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L-Histidine Induces Resistance in Plants to the Bacterial Pathogen *Ralstonia solanacearum* Partially Through the Activation of Ethylene Signaling

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Wilt disease in plants, which is caused by the soil-borne bacterial pathogen *Ralstonia solanacearum*, is one of the most devastating plant diseases. We previously detected bacterial wilt disease-inhibiting activity in an extract from yeast cells. In the present study, we purified this activity and identified one of the substances responsible for the activity as the amino acid histidine. The exogenous application of L-histidine, but not D-histidine, inhibited wilt disease in tomato and Arabidopsis plants without exhibiting any antibacterial activity. L-Histidine induced the expression of genes related to ethylene (ET) biosynthesis and signaling as well as the production of ET in tomato and Arabidopsis plants. L-Histidine-induced resistance to *R. solanacearum* was partially abolished in *ein3-1*, an ET-insensitive Arabidopsis mutant line. Resistance to the fungal pathogen *Botrytis cinerea*, which is known to require ET biosynthesis or signaling, was also induced by exogenously applied L-histidine. These results suggest that L-histidine induces resistance to *R. solanacearum* and *B. cinerea* partially through the activation of ET signaling in plants.

Keywords: Disease resistance • Ethylene • L-Histidine • Plant activator • *Ralstonia solanacearum*.

Abbreviations: ACS, 1-aminocyclopropane-1-carboxylic acid synthase; CPK, calcium-dependent protein kinase; dpi, days post-inoculation; ET, ethylene; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; NMR, nuclear magnetic resonance; PR, pathogenesis-related; SA, salicylic acid; SPE, solid-phase extraction; TMV, Tobacco mosic virus.

Introduction

The defense system of plants in response to attacks by pathogens has mainly been classified into constitutive and inducible protective responses. Defense compounds, such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA), nitric oxide and reactive oxygen species, play important roles in all of these responses (Robert-Seilaniantz et al. 2011). These compounds regulate multiple signal pathways that lead to various physiological changes in plant growth and development as well as disease resistance responses (Cheng et al. 2007, Ziegler and Facchini 2008, Vogt 2010, Kazan and Lyons 2014).

Chemical compounds that induce disease resistance in plants have recently been attracting increasing attention due to their potential to reduce the environmental burden associated with crop protection. These compounds, so-called plant activators, are characterized by their capability to induce resistance to a broad range of diseases without exhibiting direct antimicrobial activity. Many compounds have been identified as plant activators or shown to exhibit plant activator-like activity (Watanabe et al. 1979, Vernooij et al. 1995, Friedrich et al. 1996, Zimmerli et al. 2001, Noutoshi et al. 2012, Walters et al. 2013, Narusaka et al. 2015, Sun et al. 2015). Most of the plant activators identified to date are compounds that act on SA biosynthetic, metabolic or signaling pathways. For example, probenazole, a thiazole compound, induces resistance to fungal, bacterial and viral pathogens in plants through the activation of SA biosynthesis (Watanabe et al. 1979, Yoshioka et al. 2001, Nakashita et al. 2002, Iwai et al. 2007). Imprimatins have been identified as compounds that potentiate pathogen-induced cell death and induce resistance to bacterial pathogens through the accumulation of SA by inhibiting the activity of SA glucosyltransferase, an enzyme that converts SA to its metabolite SA-*O*-β-D-glucoside (Noutoshi et al. 2012). Dehydroabietinal (DA), an abietane-type diterpene, has been shown to activate systemic acquired resistance (SAR) in plants, which requires the accumulation of SA and signaling (Chaturvedi et al. 2012). In contrast, information on plant activators for JA/ET biosynthetic, metabolic or signaling pathways is limited.

Ralstonia solanacearum, a soil-borne bacterial pathogen with a wide host range, invades roots and multiplies in vascular systems, resulting in the wilting of the host plant (Buddenhagen 1964, Hayward 1991, Vasse et al. 1995, Seile et al. 1997). Only a

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few plant activators or plant activator-like compounds have been identified for this disease, including riboflavin (vitamin B2) and silicon (Liu et al. 2010, Ghareeb et al. 2011); however, the mechanism underlying the induction of bacterial wilt disease resistance by these compounds has not yet been elucidated in detail. Sclareol, a labdane-type diterpene, was isolated from tobacco as an inducer of *R. solanacearum* resistance by bioassay-guided fractionation, and sclareol-induced resistance was shown to depend partially on ET biosynthesis and signaling pathways (Seo et al. 2012). Sclareol was also demonstrated to induce resistance to a plant parasitic root-knot nematode (*Meloidogyne*) via ET-dependent and -independent defense mechanisms (Fujimoto et al. 2015).

Many host factors involved in the resistance responses of plants to *R. solanacearum* have been identified. In Arabidopsis (*Arabidopsis thaliana*), *RRS1-R*, a resistance gene that encodes a nucleotide-binding site leucine-rich repeat-type protein, regulates resistance to *R. solanacearum*. A previous study reported that *RRS1-R*-mediated resistance was partially dependent on SA (Deslandes et al. 2002). Phytohormones, including ABA and ET, mitogen-activated protein kinases (MAPKs) and a heat shock protein have been implicated in the defense responses of plants to *R. solanacearum* (Zhang et al. 2004, Hase et al. 2006, Hernández-Blanco et al. 2007, Maimbo et al. 2007, Chen et al. 2009). These findings suggest that multiple factors are involved in host defenses to *R. solanacearum*.

In the course of investigating plant activators for *R. solanacearum*, we found that a liquid fertilizer originating from a yeast cell extract exerted inhibitory effects on the development of bacterial wilt disease in tobacco plants (Obara et al. 2007). In the present study, we describe the isolation and identification of histidine as a bacterial wilt disease-inhibiting compound from a yeast cell extract and also report the results of analyses on the mechanisms underlying histidine-induced resistance to *R. solanacearum* as well as the effects of histidine on other plant diseases.

Results

Isolation of a bacterial wilt disease-inhibiting compound from the yeast cell extract

In order to investigate the bacterial wilt disease-inhibiting activity detected in the yeast cell extract, we developed a bioassay using young tomato plants and test tubes. The roots of hydroponically grown young tomato plants were soaked in the compound to be examined in a test tube for 48 h and inoculated with *R. solanacearum* 8107S, a strain with high pathogenicity towards tomato. Resistance was assessed by determining the severity of wilt in the aerial parts of the inoculated plants. Using this assay, we confirmed that a treatment with AgrevoEX, a commercially available liquid fertilizer originating from a yeast cell extract, inhibited the development of bacterial wilt disease (**Supplementary Fig. S1**). AgrevoEX was fractionated into two parts of mol. wts. >3,000 and <3,000, and each fraction was then subjected to the bioassay (**Supplementary Fig. S2A**). Activity for inhibiting the development of wilt disease was found in the fraction with mol. wt. <3,000 (**Supplementary Fig. S2B**). The active fraction was fractionated by chromatography on an aminopropyl-based solid-phase extraction (SPE) cartridge column using an acetonitrile–H_2O solvent system. Two fractions exhibited inhibitory activity, and the fraction with the highest activity, which was eluted with 30% acetonitrile in H_2O (**Supplementary Fig. S2C**), was subjected to HPLC. Inhibitory activity was detected in four fractions with retention times of ≥11 min on amide-based normal-phase HPLC (**Supplementary Fig. S3A, B**); the fraction with a retention time of 19–23 min exhibited significantly high activity, while the remaining three fractions exhibited weak activity. The most active fraction was purified further and a peak corresponding to the highest activity with a retention time of 52.0 min was collected (**Supplementary Fig. S3C, D**). Resonances in ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra for the peak were assigned to histidine.

We examined the effects of L-histidine, a natural form of histidine, on the inhibition of bacterial wilt disease using adult tomato plants. The roots of tomato plants with 5–6 true leaves were soaked in different concentrations (0.1, 0.5, 1, 5 or 10 mM) of L-histidine for 48 h and inoculated with strain 8107S. In plants pre-treated with H_2O alone as a control, wilt symptoms began to appear on their aerial parts 4 days post-inoculation (dpi) and developed rapidly thereafter, and most of the inoculated plants wilted by 8–10 dpi (**Fig. 1A**, 0 mM). The development of wilt symptoms was inhibited by L-histidinee at 5 or 10 mM (**Fig. 1A; Supplementary Fig. S4**). Consistent with the inhibited development of wilt symptoms, the growth of strain 8107S was inhibited in L-histidine-pre-treated plants (**Fig. 1B**). A treatment period of ≥48 h was required for tomato roots to manifest the inhibitory effects of L-histidine on the development of wilt symptoms (**Supplementary Fig. S5**).

Most amino acids have two optical isomers, the D- and L-forms. We investigated whether D-histidine has the same inhibitory effects on bacterial wilt disease as L-histidine. D-Histidine at 10 mM did not exhibit any inhibitory activity (**Fig. 1C**). In order to clarify whether the inhibition of bacterial wilt disease by L-histidine is due to its antimicrobial activity, strain 8107S was incubated with 5 or 10 mM L-histidine, and bacterial growth in vitro was measured. An incubation with L-histidine did not alter the growth of strain 8107S (**Fig. 1D**). We also examined whether L-histidine alters the pathogenicity of *R. solanacearum* by pre-incubating strain 8107S with L-histidine or H_2O for 48 h, followed by an inoculation of the bacterial suspension on tomato roots (**Fig. 1E**). No significant difference was observed in the development of disease symptoms between the treatments (**Fig. 1F**).

In order to examine whether L-histidine is responsible for the bacterial wilt disease-inhibiting activity detected in AgrevoEX, we measured the endogenous content of L-histidine in this liquid fertilizer. A 500-fold diluted working solution of AgrevoEX contained approximately 0.2 mmol l^{-1} L-histidine (**Supplementary Fig. S6**), a concentration which is a 20th of the minimum effective concentration (5 mM) of exogenously applied L-histidine. This suggests that L-histidine is not the sole

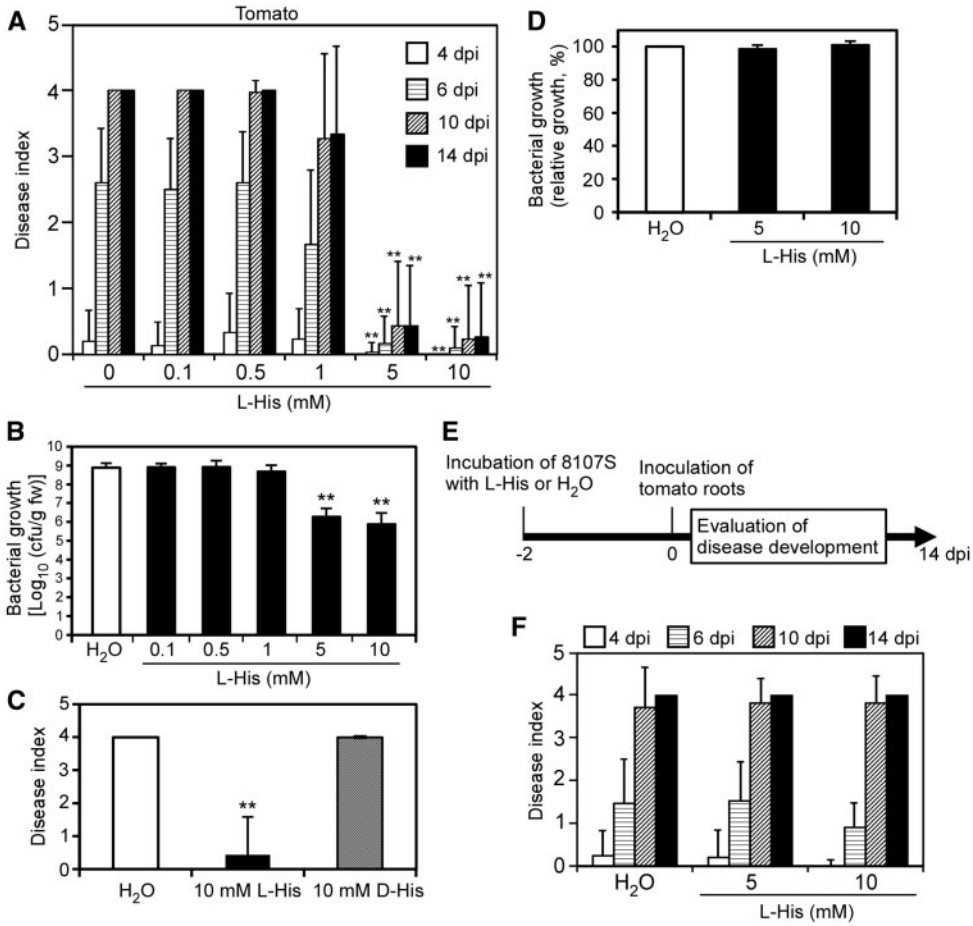

Fig. 1 Effects of exogenously applied histidine on the inhibition of bacterial wilt disease in tomato. The roots of tomato plants were soaked in the indicated concentrations of L-histidine or D-histidine for 48 h, inoculated with strain 8107S, and subjected to an evaluation of disease resistance. (A) Dose-dependent effects of L-histidine and the development of disease symptoms. (B) Dose-dependent effects of L-histidine on bacterial growth in inoculated plants 7 dpi. (C) Effects of D-histidine on the inhibition of wilt disease. The severity of wilt symptoms 14 dpi is shown. (D) Effects of L-histidine on the growth of strain 8107S in vitro. Data, which are expressed as values relative to the control set to 100%, are the means ± SD of three independent measurements. (E) Timeline of the pathogenicity assay. (F) Effects of L-histidine on the pathogenicity of strain 8107S. 8107S, cultured in medium containing H_2O instead of L-histidine, was used as a control. Data for (A), (B), (C) and (F) are the means ± SD of three independent assays, each performed with eight plants per treatment. Asterisks indicate significant differences from the H_2O (0 mM) treatment (**$P < 0.01$).

substance responsible for the bacterial wilt disease-inhibiting activity detected in AgrevoEX. However, there has been no report on the role of exogenously applied L-histidine on the induction of *R. solanacearum* resistance in plants.

The results from the antimicrobial and pathogenicity assays suggest that the inhibition of bacterial growth by L-histidine is due to host defense responses induced in the plant after the treatment with the chemical. In an attempt to elucidate the defense mechanism induced by L-histidine in more detail, we moved our assay system to *A. thaliana*. We grew Arabidopsis plants under two different growth conditions, i.e. a pot system with peat moss pots and a hydroponic system; the former system was used for the *R. solanacearum* resistance assay, whereas the latter system was used for molecular and biochemical analyses. We investigated whether exogenously applied L-histidine inhibits wilt disease caused by *R. solanacearum* in Arabidopsis. The roots of Arabidopsis Columbia (Col-0) plants grown in the pot system were soaked in 10 mM L-histidine and inoculated with RS1000, a strain that causes wilt disease in Col-0. The L-histidine treatment inhibited the development of disease symptoms and growth of the strain (**Fig. 2A, B**).

In order to identify the phytohormone involved in histidine-induced resistance to *R. solanacearum*, we analyzed the expression of genes related to phytohormone signaling or responses in Arabidopsis. The roots of hydroponically grown Col-0 plants were soaked in 10 mM L-histidine or H_2O as a control for 48 h, and induction kinetics in the treated roots were assessed for SA signaling (*AtPR-1* and *AtBGL2*), JA signaling (*AtVSP2* and *AtLOX2*), ET/JA signaling (*AtChiB* and *AtPDF1.2*) and ABA-responsive (*AtABF1* and *AtPP2CA*) genes. We also subjected the untreated leaves of plants with histidine-treated roots to the same kinetic analysis in order to examine whether exogenously applied L-histidine systemically induces host defenses. L-Histidine enhanced the accumulation of transcripts for

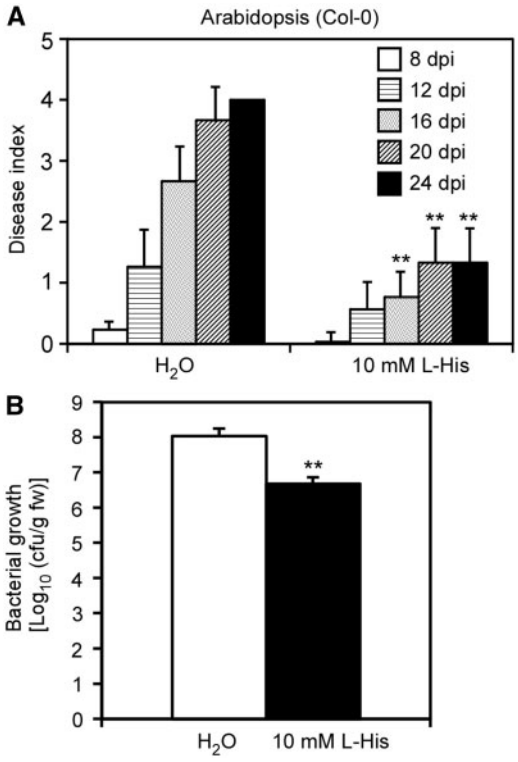

Fig. 2 Effects of exogenously applied l-histidine on the inhibition of bacterial wilt disease in Arabidopsis. The roots of Arabidopsis (Col-0) plants were soaked in 10 mM L-histidine or H_2O for 48 h, inoculated with strain RS1000 and subjected to an evaluation of disease resistance. (A) Development of disease symptoms. (B) Bacterial growth at 7 dpi. Data are the means ± SD of three independent assays, each performed with eight plants per treatment. Asterisks indicate significant differences from the H_2O treatment (**$P < 0.01$).

AtPR-1 and *AtBGL2* in roots and leaves (**Fig. 3**). L-Histidine significantly enhanced the accumulation of transcripts for *AtLOX2* and *AtVSP2* in roots, whereas it had a negligible effect on the accumulation of transcripts for these genes in leaves. The levels of transcripts for *AtChiB* and *AtPDF1.2* were increased 13- and 6-fold, respectively, by L-histidine in roots. The enhanced accumulation of transcripts for *AtChiB* and *AtPDF1.2* was also observed in leaves. The treatment with L-histidine had little or no effect on the accumulation of transcripts for *AtABF1* and *AtPP2CA* in roots and leaves. A quantitative analysis of endogenous phytohormones also showed that the levels of SA, JA and ABA in roots and leaves were not changed by the treatment with L-histidine (**Supplementary Fig. S7**). Since difficulties are associated with measuring ET from roots without applying any wound stresses that allow its production to be induced, we measured ET released from whole Arabidopsis Col-0 plants with the roots soaked in a solution containing 10 mM L-histidine. L-Histidine enhanced ET emission in a dose-dependent manner (**Fig. 4A**). D-Histidine did not exhibit this enhancing activity; it decreased ET emission. 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) is a rate-limiting enzyme of ET biosynthesis. When the roots of hydroponically grown Col-0 plants were soaked in 10 mM L-histidine, the enhanced accumulation of transcripts for *AtACS2* and *AtACS6* was observed in L-histidine-treated roots and untreated leaves (**Fig. 4B**).

In order to examine further the involvement of phytohormones, we assayed L-histidine-induced resistance to *R. solanacearum* using Arabidopsis mutants defective in the perception of specific phytohormones. The L-histidine-induced inhibition of the growth of strain RS1000 was weaker in *ETHYLENE-INSENSITIVE3-1* (*ein3-1*), an ET signaling mutant, than in wild-type (Col-0) plants (**Fig. 5A**). None of the SA signaling [*NONEXPRESSER OF PR GENES 1-1* (*npr1-1*)], JA signaling [(*CORONATINE-INSENSITIVE1-1* (*coi1-1*)] or ABA signaling [*ABA-INSENSITIVE1-1* (*abi1-1*)] mutants exhibited this attenuated resistance. We also examined whether the L-histidine-induced expression of *AtChiB* and *AtPDF1.2* is altered in *ein3-1*. The roots of hydroponically grown wild-type or mutant plants were soaked in 10 mM L-histidine or H_2O for 48 h, and induction kinetics were assessed in the treated roots. In contrast to the wild type, *ein3-1* failed to accumulate transcripts for *AtChiB* and *AtPDF1.2* in response to L-histidine (**Fig. 5B**). Except for the expression of *AtPDF1.2* in *abi1-1*, *npr1-1*, *coi1* and *abi1-1* exhibited the marked accumulation of transcripts for these genes in L-histidine-treated roots; however, the degree of induction of their accumulation varied among mutants.

In order to gain an insight into the activation mechanism of ET biosynthesis by L-histidine, we first examined the possible involvement of MAPKs because they play a role in triggering ET biosynthesis through the direct phosphorylation of ACS (Li et al. 2012). In Arabidopsis, the activation of AtMPK3 and AtMPK6 triggers ET biosynthesis. We examined whether AtMKP3 and AtMPK6 are activated by L-histidine by measuring their enzymatic activities in the roots of hydroponically grown wild-type Arabidopsis plants after the treatment with L-histidine. The kinase activities of AtMPK3 and AtMPK6 using myelin basic protein (MBP) as a substrate were not increased by the treatment with L-histidine (**Supplementary Fig. S8**). Calcium-dependent protein kinases (CDPKs/CPKs) have also been implicated in the induction of ET biosynthesis in plants (Kamiyoshihara et al. 2010, Huang et al. 2013). The effects of L-histidine on the expression of *CPK* genes were tested for two representative stress-responsible Arabidopsis *CPK* genes, *AtCPK6* and *AtCPK11* (Mori et al. 2006, Zhu et al. 2007, Boudsocq et al. 2013). L-Histidine enhanced the accumulation of transcripts for *AtCPK6* and *AtCPK11* in the roots of wild-type plants (**Fig. 6**).

Since histidine was originally identified as an inducer of the resistance of tomato to *R. solanacearum*, we investigated whether the activation of ET biosynthesis and signaling by L-histidine occurs in this plant species. L-Histidine enhanced ET emission from hydroponically grown young tomato plants (**Fig. 7A**). Consistent with enhanced ET production, the greater accumulation of transcripts for *SlACO1*, a tomato gene encoding ACC oxidase, which converts ACC to ET, was observed in roots and leaves. L-Histidine enhanced the accumulation of transcripts for *SlPti5*, an ET response factor gene (Zhou et al. 1997), and *SlPRB-1b*, an ET-responsive

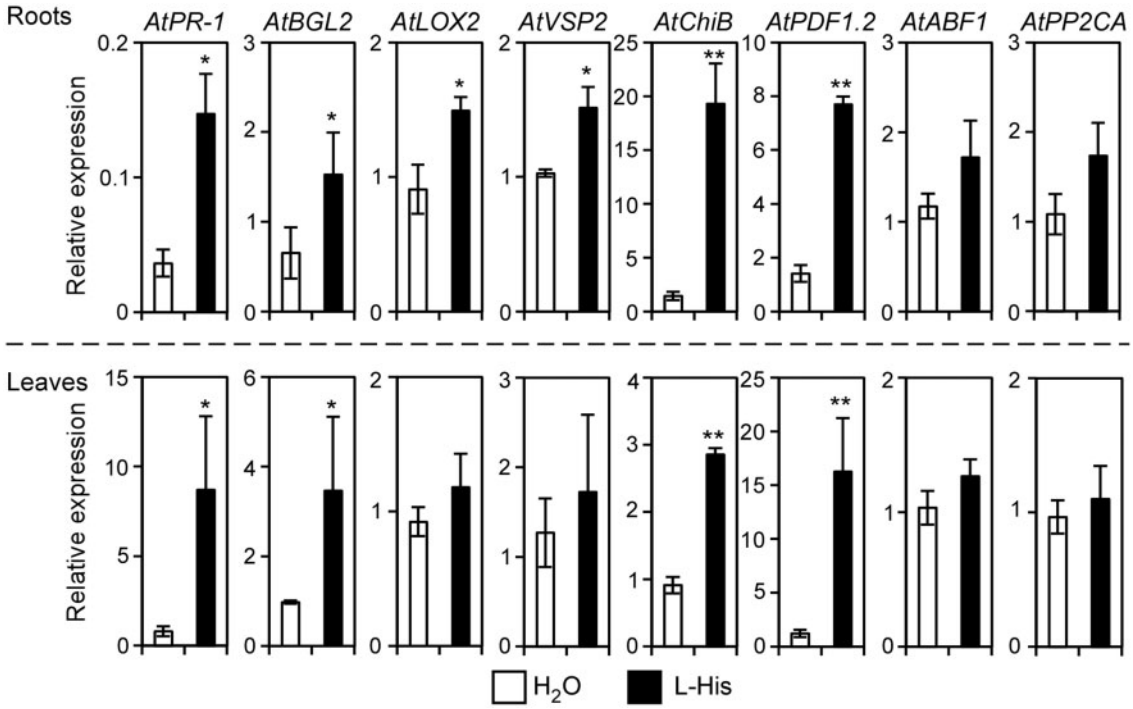

Fig. 3 Analysis of expression of defense-related genes in Arabidopsis plants after the treatment with L-histidine. Roots of hydroponically grown Arabidopsis (Col-0) plants were soaked in 10 mM L-histidine or H_2O for 48 h, and the expression levels of the indicated genes in the treated roots and untreated leaves were examined. Data are the mean ± SD of three independent measurements. Asterisks indicate significant differences from the H_2O treatment (*$P < 0.05$, **$P < 0.01$).

pathogenesis-related (PR) protein gene (Eyal et al. 1992), in roots, but not in leaves (**Fig. 7B**).

The inhibitory effects of exogenously applied L-histidine on other plant diseases was also tested for the fungal pathogen, *Botrytis cinerea* and the viral pathogen, *Tobacco mosaic virus* (TMV). Each host plant for *B. cinerea* or TMV was treated with 10 mM L-histidine or H_2O by soaking their roots in the chemical solution for 48 h, and the leaves of the treated host plant were inoculated with each specific pathogen. The sizes of lesions caused by *B. cinerea* were smaller when Arabidopsis plants were treated with L-histidine than with H_2O (**Fig. 8**). No significant difference was observed in the sizes of necrotic lesions caused by TMV or the amounts of viral RNA between treatments (**Supplementary Fig. S9**).

Discussion

In the present study, a bacterial wilt disease-inhibiting compound was isolated from a yeast cell extract and identified as histidine. The exogenous application of L-histidine did not exhibit any antimicrobial activity for *R. solanacearum* or alter its infectivity, suggesting that the inhibition of bacterial wilt disease by L-histidine was due to the host defense responses induced by this chemical. One of these responses may be mediated by an EIN3-dependent ET signaling pathway because L-histidine-induced resistance to *R. solanacearum* was attenuated in *ein3-1* mutant plants. Resistance was not completely abolished in *ein3-1*, suggesting that EIN3 is partially involved in L-histidine-induced resistance to *R. solanacearum*. Thus, the mechanism underlying L-histidine-induced resistance to *R. solanacearum* cannot be solely explained by ET. However, our results clearly indicate that ET is important for resistance. The importance of ET in host defenses to *R. solanacearum* has already been reported (Zhang et al. 2004, Hase et al. 2006, Seo et al. 2012). Further studies are required in order to identify EIN3-independent signal transduction pathways involved in L-histidine-induced resistance to *R. solanacearum*.

npr1-1, *coi1-1* and *abi1-1* mutants after the treatment with L-histidine exhibited the same degree of resistance as the wild type. This result suggests that L-histidine-induced resistance to *R. solanacearum* is independent of NPR1, COI1 and ABI1 or that they only make a minor contribution to this resistance. Despite having no induction effect on the accumulation of endogenous SA and JA, L-histidine enhanced the expression of SA- and JA-responsive genes in roots and leaves. One possible explanation is that the L-histidine-induced expression of these genes occurred through SA- and JA-independent signal transduction pathways.

AtMPK3 and AtMPK6 are involved in the induction of ET biosynthesis (Li et al. 2012). The treatment with L-histidine did not activate AtMPK3 or AtMPK6. Although the mechanisms by which exogenously applied L-histidine induce ET biosynthesis currently remain unclear, CDPKs/CPKs may be involved in the induction mechanism because the expression of *AtCPK6* and *AtCPK11* was induced by L-histidine. Further studies to

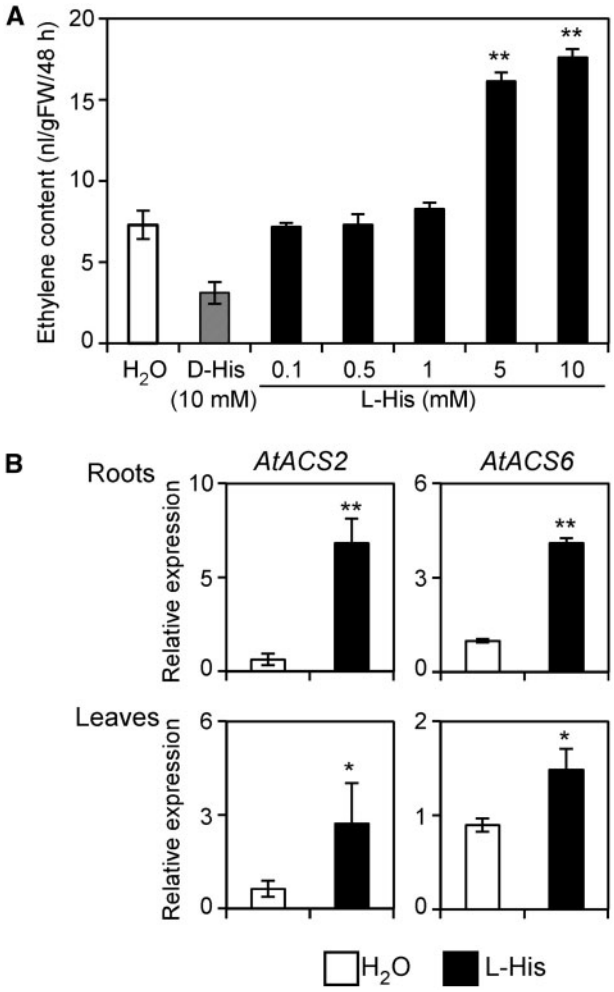

Fig. 4 Effects of exogenously applied histidine on the induction of ethylene biosynthesis in Arabidopsis. Hydroponically grown Arabidopsis (Col-0) plants were placed in a sealed vial containing the indicated concentrations of L-histidine, D-histidine or H_2O for 48 h and subjected to the measurement of ET (A) or an analysis of the expression of *AtACS* genes in the treated roots and untreated leaves (B). Data are the mean ± SD of three independent measurements. Asterisks indicate significant differences from the H_2O treatment (*$P < 0.05$, **$P < 0.01$).

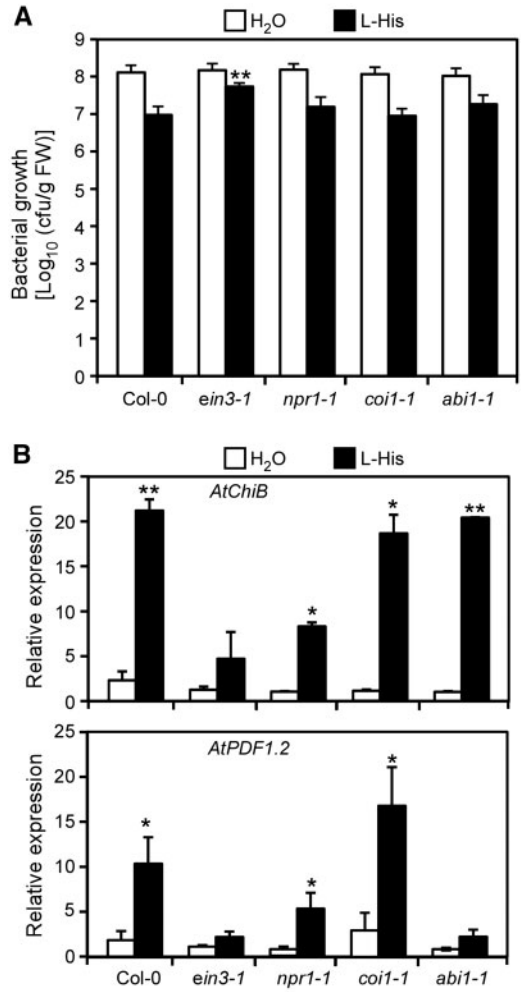

Fig. 5 Effects of L-histidine on the inhibition of bacterial wilt disease and expression of defense-related genes in Arabidopsis phytohormone mutants. (A) The roots of Arabidopsis Col-0 (WT) and mutant plants were soaked in 10 mM L-histidine or H_2O for 48 h and inoculated with strain RS1000. Bacterial growth was measured at 7 dpi. Data are the means ± SD of three independent assays, each performed with eight plants per treatment. Asterisks indicate significant differences from the L-histidine treatment in Col-0 plants (**$P < 0.01$). (B) Analysis of the expression of *AtChiB* and *AtPDF1.2* in the roots of hydroponically grown Arabidopsis Col-0 (WT) and mutant plants soaked in 10 mM L-histidinee or H_2O for 48 h. Data are the mean ± SD of three independent measurements. Asterisks indicate significant differences from the H_2O treatment (*$P < 0.05$, **$P < 0.01$).

investigate the mechanism of ET biosynthesis induction by L-histidine are required.

Exogenously applied L-histidine induced resistance to *B. cinerea*, which is a necrotrophic fungus that affects a wide variety of plant species. ET has been shown to play an important role in the resistance of Arabidopsis to *B. cinerea* (Thomma et al. 1999, Berrocal-Lobo et al. 2002). However, L-histidine had no effect on the induction of resistance to TMV, an obligate, biotrophic parasite. Thus, the ability of L-histidine to induce plant disease resistance appears to depend on the type of pathogen. The application of L-histidine to the roots of Arabidopsis plants resulted in the induction of resistance to *B. cinerea* in their aerial parts. This suggests that L-histidine exerts a systemic effect on the induction of host defenses, leading to *B. cinerea* resistance in Arabidopsis when applied to roots. Unlike Arabidopsis, tomato plants exhibited no systemic expression of ET-responsive genes such as *SlPti5* and *SlPRB-1b* in their aerial parts in response to the application of L-histidine to their roots. In tomato plants treated with L-histidine, the expression of defense-related genes such as ET-responsive genes may be differentially regulated between aerial parts and roots.

Our results indicate that exogenously applied L-histidine functions as a plant activator-like compound that activates ET biosynthesis or signaling pathways. Another such compound is sclareol, a diterpene, which has been shown to induce resistance to *R. solanacearum* and a root-knot nematode via ET biosynthesis and signaling pathways (Seo et al. 2012,

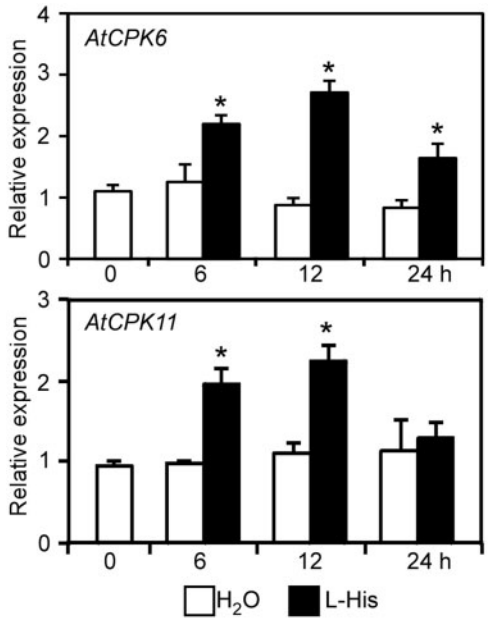

Fig. 6 Analysis of the expression of *AtCPK* genes in Arabidopsis plants after the treatment with L-histidine. The roots of hydroponically grown Arabidopsis (Col-0) plants were soaked in 10 mM L-histidine or H_2O for the indicated times, and the expression levels of the indicated genes in the treated roots were examined. Data are the mean ± SD of three independent measurements. Asterisks indicate significant differences from the H_2O treatment (*$P < 0.05$).

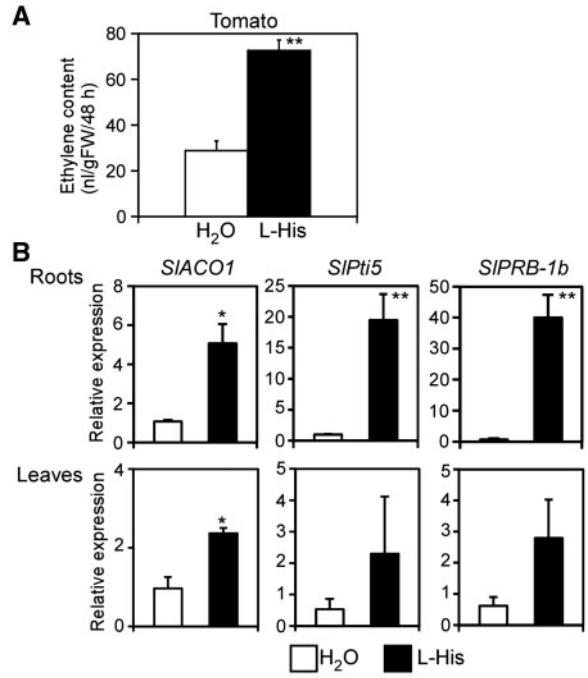

Fig. 7 Effects of exogenously applied histidine on the induction of ethylene biosynthesis in tomato. Hydroponically grown tomato plants were placed in a sealed vial containing the indicated concentrations of 10 mM L-histidine or H_2O for 48 h and subjected to the measurement of ET (A) or an analysis of the expression of the indicated genes in the treated roots and untreated leaves (B). Data are the mean ± SD of three independent measurements. Asterisks indicate significant differences from the H_2O treatment (*$P < 0.05$, **$P < 0.01$).

Fujimoto et al. 2015). L-Histidine induced the expression of *AtChiB*, an ET-responsive gene, whereas sclareol did not (Fujimoto et al. 2015). This finding suggests that L-histidine and sclareol have different modes of action, at least on the induction of ET-responsive gene expression. A previous study demonstrated that the exogenous application of amino acids inhibited bacterial wilt disease. A recent study reported that the exogenous application of the amino acid glutamate to the monocot plant rice induced resistance to *Magnaporthe oryzae*, a hemi-biotrophic fungus that causes blast disease, through the activation of host defenses in a manner depending on an SA signaling pathway (Kadonati et al. 2016). The mode of action of exogenously applied amino acids may be different between dicotyledons and monocotyledons. The addition of specific amino acids such as lysine and serine to soil resulted in the inhibition of wilt disease caused by *R. solanacearum* in tomato (Posas et al. 2010). This inhibition was suggested to be due to a decline in the bacterial population in the soil rather than the effect of resistance induced in the host plant. Although our present study clearly indicates that host defenses induced by L-histidine greatly contribute to the inhibition of bacterial wilt disease, we cannot exclude the possibility that L-histidine also affects the population of *R. solanacearum* in soil. Histidine has been demonstrated to function as a nickel chelator in nickel hyperaccumulator plants (Krämer et al. 1996). However, the physiological role of exogenous histidine in plants is not fully understood, and, thus, elucidating this role will be helpful for understanding the mechanisms underlying histidine-induced disease resistance.

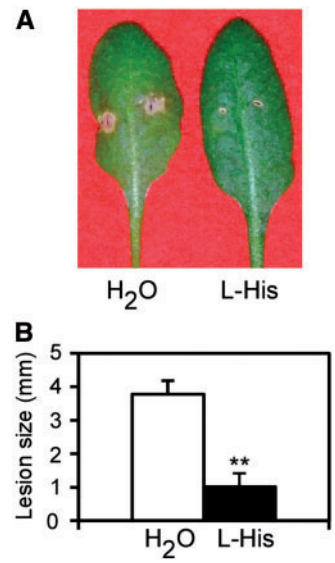

Fig. 8 Induction of resistance to *Botrytis cinerea* in Arabidopsis after a treatment with L-histidine. Arabidopsis Col-0 plants were treated with 10 mM L-histidine or H_2O by soaking their roots in the solution for 48 h, and the leaves of the treated plants were inoculated with the conidia of *B. cinerea* and photographed (A) or subjected to measurements of the diameter of necrotic lesions (B) at 6 dpi. Data are the mean ± SD of three independent assays, each performed with eight plants per treatment. Asterisks indicate significant differences from the H_2O treatment (**$P < 0.01$).

We identified histidine as a bacterial wilt disease-inhibiting compound from AgrevoEX, a liquid fertilizer originating from a yeast cell extract. However, the concentration of L-histidine in AgrevoEX was much lower than the minimum effective concentration of L-histidine when applied to roots. Thus, the bacterial wilt disease-inhibiting activity detected in AgrevoEX cannot be explained by L-histidine alone. It is possible that substances other than L-histidine in AgrevoEX have an additive or synergistic effect with the endogenous L-histidine on the induction of *R. solanacearum* resistance by this fertilizer. During the aminopropyl-based SPE step after the first fractionation by ultrafiltration, we detected bacterial wilt disease-inhibiting activity in a fraction that did not contain histidine (**Supplementary Fig. S2C**; 60% acetonitrile in H_2O), suggesting the existence of bacterial wilt disease-inhibiting compounds other than L-histidine. It will be interesting to identify these unknown compounds.

Materials and Methods

Plant materials, plant growth conditions and pathogens

The tomato plant used in the present study (*Solanum lycopersicum* cv. Ponderosa) has been described previously (Nakaho et al. 2004). All tomato plants were grown in either a hydroponic system or a pot system under a cycle of 16 h of light and 8 h of dark at 25 °C. In the hydroponic system, 7- to 10-day-old tomato seedlings were transferred to a polyethylene raft through holes (1 cm in diameter) floating on liquid fertilizer (Hyponex) diluted to 1 : 2,000, and were then grown with aeration using an air pump until they had 2–3 true leaves. Fertilizer was exchanged every 5 d. In the pot system, 7-day-old tomato seedlings were transferred to peat moss pots (Jiffy-7, Jiffy Products of America Inc.) and grown until they had 5–6 true leaves.

Arabidopsis thaliana with the Col-0 background was used and all mutants were in the Col-0 background. *coi1-1*, *npr1-1*, *abi1-1* and *ein3-1* have been described previously (Abe et al. 2008, Leon-Reyes et al. 2009, Seo et al. 2012, Fujimoto et al. 2015). Two-week-old seedlings were grown in either the pot system using Jiffy-7 pots or the hydroponic system, as described above, under a cycle of 10 h of light and 14 h of dark at 22 °C. Eight-to-ten-week-old Arabidopsis plants were used for each analysis.

Tobacco (*Nicotiana tabacum* cv. Samsun NN) plants were grown in a pot containing soil in a greenhouse under a cycle of 16 h of light and 8 h of dark at 25 °C. Approximately 5-week-old plants were used for the TMV resistance assay.

Ralstonia solanacearum (strains 8107S and RS1000) has been described previously (Seo et al. 2012). *Botrytis cinerea* has been described previously (Oka et al. 2013). TMV has been described previously (Oka et al. 2013).

Purification and identification of a bacterial wilt disease-inhibiting compound from a yeast cell extract

A 500 ml aliquot of yeast cell extract solution (AgrevoEX; Agrevo Co.) was fractionated into mol. wt. >3,000 and <3,000 using a 3 kDa mol. wt. cut-off membrane filter (Centricon; Millipore). The two fractions were subjected to a bioassay for bacterial wilt disease-inhibiting activity using a test tube as described below. Activity was detected in the fraction with mol. wt. <3,000. The active fraction was separated on an aminopropyl-based SPE cartridge column eluted with increasing concentrations of H_2O in acetonitrile. Activity was detected in a fraction eluted with 30% (v/v) acetonitrile in H_2O. The active fraction was separated on an amide-based normal-phase HPLC column (TSKgel Amide-80, particle size of 10 μm, 7.8 mm × 30 cm; Tosoh Co.) eluted with a 75% (v/v) acetonitrile/H_2O mixture at a flow rate of 6 ml min^{-1}, with monitoring at 220 nm. Activity was detected in a fraction with a retention time from 19 to 23 min. The fraction was collected and subjected to separation by the Amide-80 HPLC column using a linear gradient elution from solvent A [89% (v/v) acetonitrile in H_2O] to solvent B [75% (v/v) acetonitrile in H_2O] for >45 min, followed by a hold in solvent B for 25 min at a flow rate of 6 ml min^{-1}, with monitoring at 220 nm. A peak with the retention time of 52.0 min showed activity and was collected to yield a white powder. The purified substance was subjected to NMR and mass spectrometry (MS) analyses. ^{1}H NMR (500 MHz, D_2O) results for the purified substance were as follows: δ3.11 (1H, dd, J = 15.4, 7.8, Hβ3), 3.21 (H, dd, J = 15.9, 4.5, Hβ2), 3.86 (H, dd, J = 8.1, 5.1, Hα), 6.95 (H, d, J = 0.61, Hδ2) and 7.64 (H, d, J = 1.05, Hε1). ^{13}C NMR (125 MHz, D_2O) results for the purified substance were as follows: δ29. 2 (Cβ), 56.4 (Cα), 118.8 (Cδ2), 132.2 (Cγ), 137.1 (Cε1) and 176.1 (C). High resolution-MS: m/z calculated for $C_6H_9N_3O_2$ $[M\text{-}H]^-$ 154.0767, found 154.0567.

Bioassay of fractions using a test tube

Each fraction was evaporated to dryness, and the remaining residue was dissolved in 10 ml of methanol or H_2O. Aliquots of the solution were added to a 15 ml polypropylene test tube. H_2O alone was used as a control. One hydroponically grown tomato plant per tube was placed in the test tube until the roots were completely submerged in the solution and was then grown at 25 °C for 2 d. After briefly washing with tap water, the treated plants were inoculated by immersing their roots in a suspension [5×10^7 colony-forming units (cfu) ml^{-1}] of *R. solanacearum* strain 8107S in a new test tube at 30 °C for 24 h. After exchanging the bacterial suspension for a liquid fertilizer (Hyponex), the inoculated plants were grown under a cycle of 16 h of light and 8 h of dark at 30 °C for 7 d and subjected to the evaluation of disease development. The development of disease symptoms was evaluated using a disease index ranging from 0 to 4: 0, no wilted leaves; 1, up to 25% wilted; 2, up to 50% wilted; 3, up to 75% wilted; and 4, entirely wilted.

Chemical treatments

Stock solutions (100 mM) of L-histidine and D-histidine (Sigma) in H_2O were diluted by H_2O to various concentrations.

In order to treat plants grown in pots, plants were placed in a tray containing various concentrations of histidine until the roots were completely submerged in the chemical solution, incubated for adequate time intervals, and used in the disease resistance assay for *R. solanacearum*, *B. cinerea* or TMV.

In order to treat hydroponically grown Arabidopsis plants, plants were transferred to a Petri dish containing H_2O, with their roots submerged in H_2O, and pre-incubated for 48 h to diminish the influence of any wound stresses potentially caused by their transferral. After discarding H_2O using a pipette, a solution containing various concentrations of histidine was gently added to the Petri dish until the roots were completely submerged in the chemical solution. The roots or leaves were harvested at adequate time intervals after the addition of the histidine solution, frozen in liquid nitrogen, and stored at −80 °C until the gene expression analysis, measurements of SA, JA and ABA, and immune complex kinase assay.

Ralstonia solanacearum resistance assay

The inoculation of tomato and Arabidopsis plants grown in the pot system with *R. solanacearum* was performed as described previously (Seo et al. 2012). Strains 8107S and RS1000 were used for tomato and Arabidopsis plants, respectively. Resistance was evaluated by determining disease symptoms using the disease index as described above or measuring bacterial growth in inoculated plants. Sections (1–2 cm in length) of the middle stem of inoculated tomato plants or all rosette leaves of inoculated Arabidopsis plants were collected at the selected time points, extracted with sterilized distilled H_2O and used to measure bacterial growth as described previously (Seo et al. 2012).

Antimicrobial activity and pathogenicity assays

In order to test antimicrobial activity, strain 8107S was cultured in CPG (1% Bacto peptone, 0.1% yeast extract, 0.5% glucose and 0.1% casamino acids) medium at 28 °C for 24 h and diluted to an optical density of 0.01 at 600 nm (OD_{600}) with CPG medium containing either 10 mM L-histidine or H_2O as a control. After culturing at 28 °C for 18 h, bacterial growth was determined by measuring the OD_{600} of the bacterial culture using a spectrophotometer.

A pathogenicity assay was performed as described previously (Seo et al. 2012). Briefly, strain 8107S was cultured with 10 mM L-histidine or H_2O alone in CPG medium at 28°C for 2 d and inoculated on the roots of tomato plants grown in the pot system.

RNA extraction and real-time reverse transcription–PCR (RT–PCR)

Total RNA extracted from roots or leaves using TRIzol reagent (Invitrogen) was reverse transcribed into cDNA using random oligo-hexamers and Superscript III reverse transcriptase in accordance with the manufacturer's instructions (Invitrogen). A real-time RT–PCR analysis was performed in a two-step reaction using a SYBR Green kit (Bio-Rad) and primers specific for the indicated genes. Primers for *AtBGL2* (At3g57260), *AtPR1* (At2g14610), *AtLOX2* (At3g45140), *AtVSP2* (At5g24770), *AtPDF1.2* (At5g44420) and *AtChiB* (At3g12500) have been described previously (Fujimoto et al. 2015). We used the following primer sets: forward 5′-TCAACAACTTAGGCGGCGATAC-3′ and reverse 5′-GCAACCGAAGATGTAGTAGTCA-3′ for *AtABF1* (At1g49720); forward 5′-CGACGTCGGTTTGTGGTAGA-3′ and reverse 5′-TGCAACCGTTCTCTGCACTT-3′ for *AtPP2CA* (At3g11410); forward 5′-GGATGGTTTAGGATTTGCTTTG-3′ and reverse 5′-GCACTCTTGTTCTGGATTACCTG-3′ for *AtACS2* (At1g01480); forward 5′-GTTCCAACCCCTTATTATCC-3′ and reverse 5′-CCGTAATCTTGAACCCATTA-3′ for *AtACS6* (At4g11280); forward 5′-GCAAGTGCTTAGATCCCAATTCA-3′ and reverse 5′-CTAACTTTATTCTACCATACATAAG-3′ for *SlACO1* (X04792); forward 5′-AGACAACGATGGACGGATTG-3′ and reverse 5′-CTCCCTACACCAGCATTTCC-3′ for *AtCPK6*; forward 5′-GCATTACGGGTAATTGCTGAG-3′ and reverse 5′-GTTCCGCTGTTGTCTGTGTC-3′ for *AtCPK11*; forward 5′-GAGAGTATGGCTAGGTACGTTCG-3′ and reverse 5′-TAAGTAGTGCCTTAGCACCTCGC-3′ for *SlPti5* (U89256); and forward 5′-TTTCCCTTTTGATGTTGCT-3′ and reverse 5′-TGGAAACAAGAAGATGCAGT-3′ for *SlPRB-1b* (Y08804). We used *Atactin-2* (At3g18780; forward 5′-GGTAACATTGTGCTCAGTGGTGG-3′ and reverse 5′-GGTGCAACGACCTTAATCTTCAT-3′) and *Slactin-97* (BT012695; forward 5′-CCAGGTATTGCTGATAGAATGAG-3′ and reverse 5′-GAGCCTCCAATCCAGACAC-3′) for normalization as the standard control genes of Arabidopsis and tomato, respectively.

Phytohormone measurement

The extraction and quantification of SA and JA were performed in accordance with the procedure described by Seo et al. (2007). The extraction and quantification of ABA was performed as described previously (Müller et al. 2002).

In order to measure ET released from Arabidopsis or tomato plants, hydroponically grown plants were placed in a solution containing adequate concentrations of histidine or H_2O alone as a control, with the roots submerged in the solution in a sealed glass vial and incubated in a chamber maintained at 25 °C with 16 h of light. After 48 h, a sample was withdrawn from the headspace and analyzed for ET as described previously (Seo et al. 2007).

Protein extraction and immune complex kinase assay

Protein extraction was performed according to the procedure described by Seo et al. (2007). A 50 μg aliquot of total protein was immunoprecipitated with anti-AtMPK3 or anti-AtMPK6 antibodies, and the immunoprecipitates were subjected to the kinase assay with MBP as an artificial substrate as described previously (Seo et al. 1999, Fujimoto et al. 2015).

Assay for resistance to *B. cinerea* and TMV

The inoculation of plant leaves with a conidial suspension of *B. cinerea* was performed and resistance was evaluated by determining the diameter of lesions on inoculated leaves, as described previously (Oka et al. 2013).

The inoculation of plant leaves with TMV was performed and resistance was evaluated by determining the amount of viral RNA in inoculated leaves, as described previously (Oka et al. 2013).

Quantification of histidine

Aliquots of AgrevoEX was injected onto an HPLC column (CAPCELL PAK C18, 3 μm particle size, 4.6 mm × 25 cm; Shiseido Co.), and eluted with 50 mM sodium phosphate buffer (pH 5.8) at a flow rate of 0.5 ml min^{-1} with monitoring at 210 nm. Contents of L-histidine in AgrevoEX were determined from the standard curve.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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