D-lactic acid secreted by Chlorella fusca primes pattern-triggered immunity against Pseudomonas syringae in Arabidopsis

Sang-Moo Lee1,2, Seon-Kyu Kim3, Nakyong Lee4,5, Chi-Yong Ahn4,5 and Choong-Min Ryu1,2,*

1Molecular Phytobacteriology Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, South Korea,
2Department of Biosystems and Bioengineering, KRIBB School of Biotechnology, University of Science and Technology, Daejeon 34113, South Korea,
3Personalized Genomic Medicine Research Center, KRIBB, Daejeon 34141, South Korea,
4Cell Factory Research Center, KRIBB, Daejeon 34141, South Korea, and
5Department of Environmental Biotechnology, KRIBB School of Biotechnology, University of Science and Technology, Daejeon 34113, South Korea

Received 2 September 2019; revised 14 November 2019; accepted 9 December 2019; published online 23 December 2019.
*For correspondence (e-mail cmryu@kribb.re.kr).

SUMMARY

Biological control agents including microbes and their products have been studied as sustainable crop protection strategies. Although aquatic microalgae have been recently introduced as a biological control agent, the underlying molecular mechanisms are largely unknown. The aim of the present study was to investigate the molecular mechanisms underlying biological control by microalga Chlorella fusca. Foliar application of C. fusca elicits induced resistance in Arabidopsis thaliana against Pseudomonas syringae pv. tomato DC3000 that activates plant immunity rather than direct antagonism. To understand the basis of C. fusca-triggered induced resistance at the transcriptional level, we conducted RNA sequencing (RNA-seq) analysis. RNA-seq data showed that, upon pathogen inoculation, C. fusca treatment primed the expression of cysteine-rich receptor-like kinases, WRKY transcription factor genes, and salicylic acid and jasmonic acid signalling-related genes. Intriguingly, the application of C. fusca primed pathogen-associated molecular pattern -triggered immunity, characterized by reactive oxygen species burst and callose deposition, upon flagellin 22 treatment. The attempts to find C. fusca determinants allowed us to identify D-lactic acid secreted in the supernatant of C. fusca as a defence priming agent. This is the first report of the mechanism of innate immune activation by aquatic microalga Chlorella in higher plants.

Keywords: biological control, Chlorella fusca, defence priming, D-lactic acid, induced resistance, PAMP-triggered immunity, pathogen-associated molecular pattern.

INTRODUCTION

In the 20th century, chemical control methods were utilized mainly to manage plant diseases in modern agriculture due to their high efficacy and consistent effectiveness (Agrios, 2005). However, long-term use of synthetic agrochemicals has negatively impacted human health, causes environmental pollution, and generated chemical-resistant pathogens and herbivores (Bottrell and Smith, 1982). To overcome the limitations of agrochemicals, biological control has emerged as an alternative method of crop protection (Cook and Baker, 1983; Pal and McSpandden Gardener, 2006). Biological control agents suppress the growth of phytopathogens by producing antimicrobial compounds, inducing competition for limiting resources or physical space, and utilizing hyperparasitism (Cook and Baker, 1983; Pal and McSpandden Gardener, 2006). Biological control agents protect the host plant against phytopathogens by activating a type of
plant immunity referred to as induced resistance (Klopper et al., 1992; Pieterse et al., 1996; Fravel et al., 2003; Segarra et al., 2009).

Induced resistance is an enhanced state of plant innate immunity attained upon the application of an inducing agent against a broad spectrum of phytopathogens (Klopper et al., 1992; Pieterse et al., 1996; Ryals et al., 1996; Durran and Dong, 2004). Application of inducing agents to a plant region spatially separated from the pathogen activates induced resistance systemically throughout the plant (Klopper et al., 1992; Pieterse et al., 1996). Defence priming is an important feature of induced resistance, which increases the strength and speed of the immune response against future pathogen infection (Conrath et al., 2002; Conrath et al., 2006; Conrath et al., 2015; Mauch-Mani et al., 2017). Induced resistance is generally triggered by certain agents such as avirulent pathogens, plant growth-promoting rhizobacteria, and specific chemicals (Ross, 1961; Kuc, 1982; Klopper et al., 1992; Zimmerli et al., 2000). In many cases, bacterial cell-surface compounds and secreted compounds such as lipopolysaccharides, siderophores, 2,4-diacetylphloroglucinol, flagellin, and volatile organic compounds act as induced resistance-eliciting agents (van Peer and Schippers, 1992; Maurhofer et al., 1994; Iavicoli et al., 2003; Ryu et al., 2004; Meziane et al., 2005). However, the eukaryotic microalga itself or its derived agent that elicits induced resistance remain largely unknown.

Chlorella species are eukaryotic photosynthetic microorganisms found in freshwater, seawater, air, and soil (Liu and Chen, 2016). Because Chlorella species are rich in amino acids, carbohydrates, lipids, vitamins, and minerals, they are used as dietary supplements, feed additives, and biofuels (Liu and Chen, 2016). Chlorella culture or extract has been recently used as a biological control agent against pathogenic nematodes and fungi in various agricultural crops. Irrigation of grapevine with the dried extract of Chlorella vulgaris reduced the population of the pathogenic nematode Xiphinema index in the roots of grapevines (Bileva, 2013). Additionally, foliar spray of C. vulgaris decreased the decay rate of strawberry fruits and leafy vegetables, including lettuce, beet, and kale (Kim et al., 2014). Foliar application of C. fusca reduced the incidence of disease caused by the fungal pathogen Colletotrichum orbiculare on cucumber leaves (Lee et al., 2018; Kim et al., 2018). However, the mechanism of Chlorella-mediated biological control, such as induced resistance of host plant or direct antagonism, against pathogenic bacteria is largely unknown. In addition, despite the success of Chlorella as a biological control agent in agricultural crops, Chlorella-derived determinants involved in the interaction between Chlorella and the host plant have not been reported previously.

Here, to elucidate the molecular mechanisms underlying biological control using microalgae, we used the model pathosystems, Arabidopsis thaliana and Pseudomonas syringae pv. tomato DC3000 (Pto DC3000) and, based on previous studies, we selected C. fusca as the model microalga species. We found that foliar application of C. fusca elicited induced resistance in Arabidopsis against Pto DC3000 through priming the expression of defence-related genes involved in receptor kinases, transcription factors, and hormone signalling. Interestingly, foliar application of C. fusca primed pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), characterized by reactive oxygen species (ROS) burst and callose deposition, after PAMP treatment in Arabidopsis. Additionally, d-lactic acid was identified as a C. fusca-derived determinant, which primed induced resistance against Pto DC3000 and PAMP-triggered immunity (PTI) response upon PAMP treatment in Arabidopsis. This report is the first to describe the molecular mechanism of plant innate immunity triggered by the interaction between aquatic microalga Chlorella and the land plant Arabidopsis through the secretion of a Chlorella-derived isomeric molecule.

RESULTS
Plant protection against Pto DC3000 by spray application of C. fusca
To optimize conditions for the use of C. fusca as a biological control agent against Pto DC3000, C. fusca was...
cultivated under mixotrophic (light and glucose), heterotrophic (glucose without light), and autotrophic (light without glucose) conditions. *C. fusca* culture was sprayed on Arabidopsis thaliana ecotype Columbia (Col-0) seedlings at a concentration of $10^6$, $10^5$, or $10^2$ cells ml$^{-1}$ (Figure 1a). In leaves treated with $10^2$ cells ml$^{-1}$ *C. fusca* culture grown under mixotrophic, heterotrophic, and autotrophic conditions, the population density of *Pto* DC3000 was 5.8 x $10^5$, 1.1 x $10^4$, and 5.6 x $10^5$ colony forming units (cfu) per leaf disc (diameter = 0.8 cm) respectively at 7 days post-inoculation (dpi) of *Pto* DC3000 (Figure 1b). By contrast, the population density of *Pto* DC3000 was 3.5 x $10^8$ and 5.8 x $10^6$ cfu per leaf disc in Arabidopsis leaves pre-treated with 0.33 mM benzothiazidazole (BTH) (positive control) and BG11 medium (negative control), respectively (Figure 1b). No significant difference was observed in pathogen population density between Arabidopsis leaves treated with *C. fusca* (at two concentrations: $10^6$ and $10^5$ cells ml$^{-1}$) and those treated with BG11 medium (Figure S1). Collectively, these results indicated that mixotrophic *C. fusca* culture containing $10^6$ cells ml$^{-1}$ was the most effective against *Pto* DC3000. 

Validation of induced resistance

To investigate the mechanism of biological control by *C. fusca*, we considered four mechanisms of biological control: (1) production of antimicrobial compounds, (2) competition for limiting resources or physical space, (3) hyperparasitism, and (4) activation of induced resistance in the host plant (Cook and Baker, 1983; Pal and McSpadden Gardner, 2006). As hyperparasitism is mostly used by fungi, it was excluded as a possible mechanism of biological control used by *C. fusca*. Moreover, because *C. fusca* is an aquatic organism, competition between *C. fusca* and *Pto* DC3000 in Arabidopsis leaves was unlikely. Additionally, as *C. fusca* and *Pto* DC3000 were not spatially separated in our system, we hypothesized that *C. fusca* produces an antibacterial compound against *Pto* DC3000. To test this hypothesis, *C. fusca* was co-cultivated with *Pto* DC3000 on TSA agar medium (Figure 1c). Two antibiotics kanamycin and polymyxin B that are effective against Gram-negative bacteria inhibited the growth of *Pto* DC3000 (Figure 1c, bottom left panel). However, no inhibition zone of *Pto* DC3000 was observed around the *C. fusca* supernatant-, pellet-, and culture-inoculated disc on TSA agar medium (Figure 1c, top left panel). These results indicated that *C. fusca* exhibited no antibacterial capacity against *Pto* DC3000 in vitro.

In nature, symbiotic bacteria tightly interact with Chlorella species, affecting the growth and physiology of Chlorella (Cho et al., 2015). We observed the presence of bacteria on *C. fusca*-inoculated TSA agar medium (Figure 1c, top left panel) and *C. fusca* culture solution (Figure 1d). To determine whether Chlorella-associated bacteria exhibited antibacterial activity, nine bacterial isolates were isolated from *C. fusca* culture (Figure 1d,e). These nine isolates were classified as Microbacterium trichotecenolyticum, Brevundimonas diminuta, Rhodococcus ruber, Microbacterium hominis, Beijerinckia fluminensis, Pseudomonas azotoformans, Lysinibacillus fusiformis, Paenibacillus humicus, and Paenibacillus pasadenensis. Among these isolates, *M. trichotecenolyticum*, *B. diminuta*, and *Rhodococcus* spp. have been previously reported as phycosphere bacterial symbionts of microalgae (Watanabe et al., 2005; Lian et al., 2018; Pastore and Sforza, 2018). To investigate the antibacterial capacity of *C. fusca*-associated bacteria, each of the nine bacterial isolates was co-cultivated with *Pto* DC3000 (Figure 1c, right panel). No inhibition zone of *Pto* DC3000 was observed around discs inoculated with any of these nine bacterial isolates. This result indicates that *C. fusca*-associated bacteria do not exhibit antagonistic activity against *Pto* DC3000.

Based on the results of antagonism, we hypothesized that *C. fusca* employs the activation of induced resistance in Arabidopsis as a potential mechanism to protect Arabidopsis against *Pto* DC3000. However, the possibility that *C. fusca*-associated bacteria, but not *C. fusca*, affected induced resistance was not excluded. To assess induced resistance triggered by *C. fusca*-associated bacteria, we prepared axenic and xenic *C. fusca* cultures under mixotrophic conditions (Figure 1d,f). At 7 dpi, the population...
PAMP-triggered immunity priming by microalga Chlorella

(a) Diagram showing 0 h and 12 h time points with gene expression levels: 2066, 679, and 1357.

(b) Diagram illustrating 12 h after pathogen infection with C. fusca and plant cell structures like Nucleus and Cytosol.

(c) Graphs showing relative gene expression for 1. Receptor kinase (CRK, WRKY, PR, PDF, CHIB), 2. Transcription factor (WRKY), 3. Defense response (GST), and 4. ROS busting.

(d) Bar graphs comparing pathogen population (log CFU/leaf disc) between Col-0, crk4, and crk6 in Chlorella, BTH, and Control conditions.

(e) Additional bar graphs showing pathogen population with npr1, NahG, and jar1 conditions.

density of Pto DC3000 in Arabidopsis leaves pre-treated with xenic and axenic C. fusca cultures was $5.9 \times 10^4$ and $3.2 \times 10^6$ cfu per leaf disc, respectively (Figure 1f). By contrast, the population density of Pto DC3000 in BG11 medium-treated Arabidopsis leaves was $1.2 \times 10^6$ cfu per leaf disc at 7 dpi (Figure 1f). These results suggest that C. fusca-associated phycosphere bacteria do not contribute to the direct reduction in Pto DC3000 growth and induced resistance. Thus, in addition to Chlorella-associated bacteria, C. fusca alone is able to trigger induced resistance in Arabidopsis against Pto DC3000.

Transcriptome analysis of C. fusca-triggered induced resistance

To examine the activation of C. fusca-triggered induced resistance in Arabidopsis, we conducted RNA-seq analysis of Arabidopsis leaves treated with and without C. fusca at 0 and 12 h post-inoculation (hpi) with Pto DC3000 (Figure 2). We identified genes with $>2$-fold differences in expression between C. fusca-treated and C. fusca -untreated Arabidopsis leaves at 0 and 12 hpi with Pto DC3000. In total, 2066 and 1357 differentially expressed genes (DEGs) were identified in C. fusca-treated Arabidopsis leaves at 0 and 12 hpi, respectively (Figures 2a and S2). Additionally, 679 genes were common to both time points (0 and 12 hpi) with Pto DC3000. To analyze the functional categorization of DEGs associated with induced resistance triggered by C. fusca, we conducted gene ontology (GO) analysis of DEGs identified at 12 hpi with Pto DC3000 using the DAVID database and ReviGO software (Figure 2a). DAVID database analysis showed that numerous DEGs were associated with ‘immune system process’, ‘innate immune response’, and ‘response to bacterium’ under the biological process category at 12 hpi with Pto DC3000 (Figure S3). In addition, ReviGO analysis showed significant enrichment of DEGs involved in salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signalling under the biological process category at 12 hpi with Pto DC3000 (Figure 2a,c).

To determine the defence mechanisms induced by C. fusca, MapMan tool analysis was conducted on DEGs identified in C. fusca-treated Arabidopsis sample at 12 hpi (Figure 2b). The results showed that C. fusca triggered a series of defence mechanisms at 12 hpi by activating genes belonging to four functional groups: (1) perception (i.e., recognition of external signals by receptor kinases), (2) transcription factors, (3) defence signalling, and (4) ROS bursting. In total, 74 DEGs exhibiting receptor kinase activity were identified, including the cysteine-rich receptor-like kinase (CRK) family genes CRK4, CRK6, and CRK36, most of which were upregulated by C. fusca at 12 hpi with Pto DC3000 (Figure 2b). Additionally, 80 DEGs encoding transcription factors were identified at 12 hpi; these included transcription factor-encoding WRKY group III genes (e.g., WRKY53, WRKY54, and WRKY70) involved in plant defence. Among genes involved in multiple defence signalling pathways, C. fusca application highly upregulated 39 DEGs involved in SA and JA signalling, including pathogenesis-related 1 (PR1) and plant defence 1.2 (PDF1.2), respectively, and 28 DEGs involved in ROS burst, including glutathione S-transferase genes GSTF6, GSTF7, and GSTU4 at 12 hpi with Pto DC3000 (Figure 2b).

To validate the RNA-seq data, the expression pattern of genes in four functional groups was investigated using quantitative reverse transcription PCR (qRT-PCR). Except for expression of the Basic Chitinase (CHIB) gene, expression levels of genes involved in defence signalling, including CRKs (CRK4, CRK6, and CRK36) and WRKY group III genes (WRKY53, WRKY54, and WRKY70), as well as those involved in hormone signalling (PR1 and PDF1.2) and ROS burst (GSTF6, GSTF7, and GSTU4) were significantly upregulated by $>2$-fold in C. fusca-treated Arabidopsis leaves compared with control leaves at 12 hpi with Pto DC3000 (Figure 2c). Furthermore, C. fusca-treated Arabidopsis leaves showed defence priming of the expression of nine defence-related genes (CRK4, WRKY53, WRKY54, WRKY70, PR1, PDF1.2, GSTF6, GSTF7, and GSTU4), which did not show a difference in transcript levels at 0 hpi but were significantly upregulated at 12 hpi with Pto DC3000 (Figure 2c). In addition, no significant differences in pathogen population density were observed between C. fusca-treated and BG11 medium-treated leaves of Arabidopsis mutants crk4, crk6, npr1 (SA signalling mutant), and jar1 (JA signalling mutant), and NahG overexpression line (Figure 2d,e). Collectively, the results of our transcriptome and mutant analyses demonstrate that application of C. fusca...
PAMP-triggered immunity priming by microalga Chlorella

(a) Graph showing RLU over time with different treatments.

(b) Bar graph showing RLU 10 min after flg22 exposure.

(c) Images showing flg22 and -flg22 treatment effects.

(d) Bar graph showing number of callose with flg22 and -flg22.

(e) Images showing treatments at 0 h, Pto, and hrcC over 12 h.

(f) Bar graph showing number of callose with Pto, hrcC-12 h, and 0 h.
elicits the priming of defence-related gene expression, which triggers induced resistance against \textit{Pto} DC3000 in Arabidopsis.

**\textit{C. fusca}-triggered PTI priming**

Because \textit{C. fusca} induced the expression of CRK genes that are known to positively affect the PTI response characterized by ROS burst and callose deposition (Yeh et al., 2015; Lee et al., 2017), we examined whether \textit{C. fusca} affects flagellin 22 (flg22)-triggered PTI in Arabidopsis. ROS burst and callose deposition were detected in \textit{C. fusca}-treated Arabidopsis leaves using a luminol assay and aniline blue staining upon exposure to flg22 (Figure 3a,b). Arabidopsis leaves treated with \textit{C. fusca} (Chlorella) and BG11 medium (Medium) showed no relative light unit (RLU) signal, an indicator of ROS (hydrogen peroxide and superoxide anion) production, during the detection period (Figure 3a).

To eliminate potential side effects of \textit{C. fusca} on the expression of type III effectors and therefore is unable to repress the PTI response. At 12 h after exposure to flg22 (Medium) and subsequently treated with flg22 (Medium + flg22) (Figure 3a,b). Consistent with the ROS burst results, no difference was detected in callose deposition between leaves treated with \textit{C. fusca} (Chlorella, −flg22) and BG11 medium (Control, −flg22); however, upon flg22 treatment, callose deposition was 4.49-fold higher in leaves treated with \textit{C. fusca} and subsequently treated with flg22 (Chlorella, +flg22) than that of leaves treated with BG11 medium and subsequently treated with flg22 (Control, +flg22) (Figure 3c,d). These results suggested that \textit{C. fusca} primes PTI responses in Arabidopsis.

To validate these results using live \textit{Pto} DC3000 cells, Arabidopsis leaves were treated with a cell suspension of wild-type \textit{Pto} DC3000 rather than with flg22 (Figure 3e,f). Callose deposition was no different in \textit{C. fusca}-treated samples (Chlorella, 0 h) and the BG11 medium control (Control, 0 h). However, callose deposition in \textit{C. fusca}-treated leaves at 12 h after treatment with pathogen cell suspension (Chlorella, Pto 12 h) was 2.94-fold higher than that in the BG11 medium control (Control, Pto 12 h) (Figure 3e,f). To eliminate potential side effects of \textit{Pto} DC3000 effector proteins on the priming of callose deposition, Arabidopsis leaves were treated with live \textit{Pto} DC3000 hrcC$^-$ mutant cells (Figure 3e,f); this mutant is defective in the secretion of type III effectors and therefore is unable to repress the PTI response. At 12 h after exposure to the hrcC$^-$ mutant cell suspension, callose deposition was 2.63-fold higher in \textit{C. fusca}-treated leaves (Chlorella, hrcC$^-$ 12 h) than in BG11-treated leaves (Control, hrcC$^-$ 12 h) (Figure 3e,f).

These results indicate that \textit{C. fusca} primes the PTI response through independent of effector proteins. Taken together, our results indicated that foliar application of \textit{C. fusca} primes PTI in Arabidopsis in response to PAMP molecules.

**Identification of \textit{C. fusca} determinants conferring induced resistance**

To investigate potential determinants of \textit{C. fusca} that elicit induced resistance in Arabidopsis, the \textit{C. fusca} culture was centrifuged and the cell pellet and supernatant were applied separately to Arabidopsis leaves (Figure 4a). At 7 dpi, the population density of \textit{Pto} DC3000 in Arabidopsis leaves pre-treated with \textit{C. fusca} supernatant, cell pellet, and culture was 4.2 × 10$^5$, 7.8 × 10$^5$, and 1.2 × 10$^6$ cfu per leaf disc, respectively, whereas pathogen population density in BG11 medium-treated Arabidopsis leaves was 9.7 × 10$^5$ cfu per leaf disc (Figure 4a). Interestingly, induced resistance against \textit{Pto} DC3000 was also elicited in Arabidopsis leaves treated with the autoclaved supernatant of \textit{C. fusca} culture (Figure S4). This suggests that induced resistance is elicited by a heat-stable and secreted determinant(s) present in the \textit{C. fusca} supernatant.

To identify \textit{C. fusca} determinant(s) in the supernatant, fractionation of the supernatant was conducted using high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analyses (Figure 4b). Three organic compounds, including lactic acid, formic acid, and acetic acid were identified in the hydrophilic fraction of the supernatant. Among these three compounds, lactic acid showed the highest value of milli-absorbance units (mAU) at 12 min (Figure 4b). As lactic acid is the major compound derived from \textit{Chlorella} species during fermentation (Syrett and Wong, 1963; Begum and Syrett, 1970), we hypothesized that lactic acid functions as the major determinant in \textit{C. fusca}-mediated induced resistance against \textit{Pto} DC3000.

To assess induced resistance triggered by lactic acid, serially diluted \textit{α}-form of lactic acid, representing the racemic mixture of \textit{α}- and \textit{β}-lactic acid, was applied onto Arabidopsis leaves (Figure S5). Interestingly, the population density of \textit{Pto} DC3000 in leaves pre-treated with 10 nm \textit{α}-lactic acid was 3.2 × 10$^6$ cfu per leaf disc at 7 dpi, whereas that in the control sample was 1.3 × 10$^7$ cfu per leaf disc (Figure S5).

In conclusion, \textit{C. fusca} induces resistance against \textit{Pto} DC3000 and thereby protects Arabidopsis plants from bacterial infection. Lactic acid, a major determinant in \textit{C. fusca} supernatant, primes the PTI response to PAMPs in Arabidopsis. Further studies are required to elucidate the molecular mechanisms underlying the PTI priming effect of \textit{C. fusca}.
PAMP-triggered immunity priming by microalga *Chlorella*
aggar plate (Figure S6). These results suggest that \(\alpha\)-lactic acid is a putative determinant in *C. fusca* supernatant that elicits induced resistance in Arabidopsis against Pto DC3000.

\(\alpha\)-Lactic acid secreted by *Chlorella* acts as a defence priming determinant

Previous studies have shown that *C. pyrenoidosa* and *C. vulgaris* produce \(\alpha\)-lactate from pyruvate by the action of \(\alpha\)-lactate dehydrogenase (Gruber et al., 1974). Based on this report, we analyzed the stereoisomeric structure of lactic acid secreted by *C. fusca* using HPLC (Figure 4b). The *C. fusca* supernatant contained only the stereoisomeric \(\alpha\)-form of lactic acid (Figure 4b). Therefore, we hypothesized that \(\alpha\)-lactic acid specifically triggers induced resistance in Arabidopsis. To investigate this hypothesis, Arabidopsis leaves were treated separately with 10 \(\mu\)M each of \(\alpha\)-lactic acid and \(\lambda\)-lactic acid (Figure 4c). At 7 dpi, the population density of *Pto* DC3000 in Arabidopsis leaves pre-treated with 10 \(\mu\)M \(\alpha\)-lactic acid, \(\lambda\)-lactic acid, and BG11 medium was \(6.3 \times 10^5\), \(3.2 \times 10^6\), and \(2.5 \times 10^6\) cfu per leaf disc, respectively (Figure 4c). These results suggest that induced resistance triggered by lactic acid is dependent on its stereoisomeric structure, and \(\alpha\)-lactic acid is the most likely determinant of *C. fusca*-triggered induced resistance in Arabidopsis.

To validate whether \(\alpha\)-lactic acid affects PTI priming in Arabidopsis leaves, ROS burst and callose deposition were detected in Arabidopsis leaves treated with *C. fusca* supernatant, 10 \(\mu\)M \(\alpha\)-lactic acid, 10 \(\mu\)M \(\lambda\)-lactic acid, and BG11 medium using a luminol assay and aniline blue staining upon exposure to flg22 (Figure 5a–d). No RLU signal was observed in Arabidopsis leaves treated with *C. fusca* supernatant (Supernatant), 10 \(\mu\)M \(\alpha\)-lactic acid (\(\alpha\)-lactic acid), 10 \(\mu\)M \(\lambda\)-lactic acid (\(\lambda\)-lactic acid), and BG11 medium (Medium) during the detection period (Figure 5a,b). At 10 min after flg22 exposure, the RLU signal was 2.43- and 1.78-fold higher in leaves treated with *C. fusca* supernatant and subsequently treated with flg22 (Supernatant + flg22) and with \(\alpha\)-lactic acid and subsequently treated with flg22 (\(\alpha\)-lactic acid + flg22), respectively, than in treatment with BG11 medium control and subsequently treated with flg22 (Medium + flg22); however, no difference was observed in the RLU signal between \(\lambda\)-lactic acid + flg22 and Medium + flg22 treatments (Figure 5b). Consistent with the results of ROS burst, callose deposition in Supernatant + flg22 and \(\alpha\)-lactic acid + flg22 was 2.6-fold and 1.8-fold higher, respectively, than in the Medium + flg22 treatment (Figure 5c,d). Collectively, these data indicated that foliar application of \(\alpha\)-lactic acid primes flg22-triggered PTI responses in Arabidopsis.

In *Drosophila*, exogenous \(\lambda\)-lactic acid activates ROS production via the activation of L-lactate dehydrogenase (L-LDH) in mitochondria (Iatsenko et al., 2018). To investigate how exogenous \(\alpha\)-lactic acid affects ROS production in Arabidopsis, we evaluated the expression of mitochondrial lactate dehydrogenase genes (*D-LDH* and *L-LDH*) and two mitochondrial antioxidant enzyme-encoding genes, *alternative oxidase 1* (AOX1) and *cytochrome C oxidase subunit 2* (COX2). We compared the expression level of these genes with that of cytosolic antioxidant enzyme-encoding genes such as *catalase 1* (CAT1) and *superoxide dismutase 1* (SOD1) in leaves treated with \(\alpha\)-lactic acid at 0 and 12 h after flg22 treatment (Figure 5e). The expression levels of *D-LDH*, *COX2*, and *AOX1* oxidase were significantly upregulated in \(\alpha\)-lactic acid-treated Arabidopsis leaves (\(\alpha\)-lactic acid) by 2.66-fold, 3.87-fold, and 2.04-fold, respectively, compared with BG11 medium-treated leaves (Control) at 12 h after flg22 exposure (Figure 5e). However, no difference in *L-LDH*, *CAT1*, and *SOD1* expression levels was detected between \(\alpha\)-lactic acid and BG11 medium-treated leaves (Control) at 12 h after flg22 exposure (Figure 5e). Taken together, our results suggested that exogenous \(\alpha\)-lactic acid activates mitochondrial *D-LDH* expression and ROS generation. Taken together, these results suggested that \(\alpha\)-lactic acid secreted by *C. fusca* is a potential determinant of induced resistance and PTI priming in Arabidopsis.

**DISCUSSION**

*Chlorella*-mediated biological control against plant pathogens has been demonstrated in various crop species (Bileva, 2013; Kim et al., 2014; Lee et al., 2016; Kim et al., 2018). Nonetheless, application of *Chlorella* as a biocontrol agent in agricultural crops was not biologically relevant...
because *Chlorella* is primarily an aquatic microalga and, generally, does not inhabit soil. However, certain *Chlorella* species have been detected in soil and on plant surface (Treves et al., 2016; Zhu et al., 2018). The ubiquitous nature of *Chlorella* led us to investigate the interaction between the model plant Arabidopsis and *C. fusca*. In this study, we demonstrated the mechanisms of biological control by *C. fusca* in Arabidopsis. First, foliar application of *C. fusca* protected Arabidopsis against a bacterial pathogen *Pto DC3000* by activating plant systemic immunity, referred to as induced resistance. Second, treatment of *C. fusca* primed plant innate immunity, PTI responses in Arabidopsis induced resistance. Third, β-lactic acid in *C. fusca* supernatant was identified as a determinant of induced resistance and PTI priming.

Generally, defence priming refers to a physiological state elicited by an inducing agent that activates faster and stronger defence responses under subsequent pathogen attack (Conrath et al., 2002; Conrath et al., 2006). The priming agent β-amino butyric acid (BABA) has been intensively investigated in Arabidopsis (Zimmerli et al., 2000; Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). Treatment of BABA primed the accumulation of PR1 mRNA and callose deposition in Arabidopsis upon subsequent infection of bacterial pathogen *Pto DC3000*, pathogenic oomycete *Hyaloperonospora parasitica*, and pathogenic fungi including Botrytis cinerea, *Plectosphaerella cucumerina*, and Alternaria brassicicola (Zimmerli et al., 2000; Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). Additionally, BABA-treated Arabidopsis plants also showed enhanced expression of a PTI marker gene, FRK, and greater callose deposition after exposure to the bacterial PAMP flg22 (Singh et al., 2012). The enhanced PTI response in BABA-treated plants was reported as ‘PTI priming’ (Singh et al., 2012). In Arabidopsis, PTI priming is associated with receptor kinases such as LecRK-VI.2 and CRK (Singh et al., 2012; Huang et al., 2014; Yeh et al., 2015; Lee et al., 2017). CRK4, CRK6, and CRK36 function as positive regulators of PTI priming (Yeh et al., 2015; Lee et al., 2017). Because these three genes positively affected *C. fusca*-triggered induced resistance against *Pto DC3000* in Arabidopsis (Figure 2c, d), we hypothesized that *C. fusca* treatment induces PTI priming (Figure 3). Unlike PTI priming by BABA treatment (Singh et al., 2012), *C. fusca* and its determinant β-lactic acid primed not only callose deposition but also ROS burst (Figure 3). Apoplastic ROS play important roles in callose deposition and cell wall cross-linking to impede the penetration of pathogens (Daudi et al., 2012; O’Brien et al., 2012; Qi et al., 2017). The lack of callose deposition in peroxidase knockdown mutants *fbp1.1* and *prx34* was rescued by exogenous supply of hydrogen peroxide (Daudi et al., 2012). In addition, hydrogen peroxide activated the expression of *MYB51*, *CYP79B2*, and *CYP81F2* genes, which are required for callose deposition (Daudi et al., 2012). Therefore, our results indicated that *C. fusca* and β-lactic acid sequentially primed ROS burst and callose deposition.

Previously, ROS production by β-lactic acid has been reported in human leukocytes and *Drosophila* intestinal cells (Echigoya et al., 2012; Iatsenko et al., 2018). β-Lactic acid triggered NADPH oxidase (Nox)-mediated ROS production through L-lactate oxidation by the mitochondrial L-LDH in *Drosophila* intestinal cell (Iatsenko et al., 2018). In plants, the role of mitochondrial LDH on plant defence and ROS production is largely unexplored. Diverse plant species such as Arabidopsis, rice, and Jerusalem artichoke possess not only L-LDH, but also D-LDH, which oxidizes β-lactate (Atlante et al., 2005; Welchen et al., 2016; An et al., 2017). Based on previous reports on ROS production by β-lactic acid in animal cells, we hypothesized that exogenous β-lactic acid induces ROS production in Arabidopsis via the oxidation of β-lactate by D-LDH. Indeed, exogenous β-lactic acid activated the expression of D-LDH gene in Arabidopsis after flg22 treatment (Figure 5e). In planta, β-lactate is oxidized by D-LDH in mitochondrial intermembrane space, and electrons-derived β-lactate oxidation are delivered to the respiratory chain through cytochrome c (Atlante et al., 2005; Welchen et al., 2016). The expression of COX2 and AOX1, which encode antioxidants located in the mitochondrial membrane (Rhoads et al., 2006), was activated in Arabidopsis by β-lactic acid after flg22 treatment (Figure 5e). However, the expression of genes encoding CAT1 and SOD1 enzymes, which are largely located in peroxisomes and cytosol, respectively (Weydert and Cullen, 2010), showed no differences between β-lactic acid-treated and untreated Arabidopsis plants (Figure 5e). The mitochondrial antioxidant enzymes might be activated to catalyze mitochondrial ROS production in β-lactic acid pre-treated Arabidopsis by subsequent treatment of flg22. Therefore, our results suggest that exogenous β-lactic acid induces the generation of mitochondrial ROS through plant-specific β-lactate oxidation in response to PAMP molecules in Arabidopsis. Taken together, our results suggest that β-lactic acid in *C. fusca* functions as a PTI priming agent to enhance ROS production in Arabidopsis via the activation of β-lactate oxidation in mitochondria.

The supernatant of xenic *Chlorella* cultures contained three organic compounds: lactic acid, formic acid, and acetic acid (Figure 4b). Previous studies identified these organic acids as major products of *Chlorella* species in the presence of glucose (Syrett and Wong, 1963; Begum and Syrett, 1970). Because these organic acid compounds are also produced by bacteria in xenic *Chlorella* cultures, it is difficult to identify *Chlorella*-derived organic acids. However, among these three organic acids, only lactic acid has been identified as a *Chlorella*-specific compound, based on stereoisomeric structure analysis (Gruber et al., 1974). In nature, lactic acid exists in three forms: DL, L, and D (Maurino and Engqvist, 2015). Animals, human, and bacteria...
mainly produce the L- or Dl-form (Maurino and Engqvist, 2015). The D-lactic acid biosynthesis pathway is largely unknown, except in *Leuconostoc* spp., *Lactobacillus* spp., and *Pediococcus* spp. (Carr et al., 2002). However, *Chlorella* spp. can produce only D-lactic acid from glucose by the action of D-LDH (Gruber et al., 1974). Because we did not identify *Leuconostoc* spp., *Lactobacillus* spp., and *Pediococcus* spp. among *C. fusca*-associated bacteria (Figure 1d), D-lactic acid in the supernatant of *C. fusca* xenic culture was most likely to have been produced by *C. fusca* (Gruber et al., 1974). Additionally, previous reports of reduction in LDH activity in the dark (Perez-Garcia and Bashan, 2015) support our conclusion that D-lactic acid in mixotrophic *Chlorella* culture is a determinant of induced resistance in *Arabidopsis* (Figure 1b). Interestingly, induced resistance and ROS production were activated by lactic acid in a stereoisomer-dependent manner; Arabidopsis leaves did not respond to L-lactic acid but responded to D-lactic acid (Figure 4c). Conversely, ROS production was activated only by L-lactic acid in animal cells (Iatsenko et al., 2018). This difference between plant and animal systems may be due to the presence and expression of D-LDH genes in plants, unlike animals (Iatsenko et al., 2018; Monroe et al., 2019). The D-LDH genes are conserved in higher plants and green algae, including *Chlorella* spp. (Gruber et al., 1974; Atlante et al., 2005; Welch et al., 2016; An et al., 2017), but are absent in animals, including human and *Drosophila* (Iatsenko et al., 2018; Monroe et al., 2019). Therefore, our results suggested that D-lactic acid might be a conserved signalling molecule in photosynthetic organisms including *Chlorella* spp. and land plants including angiosperms.

To investigate the molecular basis of defence priming by *Chlorella*-mediated induced resistance, we focused on DEGs upregulated in *C. fusca*-treated *Arabidopsis* leaves at 12 hpi (Figure 2), based on previous reports (Verhagen et al., 2004; Stringlis et al., 2018). The most striking feature of our transcriptome data was the identification of the expression of the CRK family genes, which were highly upregulated in *C. fusca*-treated *Arabidopsis* (Gruber et al., 1974). Additionally, previous reports of reduction in LDH activity in the dark (Perez-Garcia and Bashan, 2015) support our conclusion that D-lactic acid in mixotrophic *Chlorella* culture is a determinant of induced resistance in Arabidopsis (Figure 1b). Interestingly, induced resistance and ROS production were activated by lactic acid in a stereoisomer-dependent manner; Arabidopsis leaves did not respond to L-lactic acid but responded to D-lactic acid (Figure 4c). Conversely, ROS production was activated only by L-lactic acid in animal cells (Iatsenko et al., 2018). This difference between plant and animal systems may be due to the presence and expression of D-LDH genes in plants, unlike animals (Iatsenko et al., 2018; Monroe et al., 2019). The D-LDH genes are conserved in higher plants and green algae, including *Chlorella* spp. (Gruber et al., 1974; Atlante et al., 2005; Welch et al., 2016; An et al., 2017), but are absent in animals, including human and *Drosophila* (Iatsenko et al., 2018; Monroe et al., 2019). Therefore, our results suggested that D-lactic acid might be a conserved signalling molecule in photosynthetic organisms including *Chlorella* spp. and land plants including angiosperms.

To investigate the molecular basis of defence priming by *Chlorella*-mediated induced resistance, we focused on DEGs upregulated in *C. fusca*-treated Arabidopsis leaves at 12 hpi (Figure 2), based on previous reports (Verhagen et al., 2004; Stringlis et al., 2018). The most striking feature of our transcriptome data was the identification of the expression of the CRK family genes, which were highly upregulated in *C. fusca*-treated *Arabidopsis* leaves (Figure 2c,d). The CRK genes represent a large subgroup of receptor-like kinases (RLKs) and harbour a C-X8-C-X2-C (DUF26) motif with unknown function in the extracellular domain (Chen, 2001). Additionally, CRK genes play critical roles in biotic and abiotic stress resistance in plants (Chen, 2001; Yeh et al., 2015; Lee et al., 2017). The CRK4, CRK6, and CRK36 genes positively regulate PTI responses and enhance resistance against *Pto* DC3000 in Arabidopsis (Yeh et al., 2015; Lee et al., 2017). *C. fusca* treatment primed the expression of CRK4, CRK6, and CRK36 in Arabidopsis after infection with *Pto* DC3000 (Figure 2b,c). Thus, our results suggest a correlation between PTI priming and CRK activation in *C. fusca*-treated Arabidopsis.
and crk6 mutants were gifted by Michael Wrzaczek at the University of Helsinki, Helsinki, Finland. Seeds were surface sterilized with 6% sodium hypochlorite for 10 min, washed five times with sterile distilled water, and then placed on half-strength Murashige and Skoog medium supplemented with 0.6% (w/v) agar and 1.5% (w/v) sucrose. The plates were incubated at 21°C under a 16 h light/8 h dark cycle for 7 days. Seedlings were transplanted in soil-less potting medium (Punon Horticulture Nursery Medium Low; Punong Co., Ltd., Gyeongju, Korea) and grown in an environmentally controlled growth room at 25°C under fluorescent lights (c. 7000 lux light intensity) and a 12 h light/12 h dark cycle.

Cultivation and application of C. fusca

C. fusca strain CHK0059 was obtained from Chang-Ki Shim at the Rural Development Administration, Wanju, South Korea, and grown under three different conditions (mixotrophic, heterotrophic, or autotrophic) in BG11 broth (Rippka et al., 1979). Under mixotrophic conditions, 1 g glucose was added to 1 L BG11 medium. Heterotrophic cultivation of C. fusca was carried out in the dark. Under all three growth conditions, C. fusca was grown at 25°C and under continuous illumination (100 μmol m⁻² sec⁻¹) in a 1 L photobioreactor containing 800 ml of autoclaved BG11 broth. To calculate the concentration of C. fusca, cell numbers were counted using a haemocytometer (INCYTO, Cheonan, Korea). When the concentration reached 10⁷ cells ml⁻¹ (exponential phase), cells were harvested and centrifuged at 4000 g for 10 min to separate the supernatant from the pellet. To obtain the axenic C. fusca culture, colonies were picked on R2A agar plates containing 50 μg ml⁻¹ kanamycin, 1 μg ml⁻¹ polymyxin B, and 5 μg ml⁻¹ vancomycin, until the colonies were bacteria-free.

To examine plant immunity induced by C. fusca, Arabidopsis leaves were sprayed with 20 ml of 10⁻³, 10⁻⁴, or 10⁻⁵ cells ml⁻¹ C. fusca culture, 0.33 mM BTH, or BG11 broth at 1 and 2 weeks after transplantation of Arabidopsis seedlings. To identify the determinant of induced resistance in C. fusca, the cell pellet and supernatant were separated from the mixotrophic C. fusca culture (10⁷ cells ml⁻¹) and applied to Arabidopsis leaves separately. The supernatant from C. fusca was filtered using a 0.45-μm syringe filter.

Inoculation of pathogen and quantification of pathogen population

P. syringae pv. tomato DC3000 (spontaneous rifampicin-resistant strain) was grown on King’s B agar plates containing 100 μg ml⁻¹ rifampicin at 30°C for 2 days. At 7 days after application of C. fusca, P. syringae pv. tomato DC3000 was suspended in distilled water. The optical density of the suspension was measured at an absorbance of 600 nm (OD₆₀₀) using a spectrophotometer (Biochrom US, MA, USA). A pathogen suspension (OD₆₀₀ = 1.0) was sprayed on Arabidopsis leaves. To determine pathogen population, C. fusca-pre-treated leaf discs (diameter = 0.8 cm) were harvested at the designated time points at 0 and 7 dpi and ground immediately in 10 ml MgCl₂. The pathogen was plated on King’s B agar plates containing 100 μg ml⁻¹ rifampicin and incubated at 30°C. The pathogen population was determined after 3 days.

Scanning electron microscopy

To investigate symbiotic bacteria on the surface of C. fusca, samples were fixed in 2.5% glutaraldehyde in 1× phosphate-buffered saline (PBS) for 10 min and post-fixed in 1% osmium tetroxide in the same buffer for 1 h. Subsequently, samples were dehydrated using a graded ethanol series and substituted by
hexamethyldisilazane. Samples were then dried and observed under a scanning electron microscope (HITACHI S4800; Hitachi, Tokyo, Japan).

RNA extraction and RNA-seq experiments

Two leaves per plant were collected from 12 Arabidopsis plants at 0 and 12 hpi. To minimize stress-induced gene expression, intact leaves were selected and immediately frozen in liquid nitrogen. Total RNA was isolated from leaves using TRIzol Reagent (Molecular Research Inc., OH, USA), according to the manufacturer's instructions as described previously (Choi et al., 2014). The quality and integrity of total RNA were confirmed by agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Fisher Scientific Inc., DE, USA). To conduct RNA-seq, a sequencing library was prepared using the TruSeq RNA Sample Preparation Kit v2 (Illumina, CA, USA), according to the manufacturer's instructions. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. The purified mRNA was fragmented and converted to cDNA. Adapters were then ligated to the cDNAs and then amplifying fragments were amplified by PCR. RNA-seq was performed using a HiSeq-2000 instrument (Illumina) to generate paired-end reads (2 × 100 bp).

RNA-seq data processing and in silico analysis

Arabidopsis thaliana reference genome sequence (v.10) was obtained from The Arabidopsis Information Resource (TAIR; assembly ID: TAIR10). Reference genome index construction and read mapping were performed using STAR (v.2.5.1b). The RNA-seq data were deposited in the NCBI Gene Expression Omnibus public database under accession number GSE89894. To determine gene expression, counts per million (CPM) mapped reads of each sample were calculated. The CPM data were normalized using quantile normalization in the R language environment (v.3.2.5). The measured gene expression values were log₂-transformed and median-centred across genes and samples. To assess differences in gene expression among samples, scatterplot matrices were performed using DAVID Bioinformatics Resources (v.6.8).

Validation of RNA-seq data using qRT-PCR

To validate RNA-seq data, expression of selected DEGs (including defence marker genes CRK, WRKY, and GST) was analyzed by qRT-PCR. To perform qRT-PCR, first-strand cDNA was synthesized from 2 μg of DNase-treated total RNA using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Enzymonics, Daejeon, South Korea). PCR reactions were performed according to the manufacturer's instructions. Expression of DEGs was analyzed using the primers listed in Table S1. A Chromo4 Real-Time PCR system (Bio-Rad, CA, USA) was used for qRT-PCR. Reaction mixtures contained cDNA template, IQTM SYBR® Green Supermix (Bio-Rad, CA, USA) and 10 μM of each primer. Thermocycler parameters used for qRT-PCR were as follows: initial polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 60 sec, and extension at 72°C for 30 sec. Conditions were determined by comparing threshold values in a series of dilutions of the reverse-transcribed product, a non-reverse-transcribed template control, and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized relative to the level of AtActin2 mRNA.

Detection of ROS

Arabidopsis leaf discs (4 mm diameter) were transferred to water in a 96-well black plate (SPL Life Sciences Co. Pocheon-si, South Korea) and incubated at room temperature overnight. Before the induction of ROS, the normalized leaf discs were floated on C. fusca culture. C. fusca supernatant, 10 μM lactic acid, and BG11 broth in a 96-well plate for 1 h and then washed twice with sterile distilled water. To trigger ROS production, leaf discs were placed in 100 μl of assay solution containing 10 ng ml⁻¹ peroxidase, 20 μM luminol, and 100 nm flg22 peptide. Light emission was measured as RLU using a Mithras Tristar2 LB 940 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany), which is a 96-well luminometer, for 1 h.

Callose deposition assay

Arabidopsis seedlings were grown on half-strength Murashige and Skoog medium supplemented with 0.6% (w/v) agar and 1.5% (w/v) sucrose at 21°C and a 16 h light/8 h dark cycle for 2 weeks. Arabidopsis seedlings were incubated in C. fusca culture, C. fusca supernatant, 10 μM lactic acid, and BG11 broth in a 50 ml tube at room temperature overnight. Subsequently, Arabidopsis seedlings were washed twice with sterile distilled water. To trigger callose deposition, Arabidopsis seedlings were incubated for 1 h in 100 nm flg22. To trigger callose deposition by live P. syringae, Arabidopsis seedlings were incubated in the cell suspension from the wild-type or hrcC mutant of Pto DC3000 (OD600 = 1). After exposure to flg22 or P. syringae, Arabidopsis seedlings in 50 ml Falcon tubes were de-stained with 95% ethanol overnight. After de-staining, the cotyledons were detached and incubated in 150 mM K2HPO4 for 30 min. Then, the cotyledons were placed in 50 ml Falcon tubes containing 150 mg K2HPO4, 0.01% aniline blue, wrapped in aluminium foil to avoid light, and stained for 2 h. Callose in the stained cotyledons was visualized under an epifluorescence microscope (Olympus BX51; Tokyo, Japan). Callose deposition was quantified using a fluorescence microscope fitted with a 4',6-diamidino-2-phenylindole (DAPI) filter (excitation wavelength: 370 nm; emission wavelength: 509 nm). The number of stained callose depositions was counted using the “analyze particles” function in ImageJ software (http://rsb.info.nih.gov/ij/).

HPLC and NMR analysis

HPLC analysis was carried out using the L-2455 system (Hitachi, Japan) equipped with a photodiode array detector. First, a preparative HPLC column (Cosmosil 5C18-MS II, 10 mm × 150 mm; Nacalai Tesque, Japan) was used with a mobile phase comprising 0.04% trifluoroacetic acid in water, a flow rate of 3 ml min⁻¹, and a detection wavelength of 222 nm. Then, an analysis column (TSK-GER ODS-100V, 4.6 mm × 150 mm; TOSH, Japan) was used with a mobile phase comprising 0.04% trifluoroacetic acid in water (A) and methanol (B) under the following gradient conditions: 100% A for 14 min and 100% B for 20 min. A flow rate of 1 ml min⁻¹ and a detection wavelength of 224 nm were used with the analysis column. H (600 MHz) and 13C (150 MHz) NMR spectra were obtained using a JEOL JNM-ECA600 600MHz FT-NMR spectrometer, with deuterium oxide as a solvent. Qualitative analyses of C. fusca supernatant were conducted by HPLC. HPLC was performed on an Aminex HPX-87 column (300 mm × 4.6 mm; particle size = 5 μm; USA) at 25°C. The flow rate was 0.6 ml min⁻¹. The mobile phase was 4 mM sulfuric acid in water for a total running time of 25 min.

The sample injection volume was 10 μl. The detection wavelength was 215 nm.

**Statistical analysis**

Data were analyzed by analysis of variance using JMP 4.0 software (SAS Institute Inc., Cary, NC, USA). Significant treatment effects were determined on the basis of the magnitude of the F-value ($P < 0.05$). When a significant F-value was obtained, the separation of means was analyzed by determination of Fisher’s protected least significant difference at $P < 0.05$.

**ACKNOWLEDGEMENTS**

This research was supported by grants from the Advanced Biomass R&D Center (ABC) of the Global Frontier Project funded by the Ministry of Science and ICT and Future Planning (ABC-2010-0029728), the cooperative Research Program for Agriculture and Technology Development (Agenda Project No. PJ011707), the Rural Development Administration, Strategic Initiative for Microbiomes in Agriculture and Food, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea (as part of the multilateral Genome Technology to Business Translation Program) (918017-4), and KRICT Initiative Program, South Korea.

**AUTHOR CONTRIBUTIONS**

S-ML and C-MR designed experiments and wrote the paper. S-MF performed the assessment of induced resistance, antagonism test, Arabidopsis mutant analysis, qRT-PCR, determinant screening, and PTI priming test. S-KK analyzed RNA-seq data. N-KL and C-YA performed cultivation of C. fusca and scanning electron microscopy. All authors discussed the results.

**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

**DATA AVAILABILITY STATEMENT**

RNA-seq data used in this study are freely available at the NCBI Gene Expression Omnibus public database under accession number GSE89894 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89894).

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

Table S1. List of primers used for qRT-PCR analysis.

Figure S1. Optimization of cell density and growth conditions of Chlorella fusca for biological control against Pseudomonas syringae pv. tomato DC3000 (Pto DC3000) in Arabidopsis.

Figure S2. Induced resistance-associated differentially expressed genes (DEGs) in C. fusca-treated Arabidopsis leaves at 0 and 12 hpi.

Figure S3. Gene ontology (GO) enrichment analysis of DEGs identified in C. fusca-treated Arabidopsis leaves at 12 hpi.

Figure S4. Activation of C. fusca supernatant-triggered induced resistance in Arabidopsis against Pto DC3000 with or without heat treatment.

Figure S5. Optimization of α-lactic acid concentration for eliciting induced resistance against Pto DC3000 in Arabidopsis.

**REFERENCES**


