HR was similarly varied in leaves at different stages. At 60 hpi, about 40% of juvenile leaves exhibited a full or occasionally a nearly full HR in response to Xoo inoculation. However, no adult or reproductive leaves produced a full or nearly full HR; rather, only 17% of adult leaves and 14% of reproductive leaves showed a partial HR (Figure 3B). DAB staining assays revealed a similar trend for H$_2$O$_2$ accumulation in these leaves (Figure 3B). These results confirm the influence of leaf stage on nonhost resistance to Xoo in N. benthamiana.

To further examine the effect of leaf stage on nonhost resistance, we investigated another pathosystem, Pst DC3000 and N. benthamiana. At 18 hpi, a strong HR occurred in lower and lower-middle leaves, while no clear HR was visible in middle-upper or upper leaves (Figure 3C).
DAB staining assays showed a high level of H$_2$O$_2$ accumulated in lower and lower-middle leaves, but no or only a very low level of H$_2$O$_2$ accumulated in middle-upper and upper leaves (Figure 3C). A bacterial count revealed that numbers of *Pst* DC3000 bacteria were about two orders of magnitude lower in lower and lower-middle leaves than in middle-upper and upper leaves (Figure 3C). Similar to *Xoo*, a *Pst* DC3000-induced HR was also delayed in upper leaves. However, at 36 hpi, all leaves displayed a severe HR, accumulated a high level of H$_2$O$_2$, and contained similar numbers of bacterial cells (Figure 3C). These data demonstrate that HR and nonhost resistance to *Pst* DC3000 are delayed in upper leaves compared with lower leaves. It is also clear that leaf stage affects nonhost resistance to early *Pst* DC3000 infection in *N. benthamiana*, and the level of this resistance is reduced from lower to upper leaves, as with nonhost resistance to *Xoo*.

Collectively, our results demonstrate that both host and nonhost resistance to diverse pathogens in *N. benthamiana* and *Arabidopsis* are affected by leaf stage, but in contrasting directions.

**Effector-triggered immunity conferred by RPS2 to *Pst* DC3000 (*AvrRpt2*) in *Arabidopsis* is significantly affected by leaf stage, like nonhost resistance to *Xoo* and *Pst* DC3000, but in contrast to host basal resistance to *S. sclerotiorum, Pstab* and *Pst* DC3000**

To examine the effect of leaf stage on effector-triggered immunity (ETI), we compared *RPS2*-conferred ETI to *Pst* DC3000 (*AvrRpt2*) in leaves at diverse stages in *Arabidopsis*. Inoculation of *Arabidopsis* leaves with *Pst* DC3000 (*AvrRpt2*) caused the HR, a hallmark of ETI. The intensity of this HR varied in leaves at different stages, with more severe HR symptoms observed in juvenile and adult leaves than in reproductive leaves. At 24 hpi, over 65% of juvenile and adult leaves exhibited a full or nearly full HR. However, no reproductive leaf showed this type HR. Instead, about 40% of reproductive leaves showed no or a weak HR (Figure 4A, B). Bacterial counts revealed that, compared with reproductive leaves, the quantity of *Pst* DC3000 (*AvrRpt2*) in juvenile and adult leaves decreased by 1.1 and 0.9 orders of magnitude, respectively (Figure 4C). These data indicate that HR and ETI conferred by *RPS2* to *Pst* DC3000 (*AvrRpt2*) in *Arabidopsis* is significantly affected by leaf stage, and is much...
stronger in juvenile and adult leaves than in reproductive leaves. This trend is similar to that observed in nonhost resistance to Xoo and Pst DC3000, but is in contrast to host basal resistance to S. sclerotiorum, Pstab and Pst DC3000.

Collectively, our results reveal that leaf stage ubiquitously influences various types of plant disease resistance to diverse pathogens, and the effect is pathosystem-dependent. Host basal resistance to S. sclerotiorum, Pstab and Pst DC3000 is much stronger in reproductive and adult leaves than in juvenile leaves, while nonhost resistance to Xoo and Pst DC3000 and ETI conferred by RPS2 to Pst DC3000 (AvrRpt2+) are stronger in juvenile and adult leaves than in reproductive leaves.

**Arabidopsis leaves at different stages exhibit striking variation in their expression of defense-related genes**

To explore the molecular mechanism underlying leaf stage-associated resistance, gene expression profiling analysis was conducted on healthy, uninfected Arabidopsis leaves at juvenile, adult, and reproductive stages. Microarray analysis revealed significantly different expression profiles in leaves at different stages. Over 1,400 genes were differentially expressed (DE) between leaves at any two stages. Among the list of genes DE by over two-fold, compared with juvenile leaves, 1,749 genes were up-regulated, and 1,552 were down-regulated in reproductive leaves, and 872 genes were up-regulated, while 607 genes were down-regulated in adult leaves. Additionally, 783 genes were up-regulated and 1,011 genes were down-regulated in reproductive leaves compared with adult leaves (Figure S1; Table S1).

Among the DE genes, over 100 were related to plant defense responses such as glucosinolate biosynthesis and metabolism, oxalic acid catabolism, reactive oxygen species (ROS) accumulation, systemic acquired resistance (SAR) and signaling, R proteins and defense signaling (Table S2). Fourteen DE genes were known to participate in glucosinolate biosynthesis and metabolism, which is essential to disease resistance in Crucifereous species (Albinsky et al. 2010). Of these 14 genes, two flavin-monooxygenas (FMO) family genes, At1g04180.1 and At1g62540.1, which encode a glucosinolate S-oxygenase that catalyzes the conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates, were up-regulated by 7-fold and 3.9-fold in adult leaves compared to juvenile leaves. Furthermore,
two *Arabidopsis* thioglucoside glucohydrolase (TGG) genes were simultaneously up-regulated. These myrosinase enzymes catalyze the hydrolysis of glucosinolates into compounds that are toxic to various microbes and herbivores. Expression of *TGG1* (At5g26000.1) increased by 2.4-fold and 3.3-fold, while that of *TGG2* (At5t25980.3) was dramatically enhanced by 40.9-fold and 115.2-fold, in adult and reproductive leaves, respectively, compared with juvenile leaves. These expression data indicate that biosynthesis of glucosinolate and its conversion to highly toxic compounds are markedly promoted in adult and reproductive leaves compared to juvenile leaves. This may at least partially account for enhanced resistance to pathogens that are sensitive to glucosinolate such as *S. sclerotiorum* (Stotz et al. 2011) and *P. syringae* (Fan et al. 2011) in adult and reproductive leaves compared with juvenile leaves.

Nineteen DE genes are involved in generating and scavenging ROS; 14 of these encode peroxidase (POD), while the rest encode ascorbate peroxidase (APX), polyamine oxidase (PAO) and glycolate oxidase (GOX). Many POD genes and a GOX gene (At4g18360.1) were down-regulated in adult and reproductive leaves, while APX5 was up-regulated by 13.4-fold in reproductive leaves compared to juvenile leaves. These data indicate that ROS accumulation is reduced in adult and reproductive leaves compared with juvenile leaves. Down-regulation of the GOX gene (At4g18360.1), which is essential for ROS generation during nonhost resistance (Rojas et al. 2012), coincides with the observation that nonhost resistance to Xoo is weaker in adult and reproductive leaves compared with juvenile leaves (Figure 3B).

Intriguingly, the *AtGER3* gene (At5g20630.1) was up-regulated by 30.3-fold in adult leaves, and by 132.8-fold in reproductive leaves, compared with juvenile leaves. *AtGER3* encodes oxalate oxidase, which catalyzes the breakdown of oxalic acid, the key pathogenicity factor of *S. sclerotiorum*. This may contribute to the weaker disease symptoms observed in adult and reproductive leaves inoculated with *S. sclerotiorum* (Figure 1A).

Compared with juvenile leaves, expression of several ETI-related genes also differed in leaves at various stages. *RIN4*, which is required for ETI conferred by *RPM1* (Mackey et al. 2002) and *RPS2* (Mackey et al. 2003) to *P. syringae*, was up-regulated in adult leaves. Two genes, *AIG1* and *AIG2L*, which are known to be induced during RPS2/AvrRpt2-induced resistance (Reuber and Ausubel 1996), were down-regulated in adult and reproductive leaves. Notably, *RPP1*, 17 TIR-NBS-LRR genes and one CC-NBS-LRR gene were significantly
down-regulated in reproductive leaves but not in adult leaves. Similarly, PROPEP2 and PROPEP3, two genes encoding precursors of damage-associated molecular patterns (DAMPs) (Ryan et al. 2007), were dramatically down-regulated in reproductive leaves but not in adult leaves by 11.4-fold and 12.8-fold, respectively, compared to juvenile leaves. These results suggest that ETI and DAMP-triggered immunity (DTI) responses vary in leaves at different stages, with reduced expression in reproductive leaves compared to adult leaves.

Transcript accumulation for the HR marker gene HIN1 was down-regulated, while that for RTM1 and RTM2, which are specifically involved in restricting the systemic movement of Tobacco etch virus (Chisholm et al. 2001), increased by about 3-fold and 6-fold in adult and reproductive leaves, respectively. Intriguingly, expression of the ARR-related genes ANAC055 and ANAC092 (Al-Daoud and Cameron 2011) was reduced by 2.8-fold and 3.6-fold in reproductive leaves, respectively, while that of ANAC092 alone decreased by 2.1-fold in adult leaves compared to juvenile leaves. The defense signaling gene EDS1, and resistance-related genes PBS3, ADR1, pEARLI1, PUB23 and DCL2 were down-regulated in reproductive but not in adult leaves compared with juvenile leaves. Additionally, five PR genes, PR1, PR2, PR4, PR5 and PR12 (PDF1.2), were greatly down-regulated in reproductive leaves by 100.0, 7.1, 3.3, 15.4 and 15.4-fold, respectively, compared to juvenile leaves. In adult leaves, only PR1, PR5 and PR12 were down-regulated by 2.3, 2.6 and 2.0-fold, respectively, while PR13 (THI2.1) was up-regulated by 4.1-fold compared to juvenile leaves.

Collectively, the change in expression level of defense-related genes was generally more dramatic in reproductive leaves than in adult leaves compared with juvenile leaves.

Among the DE genes were dozens of genes essential for biosynthesis, catabolism and the signaling of various defense-related hormones (Table S3). Most of the known genes involved in SA biosynthesis, catabolism and signaling were DE between leaves at different stages. The SA biosynthesis genes SID1, SID2 and EPS1 were significantly down-regulated in either adult leaves or reproductive leaves compared with juvenile leaves. BSMT1, which encodes a SA-methylating enzyme, was up-regulated by 3.8-fold and 22.2-fold in adult leaves or reproductive leaves, respectively. Two MES genes, whose products remove methyl groups from methyl salicylic acid (MeSA), showed opposite changes in expression: AtMES9 was down-regulated in reproductive leaves whereas AtMES7 was up-regulated. These data indicate
that SA biosynthesis is repressed, and its methylation is promoted in adult leaves and/or reproductive leaves compared to juvenile leaves.

Four ACS genes, *AtACS4*, *AtACS7*, *AtACS8* and *AtACS11*, encoding ACC synthase, showed significantly higher expression in adult leaves and/or reproductive leaves, although another, *AtACS2*, displayed lower expression in adult leaves. This indicates that ethylene biosynthesis may be enhanced in adult and reproductive leaves.

Expression of *JMT*, which encodes a JA carboxyl methyltransferase, increased by 4.7-fold and 13.0-fold in adult and reproductive leaves, respectively, compared to juvenile leaves. However, expression of *JAZ1*, a repressor of JA signaling, decreased by 2.0-fold in reproductive leaves compared to juvenile leaves. This implies that JA signaling and its methylation might be strengthened in adult and reproductive leaves.

The ABA biosynthesis gene *ABA2* was up-regulated, whereas three ABA catabolism genes, *CYP707A1*, *CYP707A2* and *CYP707A4*, and a set of ABA signaling negative regulator genes, *HAB1*, *ABI2* and *AFP1*, were simultaneously down-regulated in adult leaves and/or reproductive leaves. This demonstrates that ABA biosynthesis and signaling is probably boosted in adult leaves and reproductive leaves compared to juvenile leaves.

Additionally, about 80 genes involved in cell wall composition were DE between leaves at different stages, generally showing increased expression in adult and reproductive leaves compared with juvenile leaves (Table S4). These genes included those encoding pectolytic enzymes such as those in the pectate lyase family, pectin lyase-like superfamily, pectin esterase family, and pectin acetylesterase family, and others encoding proteins of the glycoside hydrolase family, xyloglucan endotransglycosylase family, pectin methylesterase inhibitor family, cellulose synthase family, and cell wall expansin family. This suggests that the cell wall is reinforced in adult and reproductive leaves compared with juvenile leaves, which may partially explain the weaker disease severity seen in adult and reproductive leaves inoculated with necrotrophic pathogens such as *S. sclerotiorum*, compared with juvenile leaves (Figure 1A).

Among the DE genes were also dozens of calcium signaling-related genes, and 24 WRKY transcription factor genes (Tables S5, S6). Both calcium signaling and WRKY are important in plant defense (Jiang et al. 2017; Rahman et al. 2016; Saand et al. 2015; Wang et al. 2016). Expression of most of the WRKY genes was strongly suppressed in reproductive and/or adult
leaves compared to juvenile leaves. These data support the variation of calcium-dependent and WRKY-dependent resistance between leaves at different stages.

Phytohormones regulate leaf stage-associated resistance to *S. sclerotiorum* in *Arabidopsis*

Expression of genes regulating the biosynthesis and signaling of defense-related phytohormones was altered between the leaves at various stages (Table S3). To clarify whether hormones indeed regulate leaf stage-associated resistance, numerous *Arabidopsis* mutants with altered biosynthesis or signaling of SA, ethylene, JA and ABA, were analyzed for leaf stage-associated resistance to *S. sclerotiorum*. Unlike the wild type (Col-0), which showed typical leaf stage-associated resistance, the lesion diameter in leaves of the mutants *sid1*, *sid2-3*, *ein2*, *jar1-1*, *aba1* and *aao3* was not significantly different between the different leaf stages (Figure 5A).

At 24 hpi, in wild-type plants, lesions of obviously different sizes (6.4 mm, 5.3 mm and 2.8 mm, respectively) formed in juvenile, adult, and reproductive leaves. However, in the SA-deficient mutants *sid1* and *sid2-3*, lesions of a similar size (5.8 mm, 5.9 mm and 5.4 mm in diameter in *sid1*, and 4.8 mm, 4.4 mm and 4.1 mm in diameter in *sid2-3*) were observed in juvenile, adult, and reproductive leaves (Figure 5B). This indicates that SID1-dependent and SID2-dependent accumulation of SA contributes to leaf stage-associated resistance.

In the ethylene mutants *ein3-1* and *eto2-1*, lesions with different sizes (4.3 mm, 3.6 mm and 2.1 mm in diameter in *ein3-1*, and 6.3 mm, 5.6 mm and 4.9 mm in diameter in *eto2-1*) occurred in leaves at different stages, as in the wild type. However, no leaf stage-associated resistance occurred in the mutant *ein2*. Lesions of similar sizes (4.4 mm, 5.3 mm and 4.2 mm) were observed in juvenile, adult, and reproductive leaves (Figure 5). This suggests that EIN2, but not EIN3 or ETO2, are involved in leaf stage-associated resistance.

Leaf stage-associated resistance was not observed in the JA-deficient mutant *jar1-1*. This mutant formed lesions of similar sizes (4.3 mm, 4.8 mm and 4.1 mm in diameter) in juvenile, adult, and reproductive leaves, indicating that the JAR1-dependent JA pathway contributes to leaf stage-associated resistance. In addition, four ABA-deficient mutants, *aba1*, *aao3*, *hab1-1/abi1-1/abi2-1* and *abi4*, exhibited different phenotypes of leaf stage-associated
resistance. The mutants *aba1* and *aao3* formed lesions of similar sizes (5.8 mm, 5.4 mm and 6.3 mm in diameter in *aba1*, and 4.8 mm, 4.8 mm and 4.9 mm in diameter in *aao3*) in juvenile, adult, and reproductive leaves. However, the triple mutant *hab1-1/abi1-1/abi2-1*, and the single mutant *abi4* generated lesions of significantly different sizes in leaves at different stages, as observed for wild-type control plants (Figure 5). This indicates that ABA1-mediated and AAO3-mediated ABA biosynthesis is required for leaf stage-associated resistance.

Collectively, our results reveal that the phytohormones SA, ethylene, JA and ABA contribute to leaf stage-associated resistance to *S. sclerotiorum* in *Arabidopsis*.

**Phytohormones affect leaf stage-associated nonhost resistance to *Xoo*, and ETI against *Pst* DC3000 (AvrRpt2\(^+\)) in *Arabidopsis***

To further investigate the influence of phytohormones on leaf stage-associated nonhost resistance and ETI, diverse *Arabidopsis* hormone mutants were analyzed for *Xoo*-induced and *Pst* DC3000 (AvrRpt2\(^+\))-induced HR and resistance, respectively.

In wild-type plants, severity of the *Xoo*-induced HR depended on the leaf stage. It was stronger in juvenile leaves than in adult and reproductive leaves, as observed previously. About 40% of juvenile leaves exhibited a full or nearly full HR. Neither a full nor nearly full HR was seen in reproductive leaves; rather, only 14% of reproductive leaves displayed a partial HR (Figure 6A, B). The *Xoo*-induced HR also varied in mutant leaves at different stages, as in wild-type plants, although the absolute value of HR severity differed between the same leaves of the mutants and wild-type plants (Figure 6A, B). Notably, the *sid2-3* mutant exhibited an opposite trend of HR severity in leaves at different stages compared with wild-type and 6 other mutants. In the *sid2-3* mutant, the HR was much stronger in reproductive leaves than in juvenile leaves. Nearly 60% of reproductive leaves showed a full or nearly full HR (Figure 6A, B). Furthermore, HR-induced cell death in leaves at various stages of all mutants was quantified by checking the ion leakage by measuring the conductivity of leaf tissues after inoculation with *Xoo*. These measurements revealed a similar pattern of severity for HR-induced cell death in different mutants as that observed via macroscopic HR investigation (Figure 6C). Moreover, in the same mutants, the level of bacteria in inoculated leaves at various stages was determined. In
terms of levels of *Xoo* bacteria, a similar trend was observed in inoculated areas of these mutants as in the HR assays (Figure 6D). In the *sid2-3* mutant, the quantity of *Xoo* bacterial cells in reproductive leaves was about 0.5 orders of magnitude lower than in juvenile or adult leaves, exhibiting an opposite trend of *Xoo* bacterial propagation in inoculated leaves at different stages compared to wild-type and other mutants (Figure 6D). This indicates that SID2-dependent SA accumulation contributes to leaf stage-associated nonhost HR and resistance to *Xoo* in *Arabidopsis*.

The *Pst* DC3000 (*AvrRpt2*)-induced HR in wild-type plants was much stronger in juvenile and adult leaves than in reproductive leaves, as described above. Over 60% of juvenile and adult leaves displayed a full or nearly full HR, while significant HR was not seen in reproductive leaves. The mutants *ein2*, *ein3-1*, *eto2-1* and *sid1* showed a similar trend for severity of HR in their leaves to wild-type plants, with the most severe HR seen in juvenile leaves, and the weakest HR in reproductive leaves. However, in the mutants *aba1* and *sid2-3*, HR was more severe in reproductive leaves and much weaker in juvenile leaves; the opposite trend to the wild type (Figure 7A, B). Conductivity assays revealed a similar trend of HR-induced cell death in different mutants (Figure 7C). Furthermore, coinciding with the results of HR assays, bacterial enumeration analysis showed that in the mutants *aba1* and *sid2-3*, significantly more *Pst* DC3000 (*AvrRpt2*) cells accumulated in inoculated juvenile leaves compared to inoculated reproductive leaves; the opposite trend to that seen in wild-type plants and other mutants (Figure 7D). These results demonstrate that ABA1-mediated ABA biosynthesis and SID2-dependent SA accumulation are involved in leaf stage-associated HR and ETI to *Pst* DC3000 (*AvrRpt2*).

Taken together, it is clear that phytohormones in general are important in diverse types of leaf stage-associated resistance. However, our findings indicate that the precise phytohormones involved can vary between phyto-pathosystems.

**DISCUSSION**
Leaf stage-associated resistance generally exists in plants and diversifies in different pathosystems

Leaf stage-associated resistance has been documented previously. In most examples, stronger resistance is seen in upper, younger leaves than in lower, older leaves. Among these examples are resistance to late blight (*Phytophthora infestans*) in tomato and potato (Carnegie and Colhoun 1982; Nelson 2006; Visker et al. 2003), early blight (*Alternaria solani*) in potato (Rodriguez et al. 2006), and leaf blight (*Bipolaris coicis*) in adlays (Chang and Hwang 2003). The exception is resistance to downy mildew (*Plasmodora viticola*) in grapevine, which is stronger in the lower adult leaves than in the upper, younger leaves (Reuveni 1998).

Additionally, two documented examples demonstrate a distinct effect of leaf stage on induced resistance. The level of sulfated laminarin (PS3)-induced resistance in grapevine to downy mildew (*Plasmodora viticola*) was higher in the adult leaves than in the younger leaves (Steimetz et al. 2012), while BABA-induced resistance in tomato to late blight (*Phytophthora infestans*) shows the opposite (Sharma et al. 2010).

In this study, we systemically analyzed the effect of leaf stage on various types of disease resistance – basal resistance, nonhost resistance and effector-triggered immunity (ETI), to diverse important pathogens. We found that antifungal basal resistance to *S. sclerotiorum* in *Arabidopsis* (Figure 1A) and *N. tabacum* (Figure 1B), and antibacterial basal resistance to *Pstab* in *N. benthamiana* (Figure 2A) and *Pst* DC3000 in *Arabidopsis* (Figure 2B), were stronger in leaves of adult and reproductive stages than in those of the juvenile stage. However, nonhost resistance to *Xoo* in *N. benthamiana* (Figure 3A) and *Arabidopsis* (Figure 3B), nonhost resistance to *Pst* DC3000 in *N. benthamiana* (Figure 3C), and ETI conferred by *RPS2* to *Pst* DC3000 (*AvrRpt2*) in *Arabidopsis* (Figure 4), exhibited the reverse trend.

Together with previous reports, our findings reveal that leaf stage ubiquitously influences various types of plant disease resistance to diverse pathogens, and the effect is pathosystem-dependent. Our results also indicate that, when comparing resistance phenotypes in resistance evaluation assays of a diverse genetic background, it is important to use leaves taken from the same positions from different plants.
Molecular basis of leaf stage-associated resistance

Although some pathosystems are affected by leaf stage, the molecular basis of this effect remains largely unknown. In this study, we performed a microarray expression profiling assay to reveal the gene expression profiles of leaves at different stages. Our profiling results indicate that over 100 plant defense-related genes are DE between *Arabidopsis* leaves at three different stages of maturity. These genes are involved in glucosinolate biosynthesis and metabolism, oxalic acid catabolism, ROS accumulation, SAR and signaling, R proteins and defense signaling (Table S2). The DE genes also included dozens of genes essential for the biosynthesis, catabolism and signaling of various defense-related hormones such as SA, ethylene, JA and ABA (Table S3), about 80 genes involved in cell wall composition and reinforcement (Table S4), dozens of calcium signaling-related genes (Table S5), and 24 WRKY transcription factor genes (Table S6). Differential expression of these genes in leaves at different stages provided a general basis of evidence for leaf stage-associated resistance.

It is noteworthy that some defense-related DE genes were not DE across the three leaf stages. Only 50, 78 and 67 defense-related genes were DE in the comparison between adult leaves and juvenile leaves, reproductive leaves and juvenile leaves, or reproductive leaves and adult leaves, respectively. Moreover, groups of defense-related DE genes were distinguished between the three comparisons. Most of the 31 ETI-related and five DTI-related genes were DE between reproductive and juvenile leaves, and between reproductive and adult leaves, and were overwhelmingly reduced in reproductive leaves, while they were not DE between adult and juvenile leaves (Table S2). In addition, most of the 19 ROS-related genes were not DE between reproductive and adult leaves (Table S2). These expression data support the suggestion that leaf position-associated resistance may involve different mechanisms depending on leaf position.

Careful comparison of the expression of key defense-related genes between leaves at different stages provides clues to the molecular mechanisms underlying the effect of leaf stage on resistance to some pathogens. Oxalate oxidase catalyzes break down of OA, the key pathogenicity factor of *S. sclerotiorum*. The *AtGER3* gene (*At5g20630.1*), which encodes a germin-like protein that exhibits oxalate oxidase activity, was dramatically up-regulated by 30.3-fold in adult leaves, and by 132.8-fold in reproductive leaves compared with juvenile leaves.
leaves. Meanwhile, about 80 genes involved in cell wall composition and reinforcement generally showed increased expression in adult and reproductive leaves compared with juvenile leaves (Table S4), indicating that the cell wall is reinforced in adult and reproductive leaves but not in juvenile leaves. Together, these data may explain the observation that resistance to S. sclerotiorum is stronger in adult and reproductive leaves than in juvenile leaves (Figure 1A).

A glycolate oxidase (GOX) gene (At4g18360.1) is essential to ROS generation during nonhost resistance (Rojas et al. 2012). This GOX gene was down-regulated in adult and reproductive leaves compared with juvenile leaves, which coincides with the observation that nonhost resistance to Xoo is weaker in adult and reproductive leaves than in juvenile leaves (Figure 3B).

RTM1 and RTM2, which are specifically involved in restricting the systemic movement of Tobacco etch virus (TEV) (Chisholm et al. 2001), increased by about 3-fold and 6-fold in adult and reproductive leaves, respectively. This predicts higher resistance to TEV in adult and reproductive leaves than in juvenile leaves. Meanwhile, LURP1 (At2g14560.1 and At2g14560.2), which is involved in resistance to downy mildew (Knoth and Eulgem 2008), and ATNRT3.1 (At5g50200.1), PER21 (At2g37130.2) and pEARLI1 (At4g12480.1), involved in resistance to Botrytis cinerea (Chassot et al. 2007), were DE between leaves at various stages. This suggests that leaf position can affect plants’ resistance to various pathogens.

A set of DE genes is required for SAR. Compared with juvenile leaves, expression of GLIP1 and AZII, two positive regulators of SAR (Jung et al. 2009; Kwon et al. 2009), were reduced by 4.0-fold and 3.6-fold in adult leaves, respectively, and further markedly dropped by 14.3-fold and 11.1-fold, respectively, in reproductive leaves. Meanwhile, expression of SNI1, a negative regulator of SAR (Li et al. 1999), increased by 2.9-fold and 4.8-fold in adult and reproductive leaves, respectively, compared with juvenile leaves. These data suggest that SAR might be weakest in reproductive leaves, modest in adult leaves, and strongest in juvenile leaves.

Additionally, five PR genes, PR1, PR2, PR4, PR5 and PR12 (PDF1.2), are strongly down-regulated in reproductive leaves by 100-, 7.1-, 3.3-, 15.4- and 15.4-fold, respectively. However, in adult leaves, only PR1, PR5 and PR12 were down-regulated by only 2.3-, 2.6- and 2.0-fold, respectively. These data indicate that resistance that is dependent on these PR proteins may be greatly reduced in reproductive leaves compared to that in juvenile leaves.
The effect of phytohormones on leaf stage-associated resistance is pathosystem-dependent

Dozens of genes that are required for biosynthesis, catabolism and signaling of defense-related hormones such as SA, ethylene, JA and ABA are DE between leaves at different stages (Table S3). These genes included three SA biosynthesis/accumulation genes, *SID1, SID2* and *EPS1*; the SA methylation gene *BSMT1*; the SA demethylation genes *AtMES9* and *AtMES7*; four ACC synthase (ACS) genes, *AtACS4, AtACS7, AtACS8* and *AtACS11*; a JA carboxyl methyltransferase gene, *JMT*; a JA negative regulator gene, *JAZ1*; the ABA biosynthesis gene *ABA2*; three ABA catabolism genes, *CYP707A1, CYP707A2* and *CYP707A4*; and a set of ABA signaling negative regulator genes, *HAB1, ABI2* and *AFP1*. Marked, simultaneous differential expression of these genes between leaves at different stages demonstrates that the hormones SA, ethylene, JA and ABA are involved in leaf stage-associated resistance. To our knowledge, this is the first report of the role of the ABA pathway in leaf stage-associated resistance.

Mutant analyses further reveal that SID1-dependent and SID2-dependent SA accumulation; the EIN2-dependent, but not the EIN3-dependent or ETO2-dependent ethylene pathway; the JAR1-dependent JA pathway; and ABA1-mediated and AAO3-mediated ABA biosynthesis, are required for leaf stage-associated basal resistance to *S. sclerotiorum* in *Arabidopsis* (Figure 5). However, similar analyses have demonstrated that only SID2-dependent SA biosynthesis, but not other phytohormones, contributes to leaf stage-associated nonhost resistance to *Xoo* in Arabidopsis (Figure 6); whereas SID2-dependent SA accumulation and ABA1-mediated ABA biosynthesis, but not other phytohormones, are involved in leaf stage-associated ETI to *Pst DC3000 (AvrRpt2)* (Figure 7). These results reveal that the phytohormones involved in leaf stage-associated resistance are phyto-pathosystem-dependent.

Additionally, it is notable that components involved in the biosynthesis and/or signaling of the same hormone exert different effects on leaf stage-associated resistance, even to the same pathogen. For example, the SA induction-deficient mutants *sid1* and *sid2-3* (Nawrath and Metraux 1999) exert different effects on leaf stage-associated resistance to *Xoo* (Figure 6) and *Pst DC3000 (AvrRpt2)* (Figure 7). This is probably because of difference in these genes in the extent to which they affect biosynthesis and/or signaling of the hormone, and/alternatively in the
extent to which they directly or indirectly affect defense responses other than the hormone. For instance, the SA content of sid1 and sid2-3 mutant plants differs (Nawrath and Metraux 1999). SID2/ICS1 encodes a SA biosynthesis enzyme, while SID1/EDS5 is a MATE-like SA transporter to export SA from the chloroplast to the cytoplasm (Serrano et al. 2013), although SA accumulation in sid1/eds5 mutant is significantly reduced (Nawrath and Metraux 1999; Serrano et al. 2013). It is unclear whether or not SID1/EDS5 transports other defense-related molecule(s). Recently, the role of SA in ARR was linked to its direct antibacterial and antibiofilm activity to P. syringae in Arabidopsis (Wilson et al. 2017). Therefore, it will be interesting to examine whether SA plays a similar role in leaf stage-associated resistance.

Collectively, we conclude that phytohormones are generally important in diverse types of leaf stage-associated resistance to various pathogens. However, the phytohormones involved are pathosystem-dependent.

MATERIALS AND METHODS

Plant materials

Seven days after sowing, Nicotiana tabacum and N. benthamiana seedlings were transferred to pots with a peat-based substrate, and maintained in growth chambers at 25°C with 70% relative humidity (RH) and a 16 h/8 h light/dark daily cycle. Wild-type Arabidopsis thaliana (Col-0) and mutants sid1, sid2-3, ein2, eto2-1, ein3-1, jar1-1, aba1, aao3, hab1-1/abi1-2/abi2-1 and abi4 were grown in growth chambers at 24°C with 70% RH and a 14 h/10 h light/dark daily cycle, with a light intensity of 120 μmol • m⁻² • s⁻¹. Leaves at different positions on 8-week-old Arabidopsis and N. tabacum and 5-week-old N. benthamiana plants were subjected to inoculation assays. Arabidopsis leaves at different stages are distinguishable by their leaf position, leaf shape, and trichome patterning. Juvenile leaves are at the lowest position, while reproductive leaves are located on the stems. In addition, juvenile leaves are rounder, less serrated, and have trichomes only on the upper (adaxial) surface; adult leaves have trichomes on both the upper and the lower (abaxial) surfaces. Juvenile leaves, adult leaves at the middle
position, and reproductive leaves from the stems were used for inoculation and microarray expression analyses.

**Pathogen inoculum preparation**

The bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (*Pstab*) was recovered in a Luria broth (LB) plate at 28°C from −80°C stock. Single colonies were subcultured in LB liquid media for 12 h. Bacterial cells were then collected by centrifugation (6000 r • min⁻¹, 10 min) and diluted into suspensions with an OD₆₀₀ of 0.001 using 10 mM MgCl₂ buffer. *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst DC3000*) and *Pst* DC3000 (*AvrRpt2⁺*) were incubated overnight at 28°C on King’s B medium (tryptone 20 g, K₂HPO₄ 1.5 g, MgSO₄·7H₂O 1.5 g, glycerol 10 mL, agar 15 g, pH 7.2, per litre) containing rifampicin (50 µg • mL⁻¹). Bacterial cells were collected and diluted into suspensions with an OD₆₀₀ of 0.001, similarly as described above. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was incubated in nutrient agar medium (tryptone 5 g, sucrose 10 g, yeast extract 1 g, beef extract 3 g, agar 15 g, pH 7.2, per litre). Bacterial cells were similarly collected and diluted into suspensions with an OD₆₀₀ of 0.5 using sterilized ddH₂O. The fungal pathogen *Sclerotinia sclerotiorum* was grown at 25°C on potato dextrose agar media (PDA; potato 20 g, D-glucose 20 g, agar 15 g, per litre) for 3 days.

**Pathogen inoculation and disease resistance investigation**

One leaf per plant was inoculated to avoid induced resistance in systemic leaves. For the bacterial pathogens, bacterial suspensions of described concentrations were infiltrated using a needleless syringe into leaves that were gently pre-stabbed with a sterilized needle. The plants were then maintained in the growth room at 25°C. The HR induced by *Xoo* and *Pst* DC3000 (*AvrRpt2⁺*) was investigated. HR phenotypes were sorted into three categories based on the intensity and percentage of cell yellowing and cell death in infiltrated areas: no or very weak HR (I, HR area smaller than 30% of total infiltrated area), partial HR (II, HR area larger than 30% but smaller than 90% of total infiltrated area) and full to nearly full HR (III, HR area larger than 90% of total infiltrated area) (Xu et al. 2012). HR severity is indicated as the ratio of leaves exhibiting a HR of three categories to total infiltrated leaves.
The bacterial number in inoculated leaf areas was determined as reported (Katagiri et al. 2002). Briefly, inoculated leaves were harvested and surface-sterilized. Leaf discs were punched within the inoculated areas, and extracted by grinding in sterilized dH₂O. A series of diluted samples were plated on the appropriate medium as mentioned above. Colonies grown on the plates were counted, and the bacterial numbers in the leaf samples were calculated accordingly.

For S. sclerotiorum, plugs of 2 mm (for Arabidopsis) and 4 mm (for N. tabacum) in diameter were taken from the outside circle of PDA plates containing the most actively young mycelia, and inoculated onto Arabidopsis and N. tabacum leaves for leaf stage-associated resistance analysis. Inoculated plants were maintained at high humidity. Disease symptoms including necrosis were photographed and the size of lesions was measured and statistically analyzed.

*In vitro* inoculation analyses were similarly conducted, except detached leaves were used for inoculation.

**Histochemical detection of H₂O₂**

Leaves that were inoculated with bacterial suspensions were sampled. H₂O₂ was detected using DAB staining as described (Thordal-Christensen 2003).

**Conductivity measurement**

Electrolyte leakage caused by HR was evaluated by measuring leaf tissue conductivity as described (Torres et al. 2002). Plants were inoculated with bacteria as described above. Ten minutes after inoculation, leaf discs of 5 mm in diameter were collected from the infiltrated area and washed extensively with water, and then seven discs were placed in a tube with 10 ml water. Conductivity was measured over time using a METTLER TOLEDO conductivity meter (SevenCompact S230-K).

**Statistical data analysis**

All experiments were conducted independently three times. Five wild-type and mutant plants were used for each experiment. Analysis of variance was performed using SPSS software (version 19.0, IBM, USA). Significant differences between mean values were determined using Student’s *t* test (*P*<0.05) and Duncan’s multiple range test (DMRT, *P*<0.05).
Microarray gene expression profiling analysis

Chips containing Arabidopsis genome probes (TAIR 9.0) (Roche NimbleGen, 12*135K, product No. 05543746001) were hybridized with cDNAs reverse-transcribed from RNAs extracted from juvenile, adult, and reproductive leaves of 8-week-old Arabidopsis plants. Total RNA was extracted using Trizol reagent (Invitrogen) and purified with the NucleoSpin® RNA clean-up kit (MACHEREY-NAGEL, Germany). cDNA labeled with a fluorescent dye (Cy3-dCTP) was produced using Eberwine’s linear RNA amplification method, with a CapitalBio cRNA Amplification and Labeling Kit (CapitalBio) to produce higher yields of labeled cDNA. Hybridization analyses were conducted by CapitalBio Corporation China. Digital imaging analysis was performed using NimbleScan 2.6 software. Array data were analyzed for data summarization, normalization and quality control using Robust Multichip Analysis. The normalized signal value of 400 was set as the cutoff to judge the reliability and confidence of true hybridization as either P (Present, signal value ≥400) or A (absent, signal value ≤400). To select the DE genes, an expression change of more than 2-fold and signal call as P were the criteria. Molecule Annotation System (MAS) v3.0 (http://bioinfo.capitalbio.com/mas3/) was used for functional annotation analysis.

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AUTHOR CONTRIBUTIONS

L.H.L., Y.P.X., Y.J.X. and J.Y.C. conducted the plant inoculation and resistance evaluation analyses. Y.P.X., Y.J.X. and L.H.L. designed and performed the statistical analysis and
microarray data analysis. J.Y. carried out the conductivity measurement analyses. X.Z.C. conceived of the study, and participated in its design and coordination. X.Z.C., Y.J.X. and L.H.L. prepared the manuscript.

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SUPPORTING INFORMATION

Figure S1. Scatter diagram showing genes with expression differing by over 2-fold in Arabidopsis leaves at different stages

Red and green spots represent genes with expression up-regulated and down-regulated by over 2-fold, respectively, while black spots indicate the genes with expression differing by less than 2-fold.

Table S1. Total number of differentially expressed genes in Arabidopsis leaves at different stages

Table S2. Differentially expressed genes related to disease resistance in Arabidopsis leaves at different stages
Table S3. Differentially expressed genes related to phytohormones in *Arabidopsis* leaves at different stages

Table S4. Differentially expressed genes related to cell wall formation in *Arabidopsis* leaves at different stages

Table S5. Differentially expressed genes related to calcium signaling in *Arabidopsis* leaves at different stages

Table S6. Differentially expressed genes encoding WRKY transcription factors in *Arabidopsis* leaves at different stages
FIGURE LEGENDS

Figure 1 Effect of leaf stage on antifungal basal resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana* (A) and *Nicotiana tabacum* (B)

Left: Necrotic symptoms of inoculated leaves at 24 hpi (A) or 36 hpi (B). Right: Statistical analysis of lesion diameter of leaves at different stages. Data were analyzed using SPSS. Error bars indicate SD. Significant differences are indicated by letters (DMRT, *P*<0.05) (A) or an asterisk (*) (Student’s *t*-test, *P*<0.05) (B).

Figure 2 Effect of leaf stage on antibacterial basal resistance to *Pseudomonas syringae* pv. *tabaci* (*Pstab*) in *Nicotiana benthamiana* (A) and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst DC3000*) in *Arabidopsis thaliana* (B)

Left: Symptoms of leaves inoculated with bacterial suspensions with an OD$_{600}$ of 0.001. L, lower leaves; LM, lower-middle leaves; MU, middle-upper leaves; U, upper leaves. Right: Bacterial population dynamics inside the plant leaf tissues after inoculation. Cell numbers of bacteria in the inoculated leaf tissues were determined by counting colony-forming units of the sampled tissues. Data were analyzed using SPSS. Error bars indicate SD. Letters indicate significant differences (DMRT, *P*<0.05).

Figure 3 Effect of leaf stage on nonhost resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in *Nicotiana benthamiana* (A) and *Arabidopsis thaliana* (B), and to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst DC3000*) in *N. benthamiana* (C).

Left: Hypersensitive response (HR) symptoms and H$_2$O$_2$ accumulation of leaves inoculated with *Xoo* suspensions with an OD$_{600}$ of 0.5. H$_2$O$_2$ in leaves was detected by diaminobenzidine (DAB) staining analysis. L, lower leaves; LM, lower-middle leaves; MU, middle-upper leaves; U, upper leaves. For (B), HR severity is quantified. HR is classified based on the intensity of cell yellowing and cell death in inoculated areas: I, HR area smaller than 30% of infiltrated leaf area; II, HR area larger than 30% but smaller than 90% of infiltrated leaf area; III, HR area larger than 90% of the infiltrated leaf area. HR severity is indicated as the percentage of leaves exhibiting HR over total infiltrated leaves. Right: Population dynamics of *Xoo* inside the plant...
leaf tissues after inoculation. Cell numbers of Xoo in the inoculated leaf tissues were determined based by counting colony-forming units of the sampled tissues. Data were analyzed using SPSS. Error bars indicate SD. Letters indicate significant differences (DMRT, P<0.05).

**Figure 4 Effect of leaf stage on the effector-triggered immunity conferred by RPS2 to Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) (AvrRpt2) in Arabidopsis**

(A) Hypersensitive response (HR) symptoms of Arabidopsis thaliana leaves inoculated with Pst DC3000 (AvrRpt2) suspensions with an OD\textsubscript{600} of 0.001. Photographs were taken at 24 hpi. (B) HR severity of Pst DC3000 (AvrRpt2) in inoculated Arabidopsis leaves at 24 hpi. HR classification and severity calculations are described in Figure 3B. (C) Bacterial population dynamics of Pst DC3000 (AvrRpt2) inside the plant leaf tissues after inoculation. Cell numbers of Pst DC3000 (AvrRpt2) in the inoculated leaf tissues were determined based on calculating colony-forming units in the sampled tissues. Data were analyzed using SPSS. Error bars indicate SD. Letters indicate significant differences (DMRT, P<0.05).

**Figure 5 Effect of phytohormones on leaf stage-associated resistance to Sclerotinia sclerotiorum in Arabidopsis thaliana**

(A) Necrosis symptoms of Arabidopsis hormone mutants inoculated with S. sclerotiorum. Photographs were taken at 24 hpi. (B) Statistical analysis of lesion diameter of leaves at different stages. Data were analyzed using SPSS. Error bars indicate SD. Letters indicate significant differences (DMRT, P<0.05).

**Figure 6 Effect of phytohormones on leaf stage-associated nonhost resistance to Xanthomonas oryzae pv. oryzae (Xoo) in Arabidopsis thaliana**

(A) Hypersensitive response (HR) symptoms of Arabidopsis hormone mutants inoculated with Xoo suspensions with an OD\textsubscript{600} of 0.5. Photographs were taken at 60 hpi. (B) HR severity of hormone mutants inoculated with Xoo. HR classification and severity calculations are described in Figure 3B. (C) Conductivity of the hormone mutants inoculated with Xoo. (D) Bacterial
Figure 7 Effect of phytohormones on leaf stage-associated effector-triggered immunity to Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) (AvrRpt2<sup>+</sup>) in Arabidopsis thaliana

(A) Hypersensitive response (HR) symptoms of Arabidopsis phytohormone mutant leaves inoculated with Pst DC3000 (AvrRpt2<sup>+</sup>) suspensions with an OD<sub>600</sub> of 0.001. Photographs were taken at 12 hpi. (B) HR severity of hormone-deficient mutants inoculated with Pst DC3000 (AvrRpt2<sup>+</sup>). HR classification and severity calculations are described in Figure 3. (C) Conductivity of hormone mutants inoculated with Pst DC3000 (AvrRpt2<sup>+</sup>). (D) Bacterial enumeration of Pst DC3000 (AvrRpt2<sup>+</sup>) inside the plant leaf tissues after inoculation. For details of bacterial cell determination and statistical analysis, please refer to the description in Figure 4.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7