Chitosan Oligosaccharide Induces Resistance to *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana* by Activating Both Salicylic Acid– and Jasmonic Acid–Mediated Pathways

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Chitosan oligosaccharide (COS) is an effective plant immunity elicitor; however, its induction mechanism in plants is complex and needs further investigation. In this study, the *Arabidopsis*–*Pseudomonas syringae* pv. *tomato* DC3000 (hereafter called DC3000) interaction was used to investigate the induction effect and the underlying mechanisms of COS. COS is effective in inducing resistance to DC3000 in *Arabidopsis*, and our results demonstrate that treatment with COS 3 days before DC3000 inoculation provided the most effective resistance. Disease severity in *jar1* (jasmonic acid [JA]-deficient mutant), *NahG*, and *sid2* (salicylic acid [SA]-deficient mutants) suggest both the SA and JA pathways are required for the *Arabidopsis* response to DC3000. COS pretreatment induced resistance in wild type (WT), *jar1*, and also, although to a lesser degree, in *NahG* and *sid2* plants, implying that the SA and JA pathways play redundant roles in COS-induced resistance to DC3000. In COS-pretreated plants, expression of genes related to the SA pathway (*PR1*, *PR2*, and *PR5*) and SA content increased in both WT and *jar1*. Moreover, expression of genes related to the JA pathway (*PDF1.2* and *VSP2*) and JA content both increased in WT and *NahG*. In conclusion, COS induces resistance to DC3000 in *Arabidopsis* by activating both SA- and JA-mediated pathways, although SA and JA pathways play redundant roles in this COS-induced resistance.

The interaction of *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *tomato* DC3000 (hereafter called DC3000) has been used for many years as a typical plant pathosystem to study plant defense response (Jovanovic et al. 2017; Katagiri et al. 2002; Liu et al. 2015; Naseem et al. 2014). DC3000 is a hemibiotrophic bacterium that was initially isolated from tomato (Cuppels 1986) and, thus, is not a natural pathogen of *Arabidopsis*. However, under laboratory conditions with a high bacterial titer inoculation, DC3000 invades *Arabidopsis* leaf tissue, multiplies rapidly in intercellular spaces, and causes

visible disease symptoms on infected leaves (Katagiri et al. 2002; Velásquez et al. 2017).

When pathogens attack plants, various signaling pathways are activated, including the salicylic acid (SA)- and the jasmonic acid/ethylene (JA/ET)-mediated signaling pathways (Kouzai et al. 2018; Zhang et al. 2017). In both basal and induced resistance in plants, the SA and JA/ET pathways influence each other by acting in synergistic and antagonistic ways (Betsuyaku et al. 2018; Koornneef and Pieterse 2008; Zhang et al. 2015). According to previous studies, plants that overaccumulate SA or have been treated with exogenous SA are more resistant to DC3000, suggesting that SA plays an essential role in *Arabidopsis* resistance to DC3000 (Todesco et al. 2010; Velásquez et al. 2017; Wilson et al. 2017). Generally, SA and JA pathways work in an antagonistic way; however, some studies have found that both SA- and JA-mediated pathways are required for *Arabidopsis* resistance to DC3000. During DC3000 infection, both the SA and JA pathway-related genes in *Arabidopsis* are upregulated, leading to SA and JA accumulation in infected leaves (Betsuyaku et al. 2018; Hossain et al. 2008; Khare et al. 2016; Kloeck et al. 2001).

In plant-pathogen interactions, microbe-associated molecular patterns (MAMPs) such as chitin and flagellin are perceived by plant receptors that activate plant innate immunity to defend against pathogen invasion (Poncini et al. 2017). Chitosan, composed of β -1,4-glucosamines, is one of the most abundant polysaccharides in the world. Chitosan oligosaccharide (COS) is a product of chitosan hydrolysis and is an effective MAMP that induces plant innate immunity (Yin et al. 2010, 2016). According to previous studies, COS serves as a potent immunity elicitor in many plants, including tobacco (Zhang et al. 2012), camellia (Li and Zhu 2013), wheat (Wang et al. 2015), oilseed rape (Yin et al. 2013), tomato (Zhang and Chen 2009), soybean (Valdés-López et al. 2014), *Arabidopsis* (Jia et al. 2016), and rice (Yang et al. 2017).

Chitosan's induction mechanism on fungi is revealed by inserting itself into the minor groove of DNA and activating DNA damage responses to potentiate plant immunity (Hadwiger 2015; Hadwiger and Tanaka 2017). This signal perception mode is quite different from chitin oligomers, which are recognized by receptors at the plasma membrane by affecting transcription factors without directly acting on DNA (Desaki et al. 2018; Hadwiger and Tanaka 2017; Squeglia et al. 2017). Recently, a work has revealed that fluorescence-labeled COS are enriched on the wheat leaf cell surface, and several candidates that have the potential to be COS receptors were found (Liu et al. 2018). Thus, besides acting on DNA directly, COS receptors on plant plasma membrane maybe also exist.

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Although the COS signal perception mechanism is by DNA damage responses or receptor recognition, the downstream signal transduction pathways are similar. As a broad-spectrum plant immunity elicitor, COS activates different signaling pathways for defense against pathogen invasion. Previous studies have demonstrated that COS treatment enhances the expression of JA-related genes in *Brassica napus* (Yin et al. 2006) and increases JA content in tomato (Doares et al. 1995), rice (Rakwal et al. 2002), and rapeseed (Yin et al. 2013). These results reveal that COS enhances plant immunity mainly through the JA-mediated pathway. However, when COS-treated *Arabidopsis* or tobacco plants are infected with the *Tobacco mosaic virus* (TMV), only the SA-mediated pathway is activated (Jia et al. 2016; Yafei et al. 2009). Similar results were observed in COS-pretreated rice, where infection with the *Southern rice black-streaked dwarf virus* (SRBSDV) dramatically increased PR1 transcription level (Yang et al. 2017). Thus, because of the complexity of the COS-induced immune signaling network, the topic requires further research.

In this study, *Arabidopsis*-*P. syringae* pv. *tomato* DC3000 interaction was used to further reveal the complex mechanisms of COS-induced immunity. The involvement of SA and JA-mediated pathway in COS-induced resistance to DC3000 in *Arabidopsis* were investigated by using SA- or JA-deficient mutants, including *jar1* (JA pathway-deficient mutant), *NahG*, and *sid2* (SA pathway-deficient mutants).

RESULTS

COS induces resistance to DC3000 in *Arabidopsis*.

As a broad-spectrum plant immunity regulator, COS is effective in inducing resistance against many viral or bacterial infections in

plants. According to previous studies, 50 mg of COS per liter is the most effective concentration for pretreatment to induce immunity against many plant pathogens in many plants, including tobacco-TMV, *Arabidopsis*-TMV, oilseed rape-*S. sclerotiorum*, and rice-SRBSDV (Jia et al. 2016; Li and Zhu 2013; Yang et al. 2017; Yin et al. 2008; Zhao et al. 2007). However, the optimal COS pretreatment time differs for different plant-pathogen interactions: 1 day for tobacco-TMV and *Arabidopsis*-TMV interactions and 3 days for rapeseed-*S. sclerotiorum* interaction (Jia et al. 2016; Yin et al. 2008; Zhao et al. 2007). Thus, we investigated the optimal timing for pretreatment with COS (50 mg/liter) in *Arabidopsis* in order to induce immunity against DC3000.

COS was sprayed onto *Arabidopsis* plants 1, 2, and 3 days before and 0, 1, 2, and 3 days after DC3000 inoculation. The disease phenotypes were recorded at 4 days after DC3000 inoculation. Based on the disease index and bacterial growth data, *Arabidopsis* plants treated with COS prior to DC3000 inoculation displayed reduced disease symptoms compared with those treated with COS after DC3000 inoculation. The optimal treatment time was observed at 3 days prior to DC3000 inoculation (Fig. 1A and B).

The expression of DC3000 marker genes (*avrPtoB* and *recA*) were measured to evaluate the infection status of DC3000 in infected leaves. *avrPtoB* is a DC3000 effector that enters the host cell to interfere with the kinase domains of plant pattern recognition receptor complexes, resulting in enhanced pathogen growth and enhanced DC3000 virulence (Abramovitch and Martin 2005; Chien et al. 2013; Kraus et al. 2016). *recA* is typically used as an internal reference gene when measuring relative expression of the genes in DC3000; thus, it can be used to quantify DC3000 accumulation in infected plant tissue (Scalschi et al. 2014). The

Fig. 1. The optimal treatment time for chitosan oligosaccharide (COS) in inducing resistance to *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*. COS was sprayed onto *Arabidopsis* plants for each of the 3 days before (-3d, -2d, -1d) and 3 days after (0d, 1d, 2d, 3d) DC3000 inoculation. Plants inoculated with DC3000 without COS treatment were used as control (pst). **A**, Disease index. **B**, Bacterial population per square centimeter of leaf tissues. **C**, Expression of the DC3000 marker genes *avrPtoB* and **D**, *recA* were detected. Values are the means $\pm$ standard deviation in three independent measurements. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

expression of *avrPtoB* and *recA* were consistent with the disease index and bacterial population, further confirming that pretreatment of plants with a 50 mg/liter concentration of COS 3 days before DC3000 inoculation leads to better disease control (Fig. 1C, D). Furthermore, these indexes from different levels were highly matched, so they are all usable for the symptoms calculation index in the DC3000 infection of *Arabidopsis*.

SA or JA deficiency impaired resistance to DC3000 in *Arabidopsis*.

We utilized the immune signaling pathway-deficient mutants *jar1*, *NahG*, and *sid2* to investigate the roles of SA and JA pathways in DC3000 infection of *Arabidopsis*.

Four days after DC3000 inoculation, typical disease symptoms included extensive chlorosis and multiple necrotic lesions appeared in inoculated leaves. The observed phenotypes (Fig. 2A) and disease index (Fig. 2B) demonstrated that symptoms in *jar1* (74.7%), *NahG* (71.7%), and *sid2* (74.6%) were more serious than those in wild-type (WT) plants (67.1%). The bacterial population, in DC3000-infected leaves, of *jar1*, *NahG*, and *sid2* was 1.9-, 2.8-, and 3.0-fold higher than WT, respectively, consistent with the observed infection phenotype (Fig. 2C). These results indicate that both SA and JA pathways play essential roles in *Arabidopsis*-DC3000 interactions. Furthermore, DC3000 accumulations in *NahG* and *sid2* were higher than in *jar1*, implying that the SA pathway may play a

Fig. 2. The function of chitosan oligosaccharide (COS) in the *Arabidopsis*-*Pseudomonas syringae* pv. *tomato* DC3000 interaction and disease control effect on different mutants. *Arabidopsis* pretreated with water, then inoculated with 10 mM $MgSO_4$ is noted as Mock. *Arabidopsis* pretreated with water or 50 mg of COS per liter, then inoculated with DC3000 are noted as Mock + Pst or COS + Pst, respectively. Each group contained more than 30 *Arabidopsis* plants. **A**, Disease symptoms of *Arabidopsis* leaves caused by DC3000 infection. The representative infected leaves from each treatment were photographed at 4 days after inoculation with DC3000. **B**, The disease index evaluating the disease symptoms on DC3000-infected leaves. **C**, Bacterial growth assay of DC3000 in per square centimeter of leaf tissues. Values are the means $\pm$ standard deviation in three independent measurements. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

more important role in *Arabidopsis*-DC3000 interaction than the JA pathway. Since the two SA-deficient mutants, *NahG* and *sid2*, showed a similar trend in *Arabidopsis*-DC3000 interaction, we choose to focus on *NahG* for further investigation.

The expression of *avrPtoB* and *recA* in inoculated leaves was highly consistent with the bacterial populations. The expression of *avrPtoB* in *jar1* and *NahG* was 13.1- and 59.5-fold higher than in WT, respectively (Fig. 3A). Additionally, *recA* expression in *jar1* and *NahG* was 3.8- and 50.3-fold higher than in WT, respectively (Fig. 3B). These quantitative polymerase chain reaction (qPCR) data further confirmed that both SA- and JA-mediated pathways are important for *Arabidopsis* immune response against DC3000 infection, and, moreover, the SA pathway appears to play a more critical role in the interaction.

COS pretreatment induces resistance to DC3000 in SA- or JA-deficient mutants.

We investigated the involvement of SA and JA pathways in COS-induced resistance to DC3000 in *Arabidopsis* by using *jar1*, *NahG*, and *sid2* mutants. WT plants and the different signaling pathway-deficient mutants were pretreated with a 50-mg/liter concentration of COS 3 days before DC3000 inoculation in the COS+*Pst* group, and the symptoms of inoculated leaves were significantly reduced compared with the Mock+*Pst* group (pretreated with water 3 days before DC3000 inoculation) (Fig. 2A). Additionally, the disease index of WT, *jar1*, *NahG*, and *sid2* plants in the COS+*Pst* group decreased by 19.2, 30.3, 15.4, and 13.1%, respectively, compared with the Mock+*Pst* group (Fig. 2B). Further, the amount of DC3000

bacteria in WT, *jar1*, *NahG*, and *sid2* in COS-pretreated inoculated leaves was reduced sharply compared with the Mock+*Pst* group, decreasing by 72.7, 81.7, 62.8, and 58.7%, respectively (Fig. 2C). These results suggest that COS induces resistance to DC3000 in SA- and JA-deficient mutants.

The expression of *avrPtoB* and *recA* demonstrate similar trends. In the COS-pretreated group, the expression of *avrPtoB* was reduced by 80.0, 87.2, and 74.4%, and *recA* expression was reduced by 80.3, 93.0, and 52.8%, respectively, in WT, *jar1*, and *NahG* compared with the Mock+*Pst* group (Fig. 3A and B). COS induced resistance is less effectively in *NahG* than in either WT or *jar1*, further confirmed that SA-mediated pathway plays more critical role than JA pathway in DC3000 resistance.

COS pretreatment induced resistance in WT, *jar1*, and also, although to a lesser degree, in *NahG* and *sid2* plants, implying that SA- and JA-mediated pathways play redundant roles in COS-induced resistance.

COS pretreatment enhances the expression of SA- and JA-related genes in *Arabidopsis*.

To investigate the status of the SA and JA signaling pathways in COS-pretreated *Arabidopsis* plants, the expression of SA (*PR1*, *PR2*, and *PR5*) and JA (*PDF1.2* and *VSP2*) pathway-related genes were detected.

In WT plants, the expression of *PR1*, *PR2*, and *PR5* in COS-pretreated inoculated plants were 3.6, 42.4, and 34.7-fold higher than in the Mock+*Pst* group, respectively, suggesting that the SA signaling pathway is highly activated in COS-pretreated plants (Fig. 4 A to C). Moreover, JA signaling pathway-related genes were also induced, the expression of *PDF1.2* and *VSP2* in the COS+*Pst* group were enhanced 52.5 and 22.4-fold compared with the Mock+*Pst* group (Fig. 5A and B). Thus, COS induced resistance to DC3000 in WT by activating both SA and JA-mediated pathways.

In *jar1*, the SA signaling pathway-related genes (*PR1*, *PR2*, and *PR5*) were upregulated 10.0, 16.9, and 25.3-fold, respectively, in COS-pretreated plants as compared with the Mock+*Pst* group (Fig. 4A to C). COS pretreatment did not show similar upregulation in *PDF1.2* and *VSP2* in the *jar1* mutant, due to the blocked JA pathway (Fig. 5A and B). Thus, COS induces resistance to DC3000 in *jar1* by activating the SA-dependent signaling pathway.

Furthermore, in the *jar1* mock group (Mock+*Pst*), the expression of *PR1*, *PR2*, and *PR5* was 3.5, 11.2, and 5.1-fold higher, respectively, than in the WT Mock group. This implies that the SA signaling pathway was activated in *jar1* due to the blocked JA pathway. Moreover, after COS pretreatment (COS+*Pst* group), *PR1*, *PR2*, and *PR5* expression in *jar1* was 9.9, 4.5, and 3.7-fold higher than in WT. The highly activated SA signaling pathway might be responsible for the reduced DC3000 infection in COS-pretreated *jar1* plants compared with WT. A highly activated SA pathway in *jar1* was enough for a full response of COS-induced resistance to DC3000, thus, the JA pathway plays a redundant role.

COS pretreatment highly enhanced the expression of SA signaling pathway-related genes in WT and *jar1* but not in *NahG* (Fig. 4A to C). *NahG* plants have an introduced bacterial *nahG* gene that encodes salicylate hydroxylase, which degrades SA; thus, *NahG* plants accumulate very little amount of SA. Therefore, COS pretreatment has no induction effect on *PR1*, *PR2*, and *PR5* in *NahG*. However, the expression of *PDF1.2* and *VSP2* was upregulated by 1130.0- and 12.4-fold, respectively, in the COS pretreatment group (COS+*Pst*) compared with the Mock+*Pst* group (Fig. 5A and B). Thus, COS induces resistance to DC3000 in *NahG* by activating the JA-dependent signaling pathway, revealing the redundant role of SA pathway in COS-induced resistance to DC3000.

Fig. 3. A, The expression of the marker genes of *Pseudomonas syringae* pv. *tomato* DC3000 in infected leaves *avrPtoB* and **B**, *recA*. *Arabidopsis* pretreated with water or 50 mg of chitosan oligosaccharide (COS) per liter, then inoculated with DC3000 are noted as Mock + *Pst* or COS + *Pst*, respectively. Each group contained more than 30 *Arabidopsis* plants. The expression values of the individual genes were normalized using *actin8* as an internal standard. Values are the means $\pm$ standard deviation from three technical replicates. Similar results were obtained in three biological replicates. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

Moreover, expression of both PDF1.2 and VSP2 was higher (47.0- and 2.8-fold, respectively) in the *NahG* mock group compared with WT and, additionally, was upregulated (1,010.4- and 1.6-fold, respectively) in the COS-pretreated group compared with the same treatment group in WT. These results suggest that the JA pathway is highly activated in *NahG*. However, in contrast to *jar1*, COS pretreatment was less effective at reducing DC3000 infection in *NahG*, further confirming that the SA pathway plays a more critical role than the JA pathway in DC3000 resistance.

COS pretreatment increases SA- and JA-related protein content in *Arabidopsis*.

To verify the qPCR results, the SA- and JA-related proteins NPR1 and COI1 were detected by Western blot (WB). NPR1 is a key positive regulator of the SA signaling pathway, and the activation by SA usually induces the expression of pathogenesis related genes (Mou et al. 2003; Niu et al. 2016). The WB results demonstrate that following DC3000 inoculation, COS pretreatment significantly enhanced NPR1 content in leaves of WT and *jar1*, as compared with the mock group, implying an activated SA-dependent signaling pathway (Fig. 6A).

As expected, this protein content increase after COS pretreatment did not occur in *NahG*, although the basal protein content of NPR1 is much higher than in WT and *jar1*, possibly due to a highly activated JA signaling pathway in the *NahG* mutant. It is generally accepted that NPR1 plays a complex role in SA and JA pathway crosstalk, and evidence is accumulating that nuclear-localized NPR1 regulates the SA-mediated defense response, whereas cytosolic NPR1 is required for JA-dependent pathway activation (Nie et al. 2017; Pieterse et al. 2014; Ramírez et al. 2010). The higher basal level of NPR1 in *NahG* may be due to an overactivated JA signaling pathway accumulating cytosolic NPR1.

COI1 is an essential component of the JA receptor, and its content varies depending on JA levels (Wasternack and Hause 2013; Xie et al. 1998). As expected, COI1 content in the COS+ *Pst* group is enhanced in WT and *NahG* compared with the Mock+*Pst* group; however, there was no significant increase in *jar1*, suggesting that the JA signaling pathway is activated after COS pretreatment in WT and *NahG* (Fig. 6B). Basal amounts of COI1 in *jar1* are lower than that in WT and *NahG*, due to an impaired JA biosynthesis pathway in *jar1*.

COS pretreatment upregulates SA and JA levels in *Arabidopsis*.

To further confirm the qPCR data and WB results, we measured the SA and JA content by liquid chromatography-mass spectrometry (LC-MS) in the different *Arabidopsis* genotypes under different treatments.

In the mock group without DC3000 infection, the basal SA content in *jar1* was 1.5-fold higher than WT, implying the highly activated SA pathway in *jar1*. The basal SA was 2.2-fold lower, and JA content was 4.2-fold higher in *NahG*, respectively, compared with the WT mock groups, suggesting the suppressed SA pathway and enhanced JA pathway in *NahG*.

And as expected, the COS pretreatment significantly enhanced the SA and JA content in WT by 1.8- and 1.4-fold, respectively, compared with the mock treatment group (Mock+ *Pst*). Furthermore, COS pretreatment also increased SA content in *jar1* (by 1.9-fold) and JA content in *NahG* (by 1.3-fold) (Fig. 7A and B). These results further revealing that both SA- and JA-mediated signaling pathways participate in COS-induced resistance to DC3000 in *Arabidopsis*.

DISCUSSION

COS is an effective plant immunity elicitor that is able to induce resistance to pathogens during infection in various

plants, according to previous studies. This includes tobacco-TMV, *Arabidopsis*-TMV, tomato-*Botrytis cinerea*, grapevine-*Plasmopara viticola*, and rice-SRBSDV (Jia et al. 2016; Yang et al. 2017; Yin et al. 2010; Zhang and Chen 2009). As MAMPs, the perception of COS by the host cell leads to the activation of a complicated signaling network, including the transient accumulation of signal molecules such as Ca^{2+} , NO, and H_2O_2 , the upregulation of the mitogen-activated protein kinase signaling cascade pathway, and activation of the SA and JA-mediated signaling pathways (Jia et al. 2016; Poncini et al. 2017; Yang et al. 2017; Yin et al. 2013). The COS induction mechanism is very complex and requires further investigation.

In this study, the potential defense signaling pathways in COS-induced resistance to DC3000 were investigated by using WT, *jar1*, *NahG*, and *sid2*. We found that both SA- and

Fig. 4. A, The expression of salicylic acid-mediated pathway related genes PR1, **B**, PR2, and **C**, PR5. *Arabidopsis* pretreated with water or 50 mg of chitosan oligosaccharide (COS) per liter, then inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 are noted as Mock + *Pst* or COS + *Pst*, respectively. Each group contained more than thirty *Arabidopsis* plants. The expression values of the individual genes were normalized using *actin8* as an internal standard, then compared with mock-treated wild type (WT). Values are the means $\pm$ standard deviation from three technical replicates. Similar results were obtained in three biological replicates. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

JA-mediated pathways play positive roles in reducing infection in *Arabidopsis* after DC3000 inoculation. The crosstalk between SA and JA signaling pathways is complex, and they influence each other through synergistic and antagonistic interactions. In many cases, SA and JA signaling pathways act antagonistically. For example, activation of SA biosynthesis will inhibit JA biosynthesis or signaling in many plant-pathogen interactions (Thaler et al. 2012; Zhang et al. 2015). In *Arabidopsis*-TMV or tobacco-TMV interactions, SA functions as a positive regulator of TMV resistance. According to previous studies, TMV infection or COS pretreatment enhanced SA content while decreasing JA accumulation (Jia et al. 2016).

However, in *Arabidopsis*-DC3000 interaction, both SA and JA signaling pathways were activated; thus, they may work in independent or cooperative manners without obvious antagonism. Betsuyaku et al. (2018) found that, although the SA and JA pathways are both activated in the *Arabidopsis*-DC3000 interaction, they are active in different domains around the infection site. While SA accumulates in the cells directly around DC3000 infection sites, the JA pathway is activated in cells surrounding SA-active cells (Betsuyaku et al. 2018).

DC3000 resistance was more obviously impaired in *NahG* and *sid2* than *jar1*, revealing the more critical role of the SA-mediated pathway than JA pathway in *Arabidopsis* defense to DC3000. According to previous studies, virulence systems of DC3000 usually promote infection by targeting the JA signaling pathway. For example, DC3000 is able to synthesize the polyketide toxin coronatine, which structurally mimics JA,

interfering with the JA-mediated signaling pathway (Staswick and Tiryaki 2004; Xin and He 2013; Zhao et al. 2003). This may be responsible for the major role of the SA signaling pathway in limiting DC3000 infection in *Arabidopsis*.

COS pretreatment activated both SA- and JA-mediated pathways in WT *Arabidopsis*, which all worked positively in *Arabidopsis* defense to DC3000. However, SA and JA pathways play redundant roles in COS-induced resistance to DC3000 in SA or JA pathway-deficient mutants due to the highly activated JA or SA pathway after COS pretreatment. This induction effect of COS that activates different signaling pathways under different situations makes COS work effectively in multiple plant-pathogen interactions. This phenomenon also appeared in other elicitor-treated plants, according to previous studies, such as plant growth-promoting fungus *Penicillium simplicissimum* GP17-2 (Hossain et al. 2007) and *Phoma* sp. strain GS8-1 (Hossain et al. 2008). GP17-2 or GS8-1 pretreatment were fully protective against DC3000 in *jar1*, similar to WT, and also worked effectively in *NahG* and *npr1*, although to a lesser degree. Furthermore, treatment with GP17-2 or GS8-1 resulted in enhanced expression of SA- and JA-related genes and activated both SA and JA pathways in WT, just like COS-induced resistance.

Generally, the SA signaling pathway is associated with biotroph resistance, whereas the JA/ET signaling pathway is involved in defending against necrotrophs (Robert-Seilanian et al. 2007). COS-induced resistance in the *Arabidopsis*-DC3000 (hemibiotrophic pathogen) interaction is mainly achieved by activating both SA- and JA-mediated pathways. This is quite different from *Arabidopsis*-TMV (biotrophic pathogen) interactions, since COS only activates the SA signaling pathway (Jia et al. 2016). Further, in tomato, rice, and rapeseed, COS pretreatment activates the JA-dependent signaling pathway, which results in resistance to necrotrophic pathogens (Doares et al. 1995; Rakwal et al. 2002; Yin et al. 2013). Based on these results, COS pretreatment activates different defense pathways in different plants under infection by different pathogens, activating one or both the SA-dependent and JA-dependent, signaling pathways. Thus, COS functions as an effective and broad-spectrum plant immunity elicitor in multiple plant-pathogen interactions.

The broad-spectrum disease control effects demonstrated by COS pretreatment in different plant-pathogen interactions are ubiquitous among plant immunity elicitors. For example, the well-studied pathogen-associated molecular pattern flg22 was found to induce resistance in *Arabidopsis* against DC3000 by

Fig. 5. A, The expression of jasmonic acid-mediated signaling pathway-related genes PDF1.2 and **B**, VSP2. *Arabidopsis* pretreated with water or 50 mg of chitosan oligosaccharide (COS) per liter, then inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 are noted as Mock + Pst or COS + Pst, respectively. Each group contained more than thirty *Arabidopsis* plants. The expression values of the individual genes were normalized using *actin8* as an internal standard, then compared with mock-treated wild type (WT). Values are the means $\pm$ standard deviation from three technical replicates. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

Fig. 6. A, The amount of salicylic acid- and jasmonic acid-mediated pathway related proteins, NPR1 and **B**, COI1. *Arabidopsis* pretreated with water or 50 mg of chitosan oligosaccharide (COS) per liter, then inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 are noted as Mock + Pst or COS + Pst, respectively. This was a representative experiment and was independently repeated three times using different samples from independent treatment.

activating both SA- and JA-dependent signaling pathways (Navarro et al. 2004; Wang et al. 2018). Additionally, flg22 induced resistance to *Golovinomyces cichoracearum* in *Arabidopsis* by activating the SA signaling pathway (Lu et al. 2015). In addition to flg22, other plant immunity regulators, such as harpin, *Bacillus cereus* AR156, and plant growth-promoting fungi also showed similar induction responses (Chuang et al. 2014; Hossain et al. 2008; Jiang et al. 2016; Nie et al. 2017; Wang et al. 2018).

Certainly, signaling pathways in addition to SA and JA pathways may also be involved in COS-induced resistance. COS induction mechanism is complex needs further investigation.

MATERIALS AND METHODS

Plant materials and treatment.

COS was obtained from Dalian GlycoBio Co. Ltd. (Dalian, China), with a degree of polymerization from 2 to 10 and a degree of deacetylation of 95%. *Arabidopsis* ecotype Columbia (Col-0, WT) and mutant lines, *NahG*, *sid2* (SA-

deficient mutants), and *jar1* (JA-deficient mutant) were used for experiments.

Arabidopsis plants were cultivated as described previously (Jia et al. 2016). Thirty-day-old plants, grown in soil, were used for experiments. To investigate the optimal COS pretreatment time, WT plants were sprayed with a 50-mg/liter COS solution at 3, 2, and 1 days before and 0, 1, 2, and 3 days after DC3000 inoculation. Plants inoculated with DC3000 without any COS treatment served as control.

WT, *jar1*, *NahG*, and *sid2* plants were evenly assigned into either the Mock group (spraying water 3 days before inoculating with 10 mM MgSO₄), the Mock+Pst group (spraying water 3 days before inoculating with DC3000), or the COS+Pst group (spraying a 50 mg/liter COS solution 3 days before inoculating with DC3000). Each treatment group contained at least 30 plants, and each experiment was repeated at least three times. The plants were inoculated with DC3000 according to the method described as below.

DC3000 inoculation.

DC3000 was grown in King's B medium (per milliliter, 10 mg of protease peptone, 1.5 mg of K₂HPO₄, 15 mg of glycerol) supplemented with 25 mg of rifampicin per milliliter, at 28°C. Rosette leaves of *Arabidopsis* plants were inoculated with 10 mM MgSO₄ or DC3000 suspensions in 10 mM MgSO₄, using a 1-ml syringe (without a needle). The bacterial dose infiltrated into the leaves was 1×10^5 CFU per square centimeter of leaf area (equivalent to an optical density at 600 nm = 0.002).

According to the proportion (S) of necrotic lesion area on leaves, the symptoms of infected leaves were divided into four levels: 1 ($0 < S < 0.25$), 2 ($0.25 < S < 0.5$), 3 ($0.5 < S < 0.75$), and 4 ($0.75 < S < 1$). The symptom data were obtained 4 days after DC3000 infection, using the following formula: Disease index (%) = $(\sum(\text{level} \times \text{leaves in the level}) \div \text{total leaves} \div \text{the highest level}) \times 100$. Inoculated leaves were collected at 4 days after inoculation, were weighed, were quickly frozen in liquid nitrogen, and were kept in -80°C for further experiments.

Gene expression analysis by qPCR.

Total RNA was extracted using a TRIzol reagent kit (Lab-lead) and was quantified by the Scan Drop100 (Analytik Jena AG). Equal amounts of RNA were reverse transcribed into cDNA with a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific). The diluted newly transcribed cDNA was used for gene expression detection by qPCR (qTOWER 2.2, Analytik Jena AG). The reaction volumes were 20 µl, consisting of cDNA dilutions, qPCR MasterMix, and gene-specific primers (Table 1). The gene expression data were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

WB detection.

Plant protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described previously (Jia et al. 2016). Total protein (50 µg) was loaded into the SDS-PAGE (10%) gels. Separated

Fig. 7. A, The relative level of salicylic acid (SA) and **B,** jasmonic acid (JA) in different mutants. *Arabidopsis* pretreated with water, then inoculated with 10 mM MgSO₄ is noted as Mock. *Arabidopsis* pretreated with water or 50 mg of COS per liter, then inoculated with DC3000 are noted as Mock + Pst or COS + Pst, respectively. Values were presented as means $\pm$ standard deviation from three independent measurements. One asterisk (*) indicates significant difference at $P < 0.05$, two (**) at $P < 0.01$, and three (***) at $P < 0.001$.

Table 1. Primers used for quantitative polymerase chain reaction

Gene	Locus ID	Forward	Reverse
<i>actin8</i>	AT1G49240	CACATGCTATCCTCCGTCTC	CACTTGTCCTCGGGTAAT
<i>recA</i>	—	CGGCAAGGGTATCTACCTCA	CTTTGCAGATTTCGGGGTTA
<i>avrPtoB</i>	—	CACTTGTCCTCGGGTAAT	CATGAACGCCAGGTCCTTAT
<i>PR1</i>	AT2G14610	AATGCTCAAGATAGCCCCACAAG	AATAAGTCACCGCTACCCAG
<i>PR2</i>	AT3G57260	AAAACGGAGGCCGACAAG	CGGAGGAGACGTATCAGTGGT
<i>PR5</i>	AT1G75040	ATTGACTCCAGGTGCTTCCC	GCCGTAGAAATCTTTGCCG
<i>PDF1.2</i>	AT5G44420	TCACCCTTATCTTCGCTGCTC	ATGTCCCACTTGCTTCTCG
<i>VSP2</i>	AT2G06530	TGTGAACAGGCAGATGAACC	GCGATACCGATCTCGTCAA

proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% (wt/vol) nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl, 0.05% Tween 20) for 3 to 4 h at room temperature.

Membranes were cut into two parts at 55 kDa, were probed with different antibodies, including NPR1 (regulatory protein, 66 kDa), COI1 (coronate insensitive 1, 67 kDa), and plant actin (40 kDa). The blot was incubated in the primary antibody of NPR1 or COI1 (Agrisera), at a dilution of 1:500, overnight at 4°C, followed by washing with TBST buffer three times. The blot was then incubated with the secondary antibody, HRP AffiniPure goat antirabbit immunoglobulin G (IgG) (Earthox), at a dilution of 1:1,000, for 2 h at room temperature. For plant actin detection, the membrane was probed with antiplant actin mouse monoclonal antibody (1:2,000) (Earthox) and HRP AffiniPure goat antirabbit IgG (1:2,000) (Earthox) as a secondary antibody.

SA and JA quantification by LC-MS.

The extraction and quantification of SA and JA in infected leaf samples were carried out by using the LC-MS method described previously with some modification (de Sá et al. 2014). SA and JA standards were obtained from Sigma. Chromatography conditions were modified using a hypercarb column (Thermo Fisher) (150 × 2.1 mm, 5 µm) at 35°C, and an elution gradient of 3 ml of formic acid per liter, adjusted to pH 9.0 with ammonium hydroxide (A) and acetonitrile (B) for 20 min at a flow rate of 0.2 ml/min. The details of gradient conditions were as follows: 0 min, 90% A + 10% B; 10 min, 40% A + 60% B; 14 min, 40% A + 60% B; 14.1 min, 90% A + 10% B; and 20 min, 90% A + 10% B. Q-trap 5500 (Ab Sciex) with optimized collision energy was used for SA and JA detection.

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