Serine Palmitoyltransferase, the First Step Enzyme in Sphingolipid Biosynthesis, Is Involved in Nonhost Resistance

Yoshihiro Takahashi,^{1,2} Thomas Berberich,¹ Hiroyuki Kanzaki,¹ Hideo Matsumura,¹ Hiromasa Saitoh,¹ Tomonobu Kusano,² and Ryohei Terauchi^{1,3}

¹Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami, Iwate 024-0003, Japan; ²Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba, Sendai, Miyagi 980-8577, Japan; ³Graduate School of Agricultural Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan

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An overexpression screen of *Nicotiana benthamiana* cDNAs identified a gene for the LCB2 subunit of serine palmitoyltransferase (SPT) as a potent inducer of hypersensitive response-like cell death. The pyridoxal 5'-phosphate binding site of NbLCB2 is required for its function as a cell death inducer. NbLCB2 mRNA is accumulated after infection by nonhost pathogen *Pseudomonas cichorii*. Resistance of *N. benthamiana* against *P. cichorii* was compromised by treatment with an SPT inhibitor and in NbLCB2- and NbLCB1-silenced plants. These results suggest that biosynthesis of sphingolipids is necessary for the nonhost resistance of *N. benthamiana* against *P. cichorii*.

Plants have evolved defense mechanisms to cope with a wide variety of pathogens. The defense reaction involving localized cell death, known as the hypersensitive response (HR), is one of the most important innate immune responses and acts by limiting pathogen spread. This reaction is triggered by host receptor-mediated perception of pathogen microbe-associated molecular patterns (MAMPs), or by recognition of pathogen race-specific avirulence (*avr*) factors by corresponding resistance (*R*) genes in plant (Dangl and Jones 2001; Nürnberger and Lipka 2005). Through extensive research on signaling networks leading to this innate immune response, several key proteins have been isolated and characterized (Bittel and Robatzek 2007; Chisholm et al. 2006; Jones and Dangl 2006; Mysore and Ryu 2004). However, the information is still fragmentary, and many more important components involved in innate immunity remain to be discovered. To search for such novel components, we performed high-throughput overexpression screening for cell death-inducing factors in plants and identified a cDNA from *Nicotiana benthamiana* encoding the LCB2 subunit of serine palmitoyltransferase (SPT).

Sphingolipids are membrane components in eukaryotes and some prokaryotes. SPT catalyzes the first step of sphingolipid biosynthesis, which condenses serine and palmitoyl-CoA to form 3-ketosphinganine in a pyridoxal 5'-phosphate (PLP)-dependent reaction (Fig. 1). 3-Ketosphinganine is immediately converted to dihydrosphingosine (sphinganine) by NADPH-dependent 3-ketosphinganine reductase. Dihydrosphingosine, in turn, is converted to dihydroceramide, which gives rise to ceramide. Eukaryotic SPTs are composed of membrane-associated heterodimers of subunits encoded by the *LCB1* and *LCB2* genes. LCB1 is believed to stabilize LCB2, and the SPT-active site is thought to be located at the interface of the dimerized LCB1 and LCB2 subunits (Gable et al. 2000; Yasuda et al. 2003). Both LCB1 and LCB2 subunits are conserved in plants (Sperling and Heinz 2003). Recent studies in *Arabidopsis* showed that both LCB1 and LCB2 are required for the function of SPT (Chen et al. 2006; Dietrich et al. 2008). Loss-of-function of *AtLCB1* caused embryonic lethality (Chen et al. 2006). Likewise, simultaneous knockout of two copies of *Arabidopsis* LCB2 genes, *AtLCB2a* and *AtLCB2b*, caused lethality (Dietrich et al. 2008). These works suggest that sphingolipids synthesis is essential for viability of plant cells.

Sphingolipids and their metabolites are known to generate a variety of signals in yeast and animals involved not only in maintaining cellular homeostasis but also in various stress responses such as apoptosis. Moreover, sphingolipids are key components in the membrane microdomains called lipid rafts, playing an important role in many cellular processes, including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry (Kozutsumi et al. 2002; Martin et al. 2005; Merrill 2002; Munro 2003; Obeid et al. 2002). Plant sphingolipids may also be involved in various cellular responses, including plant-microbe interactions. A partial fragment of the *LCB2* gene was isolated from potato tubers infected with *Phytophthora infestans*, and its gene expression was induced in the early stages of the HR (Birch et al. 1999). Mycotoxins such as fumonisin B1 and AAL-toxin produced by *Fusarium* and *Alternaria* spp., respectively, initiate host plant cell destruction via disruption of sphingolipid metabolism resulting from inhibition of sphinganine *N*-acyltransferase (acyl-CoA-dependent ceramide synthase) (Fig. 1) (Abbas et al. 1994; Wang et al. 1996). Cerebrosides A and C, ceramides with a sugar residue at the 1-hydroxyl moiety, isolated from rice blast fungus can be recognized as MAMPs that lead to the accumulation of phytoalexins and pathogenesis-related protein in rice (Umemura et al. 2000, 2002). *Arabidopsis* mutants in the genes encoding

Corresponding author: R. Terauchi; Telephone: +81-197-68-2911; Fax: +81-197-68-3881; E-mail: terauchi@ibrc.or.jp

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ing a putative ceramide kinase and a sphingosine transfer protein, *acd5* and *acd11*, respectively, undergo precocious programmed cell death (Brodersen et al. 2002; Liang et al. 2003). Recently, the fumonisin B1-resistant *Arabidopsis* mutant, *fbr11-1*, was revealed to be defective in the AtLCB1 gene (Shi et al. 2007). After treatment with fumonisin B1, the *fbr11-1* mutant accumulated lower amounts of sphingoid bases compared with the wild type, which seems to be the cause of fumonisin B1 resistance in *fbr11-1*. The same authors showed that direct feeding of free sphingoid bases caused cell death in *Arabidopsis*, and suggested that the balance between free and phosphorylated sphingoid bases may determine the fate of cell death or survival (Shi et al. 2007). Together, these results implicate sphingolipid metabolism in defense responses, including plant cell death. However, little is known about the physiological function of sphingolipids in plants. In particular, there is no report showing direct involvement of sphingolipids in disease resistance. In the present study, we demonstrate that sphingolipids are indeed involved in nonhost resistance.

Fig. 1. Sphingolipid biosynthesis pathway in plants. Fumonisin B1 and AAL-toxin inhibit sphinganine *N*-acyltransferase whereas myriocin prevents the formation of long-chain bases by inhibiting serine palmitoyltransferase.

RESULTS

NbLCB2 overexpression causes cell death.

We identified *NbLCB2*, a cDNA for the LCB2 subunit of SPT in *N. benthamiana* (GenBank No. AB378334), as a potent inducer of HR-like cell death using a high-throughput screening technique (data not shown) (Berberich et al. 2008; Nasir et al. 2005; Terauchi et al. 2005). Then, we tried to confirm the correlation between *NbLCB2* protein production and cell death by employing the dexamethasone (DEX) inducible expression vector pTA7001 (Aoyama and Chua 1997). cDNA inserted into pTA7001 vector was modified so that the C-terminus of the protein was tagged with HA epitope, resulting in *NbLCB2*-HA. *Agrobacterium tumefaciens* harboring *NbLCB2*-HA was infiltrated into *N. benthamiana* leaves by a needle-less syringe to establish transient transformation of the leaves. Two days later, DEX was infiltrated to induce *NbLCB2*-HA production. *NbLCB2*-HA protein was detected 4 h after DEX treatment (Fig. 2B). Leaf cell death was clearly observed 48 h after DEX treatment (Fig. 2C).

NbLCB2 (489-amino-acid protein) has a conserved GTFTKSFG motif (amino acid positions 307 to 314) corresponding to the known PLP-binding site, which is commonly found in members of the α -oxoamine synthase subfamily, such as 5-aminolevulinic acid synthase, 2-amino-3-ketobutyrate CoA ligase, and 8-amino-7-oxononanoate synthase (Fig. 2A) (Hanada 2003). To test whether the PLP-binding site of *NbLCB2* is necessary to cause cell death, we constructed a

Fig. 2. Cell death phenotype in *Nicotiana benthamiana* leaves caused by *NbLCB2* overexpression. **A**, Comparison of the pyridoxal 5'-phosphate (PLP)-binding motif of LCB2 and LCB1. Species are indicated as follows: Nb, *N. benthamiana*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*. An asterisk marks the PLP-binding lysine residue. **B**, Western blot analysis of *NbLCB2* and *NbLCB2*^{ΔPLPBS} protein. *NbLCB2* and its mutant construct *NbLCB2*^{ΔPLPBS} were transiently transformed into *N. benthamiana* leaves. Two days later, transgene expression was induced by the application of dexamethasone (DEX) (30 μ M) and the expressed proteins were detected by anti-HA-antibody. **C**, *NbLCB2* caused cell death (center leaf) but a deletion mutant of the PLP-binding site (right leaf) did not. Photos were taken 48 h after DEX application.

mutant version of NbLCB2 whereby the 8 amino acid residues corresponding to the PLP-binding site was deleted from NbLCB2 (NbLCB2^{ΔPLPBS}), and expressed it in *N. benthamiana* leaves. Expression of NbLCB2^{ΔPLPBS} did not cause leaf cell death even at 48 h after DEX treatment (Fig. 2C), while the protein was abundantly expressed (Fig. 2B). This result indicates that the PLP-binding site of NbLCB2 is essential for its function as a cell death inducer.

NbLCB2 is induced by infection by the nonhost pathogen *Pseudomonas cichorii*.

To study NbLCB2 expression in relation to defense responses, Northern blot analysis was carried out using *N. benthamiana* total RNA extracted from leaf material collected at defined time intervals after inoculation with the nonhost bacterial pathogen *Pseudomonas cichorii* or the host pathogen *P. syringae* pv. *tabaci*. The control water treatment transiently induced NbLCB2 expression after 3 h but its expression returned to the original level after 6 h (Fig. 3). A strong induction of NbLCB2 was detected after inoculation of *P. cichorii* but only a transient increase in NbLCB2 expression comparable with the water control was observed following challenge of *N. benthamiana* leaves with *P. syringae* pv. *tabaci*. These results suggest that de novo biosynthesis of sphingolipids may be important or is induced during the nonhost resistance response.

SPT inhibitor suppresses defense response.

To address the question of whether sphingolipid synthesis is required for plant defense, we analyzed bacterial growth in *N. benthamiana* using an SPT inhibitor, myriocin, which is known to block the function of SPT (Fig. 1). First, we tested whether myriocin affected the bacterial growth in liquid culture or altered plant condition. Myriocin had no significant effect on either bacterial or plant phenotype up to 5 days after treatment (data not shown). Then, we infiltrated 1 μ M myriocin together with bacteria into *N. benthamiana* leaves. Myriocin-treated leaves showed a significant inhibition of defense response against nonhost pathogen *P. cichorii* (Fig. 4A), allowing 10-fold more growth of bacteria compared with the control. However, myriocin did not affect growth of virulent pathogen *P. syringae* pv. *tabaci* in *N. benthamiana* leaves (Fig. 4B). These results suggest that sphingolipid generation mediated by SPT is necessary for nonhost resistance of *N. benthamiana* against *P. cichorii*.

Fig. 3. NbLCB2 gene expression in *Nicotiana benthamiana*. NbLCB2 gene expression was **A**, strongly induced by the nonhost pathogen *Pseudomonas cichorii* but **B**, only slightly induced by the virulent pathogen *P. syringae* pv. *tabaci*.

Virus-induced gene silencing of NbLCB2.

Loss-of-function analysis of NbLCB2 was performed by virus-induced gene silencing (VIGS) using the *Tobacco rattle virus* (TRV)-derived expression vector (Ratcliff et al. 2001). *N. benthamiana* plants infected with a TRV vector carrying a partial fragment of NbLCB2 showed abnormal leaf development, especially in the upper leaves (Fig. 5A and B). In addition, small necrotic lesions were sometimes observed on NbLCB2-silenced leaves (data not shown). These results suggest that sphingolipid biosynthesis is necessary for normal leaf development and that reduced sphingolipid synthesis resulting from suppression of NbLCB2 expression interferes with normal leaf morphogenesis. We analyzed the growth of nonhost pathogen *P. cichorii* in NbLCB2-silenced plants using the lower leaves which had less severe morphological changes and compared it with the growth in the lower leaves of control plants. NbLCB2-silenced plants allowed a higher growth (sixfold) of *P. cichorii* compared with the control 48 h after the inoculation (Fig. 5D). *P. cichorii* infection causes HR-like cell death in *N. benthamiana*. The timing and extent of cell death caused by *P. cichorii* were not significantly different between NbLCB2-silenced and control plants (Supplementary Fig. 1). We also tested the growth of the host pathogen *P. syringae* pv. *tabaci* in NbLCB2-silenced plants. There was no difference in growth rates between NbLCB2-silenced and control plants (Fig. 5E).

Gene expression and VIGS of NbLCB1.

To further confirm the involvement of SPT in plant defense, we tried to obtain the cDNA sequence of NbLCB1, a gene coding for the LCB1 subunit of SPT, by using degenerate polymerase chain reaction (PCR) primers designed according to the conserved amino acid sequences of published plant LCB1 proteins. We failed to obtain the full-length NbLCB1 cDNA but could recover a 694-bp fragment (GenBank AB453053) exhibiting a high similarity to AtLCB1 (84% amino acid identity). By using PCR primers located in this partial NbLCB1 fragment, we carried out reverse-transcription (RT)-PCR experiments to study the expression of NbLCB1. No induction of NbLCB1 after infection with *P. cichorii* as well as *P. syringae* pv. *tabaci* was detected (Fig. 6A), which is in contrast to NbLCB2 that was specifically induced by *P. cichorii* infection. Next, we performed VIGS of NbLCB1 using the infection of TRV-derived virus vector pTV::NbLCB1 harboring a partial fragment of NbLCB1. VIGS of NbLCB1 was confirmed by RT-

Fig. 4. Serine palmitoyltransferase inhibitor, myriocin, inhibits defense response against a nonhost pathogen. Bacterial growth of **A**, nonhost pathogen *Pseudomonas cichorii* and **B**, virulent pathogen *P. syringae* pv. *tabaci*, inoculated into *Nicotiana benthamiana* leaves, together with myriocin (black bars) or solution lacking myriocin (white bars). DMSO = dimethyl sulfoxide.

PCR (Fig. 6C). The phenotype of *NbLCB1*-silenced plants was almost the same as the control except for a slight downward curling of leaf edges in *NbLCB1*-silenced plants (Fig. 6B). The nonhost pathogen *P. cichorii* and the host pathogen *P. syringae* pv. *tabaci* were separately inoculated to *NbLCB1*-silenced plants (Fig. 6D and E). The growth of *P. cichorii* increased approximately 10-fold in *NbLCB1*-silenced plants compared with the control (Fig. 6D), whereas no difference was observed in growth of *P. syringae* pv. *tabaci* between *NbLCB1*-silenced and control plants (Fig. 6E). Cell death caused by *P. cichorii* showed no difference in timing or amount between *NbLCB1*-silenced and control plants. To see the effect of overexpression of *LCB1*, we cloned *Arabidopsis AtLCB1* cDNA (Chen et al. 2006) in the pTA7001 vector and transiently expressed it in *N. benthamiana* leaves. However, no phenotypic change was observed (data not shown).

Infiltration of sphingoid bases causes cell death in *N. benthamiana*.

Overexpression of *NbLCB2* caused cell death. This cell death may be caused by the accumulation of sphingoid bases in the cells owing to the enhanced activity of SPT. To test this possibility, we infiltrated three different sphingoid bases, dihydrosphingosine (DH-Sph), sphingosine (Sph), and phytosphingosine (Phyto-Sph), at a concentration of 25 μ M in the leaf blade of *N. benthamiana* by a needle-less syringe. All three sphingoid bases caused cell death around the infiltrated area (Fig. 7), suggesting that accumulation of sphingoid bases indeed triggers cell death in *N. benthamiana*.

DISCUSSION

Lipids are not only critical components of plant cell membranes but also influence pathogenesis and resistance mechanisms associated with plant–microbe interactions (Shah 2005). Some pathogens such as *Fusarium moniliforme* and *Alternaria alternata* f. sp. *lycopersici* produce toxins that target plant lipid metabolism (Wang et al. 1996). In turn, plants have evolved a mechanism to recognize microbial lipopolysaccharides, sphingolipids, and lipid-binding proteins as MAMPs for triggering defense responses (Shah 2005). In this report, we demonstrated that the overexpression of *NbLCB2*, encoding a subunit of SPT, caused cell death in *N. benthamiana* leaves. Moreover, we showed that sphingolipid biosynthesis is required in plant defense against a nonhost pathogen, *P. cichorii*.

NbLCB2 overexpression causes cell death in *N. benthamiana*.

Mycotoxins such as fumonisin B1 and AAL-toxin cause host cell death by the disruption of sphingolipid metabolism (Fig. 1). They are structurally related to sphinganine, a precursor in sphingolipid biosynthesis and, as a result, competitively inhibit de novo synthesis of sphingolipids that are necessary for a variety of cellular responses, including the production of ceramides in animal and plant cells (Abbas et al. 1994; Wang et al. 1996). A study by Spassieva and associates (2002) showed that AAL-toxin-induced cell death was associated with the accumulation of 3-ketosphinganine and sphinganine in AAL-toxin-susceptible tomato leaf. Moreover, the same proc-

Fig. 5. Virus-induced gene silencing of *NbLCB2*. **A** and **B**, Phenotype of *NbLCB2*-silenced plants 4 weeks after *Tobacco rattle virus* infection. **C**, Reverse-transcription polymerase chain reaction of *NbLCB2* and *EF1α* genes in pTV00-infected and pTV::*NbLCB2*-infected plants. Bacterial growth of **D**, nonhost pathogen *Pseudomonas cichorii* and **E**, virulent pathogen *P. syringae* pv. *tabaci* inoculated to *NbLCB2*-silenced *Nicotiana benthamiana* leaves. White bar: control plants infected with pTV00. Black bars: plant infected with pTV::*NbLCB2*. The vertical axis indicates the titer of bacteria in *N. benthamiana* leaves.

ess is blocked by the SPT inhibitor, myriocin (Spassieva et al. 2002). The authors proposed that increase in metabolic sphingolipid-intermediates such as 3-ketosphinganine and sphinganine are important for cell death caused by AAL-toxin. A recent study by Shi and associates (2007) confirmed this hypothesis by demonstrating that direct injection of dihydroshpinganine, phytosphinganine, and sphinganine into *Arabidopsis* leaves causes reactive oxygen species (ROS) production followed by cell death. The *NbLCB2*-mediated HR-like cell death observed in the current study may be caused by the accumulation of metabolic sphingolipid intermediates such as 3-ketosphinganine and DH-Sph, caused by an increase in SPT activity. This hypothesis is consistent with our observation that infiltration of dihydroshpinganine, phytosphinganine, and sphinganine in *N. benthamiana* also causes cell death (Fig. 7).

In yeast and mammalian cells, SPT is a heterodimeric enzyme consisting of LCB1 and LCB2 subunits. LCB1 and LCB2 have significant similarity to members of a subfamily of PLP-dependent enzymes such as 5-aminolevulinic acid synthase, 2-amino-3-ketobutyrate CoA ligase, and 8-amino-7-oxononanoate synthase (Hanada 2003). These proteins share a conserved PLP-binding motif, except for LCB1 (Fig. 2A). Heterologous expression of the *Arabidopsis* *AtLCB2* results in sphinganine synthesis in *Escherichia coli* (Tamura et al. 2000). However, *AtLCB2* expression in a yeast mutant *lcb2Δ* without

functional LCB2 did not result in the recovery of SPT activity (Tamura et al. 2001). Recently, Chen and associates (2006) demonstrated that co-expression of the *AtLCB1* with *AtLCB2* yields an active SPT that is able to rescue yeast *lcb1Δ* and

Fig. 7. Effects of sphingoid bases in *Nicotiana benthamiana*. Cell death induced by free sphingoid bases, dihydroshpingosine (DH-Sph), sphingosine (Sph), and phytosphingosine (Phyto-Sph). Pictures were taken 24 h after infiltration of sphingoid bases (25 μ M each).

Fig. 6. Gene expression and virus-induced gene silencing of *NbLCB1*. **A**, Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *NbLCB1* gene expression during bacterial pathogen infection of *Nicotiana benthamiana* leaves (left, *Pseudomonas cichorii*; right, *P. syringae* pv. *tabaci*). *NbLCB2* and *EF1α* were used as control. **B**, Phenotype of *NbLCB1*-silenced plant 4 weeks after *Tobacco rattle virus* infection. **C**, RT-PCR results of *NbLCB1* and *EF1α* genes in pTV00-infected and pTV::NbLCB1-infected plants. Bacterial growth of **D**, nonhost pathogen *P. cichorii* and **E**, virulent pathogen *P. syringae* pv. *tabaci* inoculated into *NbLCB1*-silenced *N. benthamiana* leaves. White bar: control plants infected with pTV00. Black bars: plant infected with pTV::NbLCB1.

lcb2Δ single- and double-knockout mutants. This result indicates that plant SPT functions as a heteromeric enzyme that requires both LCB1 and LCB2 for the optimal activity. Probably, exogenously expressed NbLCB2 enhanced SPT activity in our experiments by interacting with endogenous NbLCB1. It is known that a change in SPT activity in response to UVB irradiation parallels the change in the *LCB2* mRNA level but not the *LCB1* mRNA level in cultured human keratinocytes (Farrell et al. 1998). Similarly, our gene expression study indicated that *NbLCB2* was highly induced whereas *NbLCB1* was not inducible by the challenge with nonhost pathogen *P. cichorii* (Figs. 3A and 6A). From this result, we propose that plant cells can also regulate the amount of the LCB1/LCB2 complex through transcriptional regulation of the *LCB2* gene even without parallel regulation of the *LCB1* gene. Therefore, we hypothesize that overexpression of *NbLCB2* enhanced the SPT activity which, in turn, triggered plant cell death.

Sphingolipids are involved in leaf morphology.

It has been shown that the SPT-defective mutants in yeast or mammalian cells require external sphingolipids for cell growth (Hanada et al. 1992; Pinto et al. 1992). Complete loss of SPT activity in fruit fly results in embryonic lethality (Adachi-Yamada et al. 1999). A similar result was obtained in the knockout mutants of *AtLCB1* (Chen et al. 2006) as well as *AtLCB2* (Dietrich et al. 2008). Partial RNAi suppression of *AtLCB1* expression was accompanied not only by reduced cell expansion but also by curled leaf blades and twisted leaf tips. However, major sphingolipid content on a mass basis was not significantly altered (Chen et al. 2006). Our *NtLCB1*-silenced plants also showed a phenotype similar to *AtLCB1*-knockdown plants with curled leaf edges (Fig. 6B). *NbLCB2*-silenced plants sometimes showed necrotic lesions. This seems to be unrelated to cell death phenotype caused by *NbLCB2* overexpression. We speculate that cell death in *NbLCB2*-silenced plants is caused by a shortage of sphingolipids necessary for the maintenance of cellular viability as observed in *Arabidopsis* (Dietrich et al. 2008). Interestingly, *NbLCB2*-silenced plants had changed leaf morphology but no significant change in stem length (Fig. 5A and B). Perhaps, sphingolipid signaling is not necessary for stem elongation. Future studies will shed light on the molecular mechanisms underlying the function of *NbLCB2* in plant morphogenesis.

Biosynthesis of sphingolipids is important for plant nonhost resistance.

NbLCB2 mRNA is induced at an early stage of infection of nonhost pathogen *P. cichorii*. Moreover, results of SPT inhibitor treatment and *NbLCB2*- and *NbLCB1*-silencing experiments suggested that sphingolipid biosynthesis is important for suppression of growth of nonhost pathogen *P. cichorii* in *N. benthamiana*. The quantitative nature of reduction of resistance in SPT-inhibitor-treated and *NbLCB2*- and *NbLCB1*-silenced plants may suggest that SPT is not a sole key regulator but one of several regulators of nonhost resistance. We attempted to identify de novo sphingolipid metabolites generated during pathogen infection. *N. benthamiana* leaf disks infected by *P. cichorii* were incubated with ¹⁴C-serine. Total sphingolipids and long-chain bases were extracted and analyzed by thin-layer chromatography in a similar method described by Spassieva and associates (2002). However, we could not detect any signal of de novo sphingolipid products derived from *N. benthamiana*, presumably because ¹⁴C-serine was preferentially incorporated into bacterial pathogen growing in the intercellular space of plants. Similarly, we failed to identify the sphingolipid products derived from *N. benthamiana* leaves by *Agrobacterium*-mediated transient overexpression of *NbLCB2*.

What is the possible role of sphingolipids in defense responses? In animals, sphingolipid metabolite Sph-1-phosphate (S1P) is known as a signaling molecule (Spiegel et al. 2002). It was recently shown that S1P is a signaling molecule involved in ABA-dependent guard cell closure in plants, the signal being transduced through the sole prototypical G-protein α-subunit (Coursol et al. 2003; Ng et al. 2001). Interestingly, G-protein α-subunit is known as a key molecule for disease resistance in rice (Suharsono et al. 2002). Moreover, Shi and associates (2007) suggested that the balance between free and phosphorylated sphingoid bases determines the fate of cell death or survival in *Arabidopsis*. Further studies are needed to elucidate how the relative amount of sphingolipid intermediates could be perceived by plant cells and converted into signals that determine cell death and defense responses.

The regulation of sphingolipid biosynthesis and the function of sphingolipids are still largely uncharacterized in plants. In this study, we demonstrated for the first time that sphingolipids play a crucial role in plant innate immunity. Our findings may lead to further elucidation of molecular mechanisms in plant-microbe interactions.

MATERIALS AND METHODS

Plant materials and screening of cell death-inducing factors.

N. benthamiana plants were grown in a glasshouse at 23°C. cDNA library construction and screening of cell death-inducing factors by toothpick inoculation of *Agrobacterium tumefaciens* clones were reported previously (Nasir et al., 2005).

Agrobacterium-mediated transient overexpression of NbLCB2.

A DNA sequence corresponding to HA tag (YPYDVPDYA) was added to the 3' end of the open reading frame (ORF) of *NbLCB2* by PCR, resulting in *NbLCB2*-HA cDNA. This fragment was cloned into *Xho*I and *Spe*I sites of the GVG-vector pTA7001 (Aoyama and Chua 1997) and transformed into *A. tumefaciens* GV3101. *A. tumefaciens* was grown in L broth. The bacterial cells were harvested and resuspended in 10 mM MgCl₂, 10 mM MES (morpholineethanesulfonic acid) (pH 5.6), and 150 μM acetosyringone, and kept at room temperature for 2 h. For transient expression, separate cultures containing pTA7001-derived constructs and 35S-p19 (Voinnet et al. 2003) were mixed in a 1:1 ratio. The culture was then infiltrated to the underside of 4-week-old *N. benthamiana* leaves. Two days after *A. tumefaciens* infiltration, DEX (30 μM in 0.1% ethanol) was infiltrated to induce transgene expression. *NbLCB2*^{ΔPLPBS}, a deletion mutant of the PLP-binding site of *NbLCB2*, was generated by the overlap extension PCR method.

Immunoblot analysis.

For detection of NbLCB2-HA protein, equal amounts of leaf samples were ground under liquid nitrogen and extracted with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After centrifugation, the supernatants were used for 10% SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). After binding of the anti-HA antibody (Roche), the localization of antigen was detected by a secondary antibody (Sigma, St. Louis) and visualized by chemiluminescence using the ECL system (GE Healthcare, Bucks, U.K.).

RNA gel blot analysis.

Total RNA was isolated according to the method of Nagy and associates (1988). Aliquots (10 μg each) were separated by

electrophoresis on formaldehyde 1% agarose gels and blotted onto nylon membrane (Hybond N+; GE Healthcare). Hybridization was performed as described previously (Nasir et al. 2005).

VIGS of *NbLCB1* and *NbLCB2*.

A cDNA fragment corresponding to the nucleotide positions 1 to 527 of *NbLCB2*, whereby the first nucleotide of the first codon was set to position one, or 452-bp fragments of *NbLCB1*, were cloned into the TRV vector pTV00 (Ratcliff et al. 2001) in antisense orientation, resulting in pTV:*NbLCB2* or pTV:*NbLCB1*, respectively. VIGS assays were performed by following the protocol described by Ratcliff and associates (2001). Confirmation of gene silencing of *NbLCB1* or *NbLCB2* were made by RT-PCR using the primer pair for *NbLCB1* (forward 5'-AAGTTACTTGAGACATGCACCAGG-3', reverse 5'-CCTCCTTCGGTGGCCAATGCATGT-3'), *NbLCB2* (forward 5'-TCTCATCAGAGATCTCCGATTCTG-3', reverse 5'-AACAACAGCTCGGAACTCCTTCG-3'), and *EF1α* (forward 5'-CATCAACATTGTGGTCATTGGCCAC-3', reverse 5'-ATCTGGTCAGAGCCTCAAGAAGAGT-3').

Inoculation of *P. cichorii* and *P. syringae* pv. *tabaci*.

P. cichorii (Hikichi et al. 1998) and *P. syringae* pv. *tabaci* (Taguchi et al. 2001) were grown at 28°C in nutrient broth medium (Difco Laboratories, Detroit) containing rifampicin (20 µg ml⁻¹) overnight. After centrifugation, bacterial cells were resuspended in 10 mM MgCl₂ (optical density at 600 nm = 0.01 and 0.0001 for *P. cichorii* and *P. syringae* pv. *tabaci*, respectively). Bacterial suspensions were infiltrated into leaves using a needleless syringe. The increase in the numbers of bacteria was estimated in leaf disks as described by Coemans and associates (2008).

Chemicals.

Myriocin and sphingoid bases were purchased from Calbiochem and Sigma, respectively, and dimethyl sulfoxide was used for the preparation of stock solutions. The same concentrations of solvents were used as control in this experiment.

Detection of cell death.

Cell death was examined by Evans Blue staining. Leaf disks were vacuum infiltrated in 0.1% Evans Blue for 10 min and maintained for 30 min under vacuum. After staining, leaf disks were washed for three times (15 min each) with a water containing 0.05% Tween 20. Leaf disks were homogenized and bound dye was extracted with 1% SDS. The extracted dye was measured spectrophotometrically at 600 nm.

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