Studies on Arabidopsis ERF9 and NSL2 involved in stress responses.

（環境ストレスに関与するシロイヌナズナ ERF9 と NSL2 の研究）

by

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A Dissertation
Submitted to the Graduate School of Life Science
Hokkaido University
in partial fulfillment of the Requirements for the Degree of Doctor of Life Science

Division of Cellular Life Science
Graduate Course of Biosystem Science
Graduate school of Life Science
Hokkaido University, Sapporo, Japan

December 2013

Acknowledgment

I wish to express my sincerely gratitude to Dr. Junji Yamaguchi, Professor of Graduate School of Life Science, Hokkaido University, for devoted guidance, pertinent advice, significant discussion, kindly suggestion and critical review throughout my PhD study and the writing of this thesis.

I am very grateful to Drs. Yukako Chiba, Takeo Sato, Yutaka Asada for their technical advises and significant discussion. I am greatly thankful to all members in my laboratory and Hokkaido University for their assistance, help, and friendliness.

I would like to express my heartfelt thanks to my family for their encouragement and kindness all of my life.

December 2013

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Arabidopsis NSL2 regulated cell death were modulated by chloroplast enzyme activity and protein degradation.

Chapter 1

General introduction

General introduction

Plant adjusts to changes in environmental condition

Plants are many environmental stress, biotic stress and abiotic stress. The heat, cold, drought (Levitt *et al* 1972; Guy *et al* 1999), and salt stress are categorized as abiotic stress. The havibolus, virus, bacteria and nematode are biotic stress. Theses stress exists in the surroundings of the planta. However plant had adjusted their environmental stress from long time ago. One of the mechanisms that adjustment of their environment is cell death. Cell death is related to many life phenomena. About the histogenesis, the portion of the hand in an animal is guided by cell death. The morphogenesis, early embryo formation is related cell death as same as plant. For the plant and animal, programmed cell death (PCD) is an essential of development and of responses to abiotic stress or pathogens (Hofius *et al* 2007).

The cell death is important role for plant stress responses

Cell death in plants is related to development, senescence, resistance and morphogenesis (Thomas *et al* 2003). The activation of plant resistance to their invaders, the cell death is powerful tool for defense to invaders (Soosaar *et al* 2005). According to the TAIR (Arabidopsis Information Resource ;http://www.arabidopsis.org/) database, there are about 150 genes in connection with cell death. DEAR1 (DREB and EAR motif protein 1) and NSL2 (Necrotic Spotted Lesion 2) are cell death related genes. DEAR1 overexpressor showed constitutive cell death phenotype (Thurhui et al 2009) as same as *nsl2* (Morita-Yamamuro *et al* 2005). The studies on the plant cell death have been considerably. About the cell death, there are three major types in animals, Apoptosis, autophagic cell death and necrosis (Kroemer *et al* 2009).

Plant resistance for pathogen

Plants are continually exposed to a many numbers of pathogens and, as a result,

they have evolved defense mechanisms to recognize and defend themselves against the invading pathogens (Atkinson *et al* 2011). The plant defense response induced a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defense related genes [e.g. PR (pathogenesis-related) genes or PDF1.2 (plant defensin)], and the oxidative burst (Attack 1995). Host resistance is often governed by resistance genes, the products of which interact with the specific elicitors made by the avirulence genes of pathogens (Flor *et al* 1971; Hammond-Kosack *et al* 1997).

Plant defense signaling

The plant signaling components are involved during the induction of plant defense. An invading pathogen has to avoid plant defense signaling components to cause disease successfully. Some plant hormones modulating inducible defenses are salicylic acid (SA) and jasmonic acid (JA)/ ethylene (ET) (Pieterse *et al* 2009). For example, the ET is an important signaling component during plant-pathogen interactions. ET perception is often required for basal resistance against pathogens and it can also induce disease resistance in plants (Merchante *et al* 2013). An ethylene-insensitive tobacco has been shown to lack non-host resistance against several soil-borne fungi (Kroemer *et al* 2008).

Plant resistance to necrotrophic and biotrophic pathogens

The plant invaders were classified into necrotroph and biotroph (Glazebrook *et al* 2005). Necrotrophic pathogens destroy plant cells, often through the production of phytotoxins, after which they feed on the contents. Biotrophic pathogens derive nutrients from living plant tissues, commonly through specialized feeding structures that invaginate the plant cells without disrupting (Glazebrook *et al* 2005). The plants possess resistance for biotrophic pathogens are mediated by SA dependent pathway (Loake *et al* 2007). On the other hand, the resistance to necrotrophic pathogens are mediated by ET and JA pathway (Turner *et al* 2002).

Cross Talk in Defense Signaling

SA pathway and ET/JA pathway are cross talking (Koornneef et al 2008). Some studies have reported that exogenous SA treatment suppresses the expression of JA induced genes (Pena-Cortés *et al* 1993; Doares et al 1995). When the herbivore attacked to plant, the JA signal is activated. At the same time, SA synthesis related *NPR1* expression is suppressed and SA production is negative regulated. These results suggested the regulatory role of SA in cross talk with JA/ET pathway (Koornneef *et al* 2008).

Interaction defense response with abiotic stress and other plant hormone

The SA and JA/ET pathway are affected not only biotic stress but also abiotic stress (Pieterse *et al* 2009). Abscisic acid (ABA) produced by cold stress or drought stress for adjustment of environment. In defense responses, ABA related to the SA and ET/JA pathway, as it was shown to suppress JA/ET dependent gene expression and to affect JA biosynthesis and resistance to necrotrophic pathogens (Adie *et al* 2007; Anderson *et al* 2004). Moreover, auxin to affect JA biosynthesis too (Nagpal *et al* 2005).

About the DEAR1

The he Arabidopsis DEAR1 (DREB and EAR motif protein 1; At3g50260) gene has a protein containing significant homology to the DREB1/CBF (dehydration-responsive element binding protein 1/C-repeat binding factor) domain and the EAR (ethylene response factor-associated amphiphilic repression) motif (Tsutsui *et al* 2009). The cold induced CBF/DREB1 transcriptional pathway plays a critical role in modulating cold and drought stresses responses in Arabidopsis thaliana (Zhou *et al* 2011). The transcription factors CBF/ DREB1 specifically interact with the DRE/CRT cis-acting element and control the expression of many stress-inducible genes in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki 1994; Zhou *et al* 2011).

The overexpressing *DEAR1* showed a dwarf phenotype and HR-like cell death,

together with constitutive expression of *PR* and *PDF1.2* genes, with accumulation of plant resistance-related hormone such as SA and JA (Tsutsui *et al* 2009). DEAR1ox also showed resistance to P. syringae pv. tomato DC3000. In addition, DEAR1 ox was increased sensitivity about cold stress as compared with the plant of a wild type. At the same time, DREB1/CBF family genes were suppressed in DEAR1ox (Tsutsui *et al* 2009).

About the NSL2

NSL2 have a MACPF domain in a N-terminal region. The MACPF containing proteins have important roles in bacterial pathogenesis and the immune system in mammalian (Gilbert *et al* 2012). MACPF/ CDC family has conserved mechanism within their domain. The family proteins fold oligomerization into a ring with the targeting of membranes within which the majority contribute to the formation of a pore (Rosado *et al* 2008). About the *planta*, there are little known to the mechanisms of MACPF domain-containing protein. In Arabidopsis, there are four MACPF domain containing protein, NSL1, 2, 3 and 4. (At1g28380, At1g29690, At1g14780, At4g24290). The lack of NSl1 and 2 shows cell death phenotype, but other genes had not known (Morita-Yamamuro *et al* 2005; Noutoshi *et al* 2006). The *nsl1* and *nsl2* has a resistance for biotrophic pathogen p. syringe pv tomato, indeed pathogen resistance genes were activated and accumulation of SA. Excepted the pathogen resistance phenotype dwarfism was shown (Morita-Yamamuro *et al* 2005; Noutoshi *et al* 2006).

Outline of this thesis

The main goal of the work described in this thesis was to understand the genetic and molecular mechanisms of induced cell death to environmental stress. In order to clarify plant induced cell death mechanisms. I used *DEAR1* overexpressor and *nsl2* mutant in this study. Theses mutant showed cell death phenotypes. I clarified the part of cell death mechanisms by analyzing of these plants. In chapter 2, I focused in the role for DEAR1 gene to biotic stress.

DEAR1 mediated ERF9 expression by binding to ERF9 promoter. Moreover, ERF9 bound to PDF1.2 promoter resin and negative regulated to the ERF9 expression.

In chapter 3, I focused the mechanisms of nsl2 induced cell death. NSL2 is MACPF domain containing protein. Previously MACPF containing protein was known as a mediator of naturel immunity in mammalian cells. There is little known the role of NSL2 and other NSLs in Arabidopsis. In this chapter, I clarified the mechanisms of *nsl2*induced cell death was regulated chloroplast enzyme and protein.

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Chapter 2

The Arabidopsis transcriptional repressor ERF9 participates in resistance against necrotrophic fungi

Summary

Plant defense hypersensitive response (HR) mediated by plant hormones, such as salicylic acid (SA), jasmonic acid (JA) and ethylene. We previously isolated Arabidopsis DREB AND EAR motif protein 1 (*DEAR1*), and showed that its overexpression resulted in a dwarf phenotype and lesion-like cell death, accompanied by elevated expression of pathogenesis-related(PR*)* genes. Here, we show that transgenic Arabidopsis overexpressing DEAR1 (DEAR1ox) has enhanced resistance to the necrotrophic fungus *Botrytis cinerea* (*B. cinerea*), indicating that it represses negative regulators of plant defense responses, such as transcriptional repressors of the ERF (ethylene response factor) family. Knockout mutants of ERF9 (*erf9*), which were downregulated in DEAR1ox plants, caused the upregulation of pathogen-inducible plant defensin (*PDF1.2)* genes at the transcriptional level, which serve as a well-characterized of the JA and ethylene signaling pathways and enhanced resistance to *B. cinerea*. Biochemical assays demonstrated that the ERF9 binds the GCC box, a cis-element contained in the promoters of the *PDF1.2* gene that possesses transrepression activity. Moreover, infection with *B. cinerea* resulted in upregulation of *PDF1.2* expression, which coincided with suppression of *ERF9* under the control of the *DEAR1* gene. These results indicate that the transcriptional repressor ERF9 participates in plant defense mechanisms against necrotic fungi mediated by the DEAR1-dependent ethylene/jasmonic acid signaling pathway.

Introduction

The induction of plant defense responses upon pathogen infection involves the action of several signaling molecules, including salicylic acid (SA), jasmonic acid (JA), and ethylene (Glazebrook *et al* 1996). Signaling pathways mediated by JA and ethylene mainly contribute to defense against necrotrophic fungi. The *Ein2* (*ethylene insensitive 2*) mutant, which eliminates the ethylene-induced triple-response, is susceptible to infection by necrotic fungi *Alternaria brassicicola* and *B. cinere* (Berrocal *et al* 2002; Thomma *et al* 1999). Furthermore, the *coi1* (*coronatin insensitive 1*) mutant, which is resistant to JA, is also susceptible to infection by *B. cinerea, Plectosphaerella cucumerina,* and *Alternaria brassicicola* (Berrocal *et al* 2002; Lorenzo *et al* 2004).

The Apetala 2/ethylene response factor (AP2/ERF) family of Arabidopsis thaliana is a large group of plant-specific transcription factors that are characterized by a conserved DNA-binding domain, and are important in defense responses mediated by JA and ethylene (Fujimoto *et al* 2000; Nakano *et al* 2006). Among its four major subfamilies; namely, AP2, RAV, ERF, and dehydration-responsive element-binding protein (DREB), the ERF protein has a conserved domain that binds to sequences containing the AGCCGCC motif (GCC box) (Nakano *et al* 2006). The GCC box is reportedly a major *cis*-acting element in infection inducible genes involved in JA and ethylene-mediated defense responses, such as pathogen-inducible plant defensin 1.2 (*PDF1.2)* and pathogenesis-related 3 (*PR3)*, which encodes chitinase (Nakano *et al* 2006; Brown *et al* 2003; Hao *et al* 1998). The Arabidopsis thaliana ERF transcription factor 1A (AtERF1) is synergistically induced by JA and ethylene, and integrates signals from these two plant hormones, designated as the ethylene/JA signaling pathway. Moreover, constitutive expression of the *AtERF1* gene has a positive effect on *PDF1.2* expression, and confers resistance to necrotrophic fungi (Berrocal *et al* 2002; Lorenzo *et al* 2003). On the other hand, AtERF5 negatively regulates chitin-mediated defense responses against the fungal pathogen *Alternaria brassicicola*, and positively regulates defense against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000, mediated by the SA signaling pathway (Geon *et al* 2012).

Arabidopsis has eight proteins belonging to subgroup VIII in the ERF family, all of which contain the EAR (ERF-associated amphiphilic repression) motif, which is responsible for transcriptional repression (Nakano *et al* 2006; Hiratsu*et al* 2003; Ohta*et al* 2001). AtERF4 and AtERF7 inhibit expression of

ethylene-responsive genes, thereby reducing ethylene sensitivity through suppression of abscisic acid (ABA)-responsive genes (Yang *et al* 2005). Previously, we identified the Arabidopsis *DEAR1* (DREB AND EAR motif protein 1) gene, which encodes the DREB (dehydration-responsive element binding) domain and EAR motif (Tsutsui *et al* 2009). DREB is a conserved domain among members of subgroup I-IV in the ERF transcription factor family, and binds to the DRE/CRT (dehydration-response element/C-repeat) sequence $^{A}/_{G}$CCGAC. The DRE/CRT sequence is a major *cis*-acting element in cold-inducible genes, such as *RD29A* and *COR15A* (Nakano *et al* 2006; Yamaguchi-Shinozaki *et al* 1994). We previously showed that *DEAR1* mRNA accumulates in response to both pathogen infection and cold treatment (Tsutsui *et al* 2009). In addition, transgenic Arabidopsis overexpressing DEAR1 (DEAR1ox) showed a dwarf phenotype and lesion-like cell death, together with constitutive expression of *PR* genes, including *PDF1.2,* and accumulation of SA. DEAR1ox plants also showed limited *P. syringae* pathogen growth, consistent with an activated defense phenotype. Indeed, transient expression experiments demonstrated that the DEAR1 protein repressed DRE/CRT-dependent transcription, which is regulated by low temperature. Induction of the DREB1/CBF family of genes by cold treatment was suppressed in DEAR1ox plants, leading to reduced freezing tolerance (Tsutsui *et al* 2009). These results suggest that DEAR1 has an upstream regulatory role in mediating crosstalk between signaling pathways for biotic and abiotic stress responses (Tsutsui *et al* 2009). Furthermore, microarray analyses ERF9 downstream target of DEAR1 (Tsutsui *et al* 2009), and ERF9 and ERF14 were reported to inhibit the expression of *PR* genes and confer colonization of *Piriformospora indica* (Iris *et al* 2010; Camehl *et al* 2010). The possibility of cell death mediate genes, VIII group genes were reported. When the transient expression of VIII group belonging genes in the tobacco, more than half genes induced cell death, that was contained ERF9 (Ogata *et al* 2010).

In this study, we further investigated the relationship between DEAR1 and ERF9, with special emphasis on resistance against necrotrophic fungal infection. We found that infection of Arabidopsis *by B. cinerea* promoted DEAR1 expression and suppressed ERF9. We also demonstrated that the *erf9* knockout mutant enhanced resistance to *B. cinerea,* and showed that ERF9 directly binds to and dominantly represses *PDF1.2* gene expression. Taken together, these data indicate that ERF9 acts as a negative regulator of plant defense mechanisms

mediated by the DEAR1-dependent ethylene/JA pathway.

Materials and Methods

Plant materials and growth conditions

For germination of *Arabidopsis thaliana* (ecotype Columbia-0) wild-type and mutants, seeds were surface-sterilized and placed on MS medium supplemented with 2% sucrose (germination-inducible medium, GM). After cold treatment for 2 d to synchronize germination, seeds were transferred to 22°C and 50% relative humidity under a 16-h light/8-h dark cycle (Asada *et al* 2011). Seeds of the *erf9-1* and *erf9-2* mutant were obtained from the Arabidopsis Biological Resource Center (ABRC, stock numbers SALK_043407 and CS854716). The T-DNA insertion homo line was confirmed by genomic DNA PCR.

Transcript level analysis

Total RNA (0.7 μg), which was isolated from seedlings using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used as a template for first-strand cDNA synthesis using ReverTraAce-α-® reverse transcriptase (TOYOBO, Osaka, Japan). First-strand cDNA (0.5 μl) was then assayed for gene-specific DNA fragments using the primer pairs listed in Table S2, designated as DEAR1-1 and DEAR1-2, ERF9-1 and ERF9-2, ERF1-1 and ERF1-2, and ACT2 and PDF1.2 primer, as previously described (Zhang *et al* 2011; Sato *et al* 2011). Primers were also used to amplify DNA fragments of *DEAR1*, *ERF9*, *PDF1.2*, *ERF1*, and *ACT2* from their cDNAs by PCR. PCR was performed with optimized cycling conditions for each gene using *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA). The amplified fragments were electrophoresed on 1.2% (w/v) agarose gels and visualized by ethidium bromide staining. Quantitative real-time PCR (qPCR) was performed with the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TAKARA RRA420A) on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, forest City, CA, USA) according to the manufacturer's instructions. Expression level of *ACT2* was used for signal normalization. Primers used for qPCR are described in Table S1. Relative gene expression was calculated using the delta delta CT method (Livak *et al* 2001).

B. cinerea and P. syringae infection

The *B. cinerea* strain ET1 was incubated at 20°C on potato dextrose medium

containing 2% (w/v) agar (Wako, Osaka, Japan) under continuous dark conditions for one week, and was then scratched off with a brush. Thereafter, it was grown under continuous irradiation with blacklightblue (BLB) for two weeks. Spores were collected, suspended in half-strength potato dextrose broth, and adjusted to approximately 1 x 10^5 conidia/ml. Then, 5 μl of the suspension was spotted on each rosette leaf. Infection with *P. syringae* was performed according to the method by Tsutsui *et al.* (Tsutsui *et al* 2009), and leaves were stained with trypan blue to measure the lesion area caused by the fungi as previously described (Morita-Yamamuro *et al* 2005).

Electrophoretic Mobility Shift Assay (EMSA)

The coding region of DEAR1 and ERF9 gene were cloned into the pENTR/D-TOPO vector and sequenced. After sequencing, the gene was cloned into pYU1274 destination vector (Gateway-compatible version of pGEX4T-1 provided by Dr. Yoshihisa Ueno, National Institute of Agrobiological Sciences). by LR reactions and was expressed in *Escherichia coli* BL21 (DE3) *pLysS* cells. Both strands of the following oligonucleotides were synthesized: wild-type (DRE: AGCTATTCATATTGGGCCGACAAAAAAAAA), and mutant (mDRE : AGCTATTCATATTGGGCTTTAAAAAAAAA) for DERA1; GCC box, (GCC: CAGATTAACCAGCCGCCCATGTGAACG) and mutant GCC box (mGCC: CAGATTAACCATCCTCCCATGTGAACG), with underlined letters indicating replacement of G with T. The EMSA was performed using the digoxigenin Gel Shift Kit, 2nd generation (Roche Diagnostics) according to the manufacturer's instructions. Protein and labeled DNA were incubated with binding buffer at room temperature for 15 min and separated on an 8% polyacrylamide gel and transferred to a positively charged nylon membrane. The membrane was cross-linked with UV and probed with as sheep anti DIG antibody and detected using the chemiluminescent reagent (Roche Diagnostics) by exposing the membrane.

Transient expression

To generate the reporter gene construct, an approximate 500-bp region containing the GCC box sequence from the Arabidopsis *PDF1.2* gene was amplified using PDF1.2 pro-1 and PDF1.2 pro-2 primers (Table S2), placed upstream of the minimal TATA box from the cauliflower mosaic virus (CaMV) 35S promoter, and fused transcriptionally to the β-glucuronidase (GUS) gene.

For effector plasmids, the coding region of each AtERF gene was replaced in pUGW2 (Nakagawa *et al* 2007) by LR recombination (Invitrogen). Transient assays were performed using the polyethylene glycol (PEG) method (Tsutsui *et al* 2009; Shimada et al 2005). The Renilla LUC gene (Promega Madison, WI, USA) under the control of the CaMV 35S promoter (pREX-LUC) was used as a reference reporter gene. After transformation, the samples were washed three times with JPL medium (0.4 M D-mannitol, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES/KOH, pH 5.6), incubated in a growth chamber at 22°C overnight, and quantified for luciferase (LUC) and beta-glucuronidase (GUS) activities.

Chromatin immunoprecipitation assay

Chromatin samples were prepared as described previously (Saleh *et al* 2008). 35S:DEAR1-GFP overexpression lines (Tsutsui *et al* 2009) and 35S:GFP (as a control) were used. About 4 g of each plant were fixed with 1% formaldehyde contain cross-linking buffer for 10 min and extracted DNA for immunoprecipitation. The chromatin samples served as the input controls were incubated overnight at 4°C either with or without anti-GFP or anti-myc monoclonal antibody beads (MBL). The eluted DNA was analyzed by semi-qPCR and qPCR using the following specific primers: ERF9-c1, ERF9-c2 (Supplementary Table S2-1), ACT2-c3 primers as previously described (Dong *et al* 2010). The amplified bands were visualized on a 2.5% agarose gel. ChIP-qPCR data was analysed by delta Ct method (Livak *et al* 2001). Each ChIP samples were normalized by input value.

Results

DEAR1 expression confers resistance to the necrotic fungus *B. cinerea* and stimulates *ERF9* expression

We previously demonstrated that transgenic Arabidopsis overexpressing DEAR1 (DEAR1ox) showed accumulation of SA and promoted *PR* gene expression, leading to restriction of hemibiotrophic bacterial growth (e.g., *Pseudomonas syringae* DC3000 pv. *Tomato*). Moreover, DEAR1ox showed elevated expression of *PDF1.2* (Tsutsui *et al* 2009), indicating that DEAR1 integrates signaling from JA, ethylene, and SA, leading to pathogen resistance. To determine if DEAR1ox also confers resistance to necrotrophic fungi, we carried out infection analysis using *B. cinerea*. Specifically, mature leaves from 3-week-old wild-type and DEAR1ox plants were inoculated with 1 x 10^5 spores per ml of *B. cinerea*. Disease symptoms characterized by large lesions were observed in the wild-type plant after trypan blue staining, whereas such lesions were small and restricted to the inoculation site of the DEAR1ox plant (Fig. 2-1a). In addition, the size of the necrotic area caused by the fungus in DEAR1ox was half of that observed in the wild-type plant (Fig. 2-1b). The *PDF1.2* gene, which encodes pathogen-inducible plant defensin and is induced during the response to necrotic fungal attack (Penninckx*et al* 1998), was transcriptionally activated in DEAR1ox, indicating that *DEAR1* overexpression is sufficient to increase resistance to the necrotic fungus *B. cinerea.*

Since DEAR1 contains a DREB domain and an EAR motif, it is predicted to control transcriptional repression of downstream target genes possess a DRE/CRT sequence in their promoter region. In a previous study (Tsutsui *et al* 2009), microarray analysis of DEAR1ox revealed that *ERF9* (At5g44210), one member of the ERF gene family, is a candidate target gene of *DEAR1*, since the *ERF9* gene has a DRE motif in the promoter region and is transcriptionally repressed in DEAR1ox plants (Table S2-1). Therefore, we evaluated the *ERF9* gene as a potential downstream target of *DEAR1*.

ERF9 is induced by the plant hormone and wounding

ERF family members were originally reported as ethylene-inducible genes (Fujimoto *et al* 2000; Ohme-Takagi *et al* 1995. Treatment with ACC (1-aminocyclopropane- 1-carboxylate), a precursor of ethylene biosynthesis that promotes its production, activated expression of *AtERF1* (At3g23240), a typical

ethylene-inducible gene (Hao *et al* 1998; Penninckx *et al* 1998) as well as that of *ERF9* (Tsutsui *et al* 2009; Zhang *et al* 2011). Although *AtERF1* was up regulated, *ERF9* was down regulated in DEAR1ox (Table S1).

We also investigated expression patterns of *DEAR1* and *ERF9* under various stress conditions (Fig. 2-2). For expression analysis, mRNA was extracted from 14-day-old seedlings of WT plants after hormone treatments (MeJA and SA) and wounding. *DEAR1* transcript was dramatically increased after all the treatments. *ERF9* was suppressed at 3-6 hours after all the treatment, but the transcript level restored to the initial level at 12 hours when the DEAR1 transcript reduced. These results suggest that both genes are transcriptionally associated with various stress treatments.

Knockout of the ERF9 gene confers resistance to necrotic fungi

To determine the physiological role of ERF9, we analyzed allelic homozygous T-DNA insertion mutants on a Columbia-0 background, *erf9-1* and *erf9-2* (Fig. 2-3a). Expression analysis showed that the mutant did not produce *ERF9* mRNA (Fig. 3b), and morphological analysis of the *erf9* mutant plants showed no differences when compared to the wild-type plant (Fig. 2-3c). However, a different response against necrotic fungi was observed in the mutant (Fig. 2-4a). In spot-inoculated wild-type plants, inoculation of *Botrytis* resulted in increased disease symptoms characterized by large lesions surrounded by extensive chlorosis (Fig. 2-4b). In contrast, although inoculation of fungal spores in *erf9* mutants also led to the formation of disease lesions, the lesions were largely restricted to the inoculation site, indicating that the mutant had increased resistance to the fungal pathogen. *B. cinerea* invades host plant cells through three steps: germination, formation of appressorium, and penetration through the plant cell wall and plasma membrane. Hyphae grown from the first appressorium do not penetrate plant cells, but hyphae grown from the second appressorium penetrate plant tissues (Mendgen *et al* 1996). Restriction of disease symptoms was also demonstrated by trypan blue staining (Fig. 2-4c). In addition, the size of the necrotic area caused by the fungus in the *erf9* mutants was smaller than that in the wild-type (Fig. 2-4d). These results indicate that knockout of the *ERF9* gene is sufficient to increase resistance to the necrotic fungus *B. cinerea*, suggesting that the underlying processes of appressorium formation and penetration were inhibited in the mutant plant, since the *PDF1.2* gene was activated (Fig. 2-3b).

Gene expression analysis after *B. cinerea* infection was also examined (Fig.

2-5), and the results showed that *DEAR1* and *ERF1* gene expression did not significantly differ between the wild-type and *erf9* mutant. However the expression level of *PDF1.2* was upregulated 0 and 12 hours after infection in the *erf9* mutant compared to wild-type. Additionally, the GCC box-containing *CHIB* gene, another marker gene of resistance, was also transcriptionally upregulated 12 and 24 hours after infection in the *erf9* mutant. These data indicate the possibility that the *PDF1.2* gene and other GCC box-containing resistance genes are transcriptionally regulated by ERF9.

We also examined resistance to pathogenic bacteria *S. syringae* pv. *tomato* DC3000 in the *erf9* mutant (Fig. S2-1). A marked increase in bacterial numbers was detected on wild-type leaves on day 2 after inoculation, while no significant decrease in bacterial numbers was observed in the *erf9* mutant, indicating that the *erf9* mutant does not show a resistance phenotype. Taken together, we conclude that ERF9 is associated with resistance to necrotic fungal infection.

DEAR1 binds to the genomic region of ERf9 promoter.

To determine whether *ERF9* as direct target of DEAR1, we attempted EMSA and chromatin immunoprecipitation (ChIP) approach. We used 5 or 10 μg of GST (Glutathione S-transferase) fused DEAR1 protein in this assay. Dig-labelled DNA (4 ng) was loaded each line. The possibility of non-specific binding of GST protein was also eliminated as no shifted bands were observed when the GST protein was incubated with the wild type probe. The affinity of protein-DNA complexes was detected when GST-DEAR1 fusion protein was incubated with wild type probe (Fig. 2-6a, 6b). These results indicate that the interaction between wild type probe and the DEAR1 was specific.

Furthermore, we determined the binding efficiency of DEAR1 to ERF9-c1 and ERF9-c2 fragments by semi-qPCR and qPCR assay. The ChIP assay using 35S:DEAR1-GFP transgenic plants which express a GFP-tagged DEAR1 protein (Tsutsui *et al* 2009) and 35S:GFP plant was used as a control. Specific immunoprecipitation was conducted with an anti-GFP antibody and an anti-myc anti-body (as non-specific control). Actin was used as a control for the non-DRE fragment (Fig. 2-6c). Consistent with both PCR results, DEAR1 was enriched at the ERF9-c2 fragment than ERF9-c1, indicating that the DEAR1 binds to ERF9-c2 fragment with higher efficiency than ERF9-c1 fragment (Fig. 2-6d, 6e). These results indicate that the DEAR1 directly interacts to DRE *cis*-element in the *ERF9* promoter region.

ERF9 protein is located in the nucleus and exhibits DNA-binding activities to the PDF1.2 gene

The ERF9 protein contains the ERF and the EAR motifs, and is therefore predicted to function as a transcriptional repressor of genes containing the *cis*-acting GCC box within their promoter, as well as the *PDF1.2* gene (Table S2-1). To analyze whether ERF9 has GCC box binding activity, we purified ERF9 protein as a GST fusion protein (ERF9) expressed in *E. coli,* and performed EMSA. As shown in Figure 7, ERF9 was able to bind to a 27-bp oligonucleotide that contained the wild-type GCC box sequence (AGCCGCC), whereas competitive activity was abolished when both G residues within the GCC box were replaced with T residues (ATCCTCC). These data indicate that ERF9 has GCC box-specific binding activity. Since ERF9 is a putative transcription factor, it is likely to be localized in the nucleus. Thus, to verify its subcellular localization, we generated an N-terminal translational fusion of the ERF9 cDNA with the sequence encoding green fluorescent protein (GFP). The plasmid containing 35S:GFP-ERF9 was introduced into *A. tumefaciens* (GV3101pMP90), which then infiltrated into *N. benthamiana*. The recombinant protein accumulated exclusively in the nuclei of epidermal cells, whereas GFP protein alone accumulated in both the cytoplasm and the nucleus because of its small size (Fig. S2-2). These results demonstrate that the ERF9 protein is localized in the nucleus, supporting its predicted role as a transcriptional regulator.

ERF9 suppresses PDF1.2 gene transcription

The ability of ERF9 to regulate transcription in plant cells was evaluated using transient expression assays (Fig. 2-7c, d). Specifically, a reporter plasmid with the native *PDF1.2* promoter containing the GCC box sequence fused to GUS, and an effector plasmid consisting of ERF9 or AtERF1 cDNA under the control of CaMV 35S promoter (Fig. 2-7c), were delivered to protoplasts prepared from Arabidopsis cultured cells T87 by the polyethylene glycol method (Tsutsui *et al* 2009). Expression of the AtERF1 protein in protoplasts resulted in activation of GUS reporter gene expression (Fig. 2-7d), consistent with a previous report, which demonstrated that AtERF1 functions as a transcriptional activator of the *PDF1.2* gene. In contrast to AtERF1, expression of ERF9 repressed transcription of the reporter gene. Furthermore, coexpression of ERF9 and AtERF1 with the reporter construct resulted in similar repression as expression of

ERF9 alone. These results indicate that ERF9 dominantly represses transcription of the *PDF1.2* gene, which is consistent with the *erf9* knockout mutant showing upregulation of the *PDF1.2* gene (Fig. 2-2b).

Discussion

To clarify the function of ERF9 in plant defense responses mediated by DEAR1, we examined resistance to the necrotic fungus *B. cinerea* in the *erf9* mutant. The DEAR1 overexpressor plant exhibited resistance to *B. cinerea* (Fig. 2-1a, b), suggesting that DEAR1 is a positive regulator of the JA-mediated pathway. We demonstrated that the ERF9 gene is transcriptionally regulated by *B.cinerea* infection and plant hormone treatment (Figs. 2-3, 5), and that the *erf9* mutant shows increased resistance against *B. cinerea* and activation of *PDF1.2* gene (Figs. 2-4, 5). Furthermore, we also demonstrated that the ERF9 protein binds to the GCC box in the promoter of the *PDF1.2* gene (Fig. 2-7b), and directly represses transcription of the gene (Fig. 2-7d). Therefore, we concluded that the ERF9 protein is a negative regulator of resistance against *B. cinerea* under the control of the upstream regulator DEAR1. Taken together, we propose the following model of action for DEAR1 and ERF9 during fungal infection (Fig. 2-8). When the plant is not subject to fungal attack, ERF9 regulates the ethylene/JA defense pathway, including *PDF1.2* expression. Once the plant is infected by necrotrophic fungi, DEAR1 expression is induced and directly represses *ERF9* expression, leading to activation of the defense pathway. In addition to the DEAR1 dependent pathway, ERF1 (and other ethylene/JA-inducible ERF proteins) is also induced during fungal infection (Liu *et al* 1998; Sakuma *et al* 2002), which in turn, promotes expression of the *PDF1.2* gene and other GCC box-containing genes, ultimately leading to enhanced resistance against fungal infection. Finally, ERF9 may function to inhibit defense activation through the ethylene/JA signaling pathway once the initial infection has been successfully restricted.

Indeed, sustained activation of a defense response is a metabolically expensive process; therefore, plants have evolved feedback mechanisms to keep such responses under tight control during normal growth and development. One key method for controlling defense-related gene expression is to use molecular brakes or repressors. Thus, DEAR1 and ERF9, two repressors related to plant defense against fungi, might be serially connected to act as brakes of defense activation.

Arabidopsis has approximately 120 ERF genes that are divided into two major subfamilies: ERF and DREB (Nakano *et al* 2006). Figure 2-2 shows the further division of ERB and DREB into ten groups according to the report by Nakano *et al.* (Nakano *et al* 2006). The DREB subfamily comprises groups I to IV, while the

ERF subfamily includes groups V to X. DREB1A (group III) and DREB2A (group IV) binds to the DRE sequence and plays important roles in cold and hydration tolerance (Liu *et al* 1998; Sakuma *et al* 2000; Sakuma *et al* 2006). DEAR1 (group II) and ERF9 (group VIII) contains an AP2/ERF domain. In addition, DEAR1 contains the CMII-1 motif, which is similar to the CMIII-1 motif that is conserved in Groups II and III. The other motif in DEAR1 is the EAR motif, which is responsible for transcriptional repression (Tsutsui *et al* 2009). Indeed, we demonstrated that DEAR1 expression suppresses cold tolerance and leads to plant defense responses (Tsutsui *et al* 2009).

The ERF subfamily is important in ethylene or JA-mediated stress responses and in the plant defense system. ERF proteins, such as ERF1 and ERF2, activate *PDF1.2* expression. Furthermore, overexpression of these genes results in enhanced plant defense responses and constitutive ethylene or JA responses (Berrocal *et al* 2002; Ohta *et al* 2001; Sakuma *et al* 2006). ERF3 and ERF4 (group VIII) dominantly repress gene expression and plant defense systems (Hiratsu *et al* 2003; Sakuma *et al* 2006; McGrath *et al* 2005). All members of group VIII contain the AP2/ERF domain and the EAR motif. In addition, ERF3, ERF4, ERF7, ERF8, and ERF11 have another motif, CMVIII-2 (Nakano *et al* 2006), indicating that these proteins have a role that differs from that of other members of group VIII. Here, we report that ERF9, which belongs to group VIII and does not contain the CMVIII-2 motif, also contributes to plant defense responses.

Acknowledgments

We are grateful to Dr. Yutaka Asada, Mr. Wataru Kato, and Kazuma Takahashi for their helpful comments and technical assistances and also to Drs. Nakagawa and Mano for providing the pUGW2 vector. This work was supported by Grants-in-Aid for Scientific Research (no. 23380198, 24114701, 25112501) (JY), and by the Cooperative Research Grant of the Gene Research Center the University of Tsukuba. This work was also supported by Grants-in-Aid to YM for Scientific Research for Plant Graduate Students from the Nara Institute of Science and Technology by The Ministry of Education, Culture, Sports, Science, and Technology in Japan (2012-2013).

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Figure and Legend

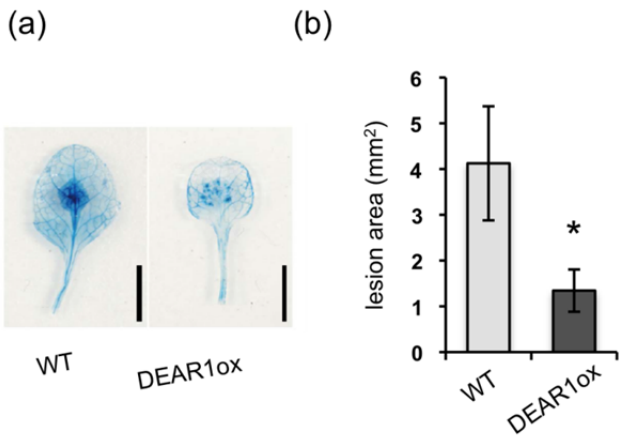

Figure 2-1.

Figure 2-1. Resistance to *Botrytis cinerea* in the DEAR1 overexpressor (DEAR1ox).

(**a**) Two days post-inoculation, macroscopic symptoms of rosette leaf of wild-type (WT) and DEAR1ox 2 were visualized by trypan blue staining. Scale bar: 1 cm. (**b**) Two days post-inoculation, the lesion area was measured in 3-week-old WT and DEAR1ox plants (n = 5; error bars indicate S.D.). Asterisks indicate statistical significance at $P<0.05$ using the t-test. The experiment was repeated three times with similar results.

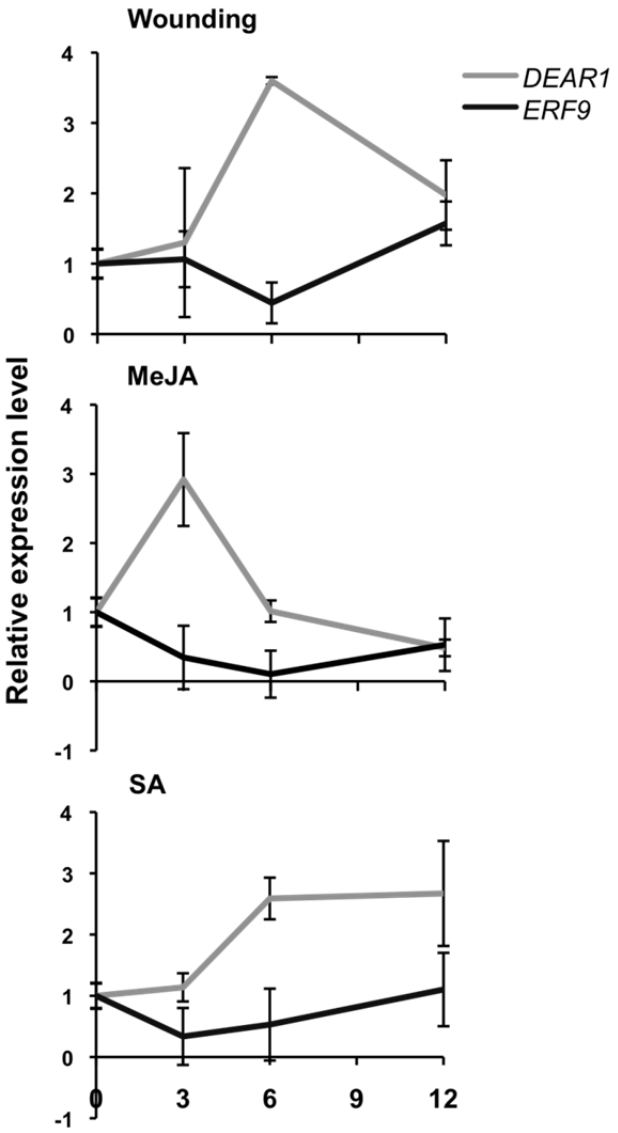
Wounding
MeJA
SA
DEAR1
ERF9
Relative expression level
4
3
2
1
0
-1
0
3
6
9
12

Figure 2-2.

Figure 2-2. The expression analysis of *DEAR1* and *ERF9* using qPCR.
Arabidopsis leaf samples were collected 0, 3, 6 and 12 hours treatments with wounding, MeJA (50 mM) and SA (100 mM). Transcript levels for *DERA1* and *ERF9* genes were normalized to the expression of *ACT2* measured in the same samples of 0 hour as 1. Error bars indicate standard deviation (n = 3).

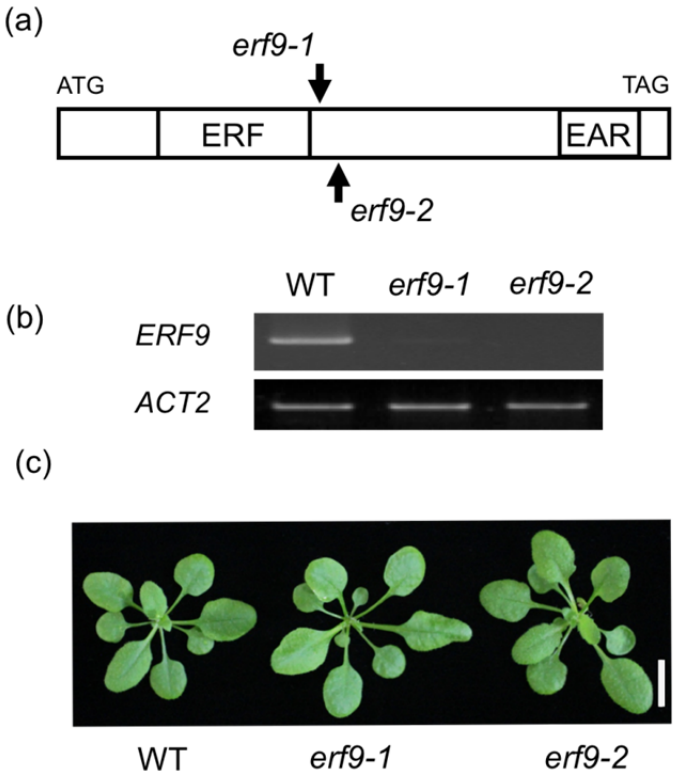
(a)
erf9-1
ATG
TAG
ERF
EAR
erf9-2
(b)
WT
erf9-1
erf9-2
ERF9
ACT2
(c)
WT
erf9-1
erf9-2

Figure 2-3.

Figure 2-3. Phenotypes of the *erf9* mutants.

(**a**) The ERF9 cDNA structure contains the ERF and EAR motifs. The T-DNA was inserted in the ERF motif designated as *erf9-1* and *erf9-1*. (**b**) Expression analysis of *ERF9* and *PDF1.2* genes in *erf9-1* and *erf9-2* Total RNA from 3-week-old wild type (WT) and *erf9* plants was isolated, and expression was analyzed by PCR. *ACT2* was used as a control. (**c**) Macroscopic phenotypic analysis of 3-week-old WT, *erf9-1* and *erf9-1* mutants. Scale bar: 1 cm.

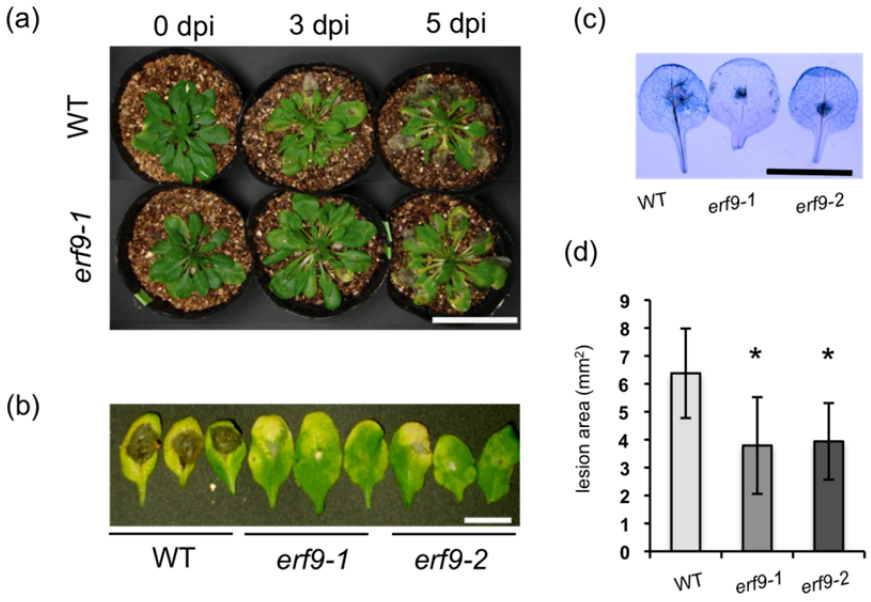
(a)
0 dpi
3 dpi
5 dpi
WT
erf9-1
(b)
WT
erf9-1
erf9-2
(c)
WT
erf9-1
erf9-2
(d)
lesion area (mm²)
0
1
2
3
4
5
6
7
8
9
*
*
WT
erf9-1
erf9-2

Figure 2-4.

Figure 2-4. Resistance of *erf9* mutant against *B.cinerea* infection.

(a) Spore suspension was spotted on each rosette leaf of 7- or 8-week-old plants of the wild-type (WT) and *erf9-1* mutant, and disease responses were observed 0, 3, and 5 days post-inoculation (dpi). Scale bar: 5 cm. (b) Rosette leaves at 4 dpi. Scale bar: 1 cm. Macroscopic symptoms (c) of rosette leaf of 3-week-old plants of WT, *erf9-1 and erf9-2* mutants at 2 dpi were visualized by trypan blue staining, and the lesion area. Scale bar: 1 cm. (d) was measured (n = 5; error bars indicate S.D.). Asterisk in (d) indicates statistical significance at $P<0.05$ using t-test. The experiment was repeated two times with similar results.

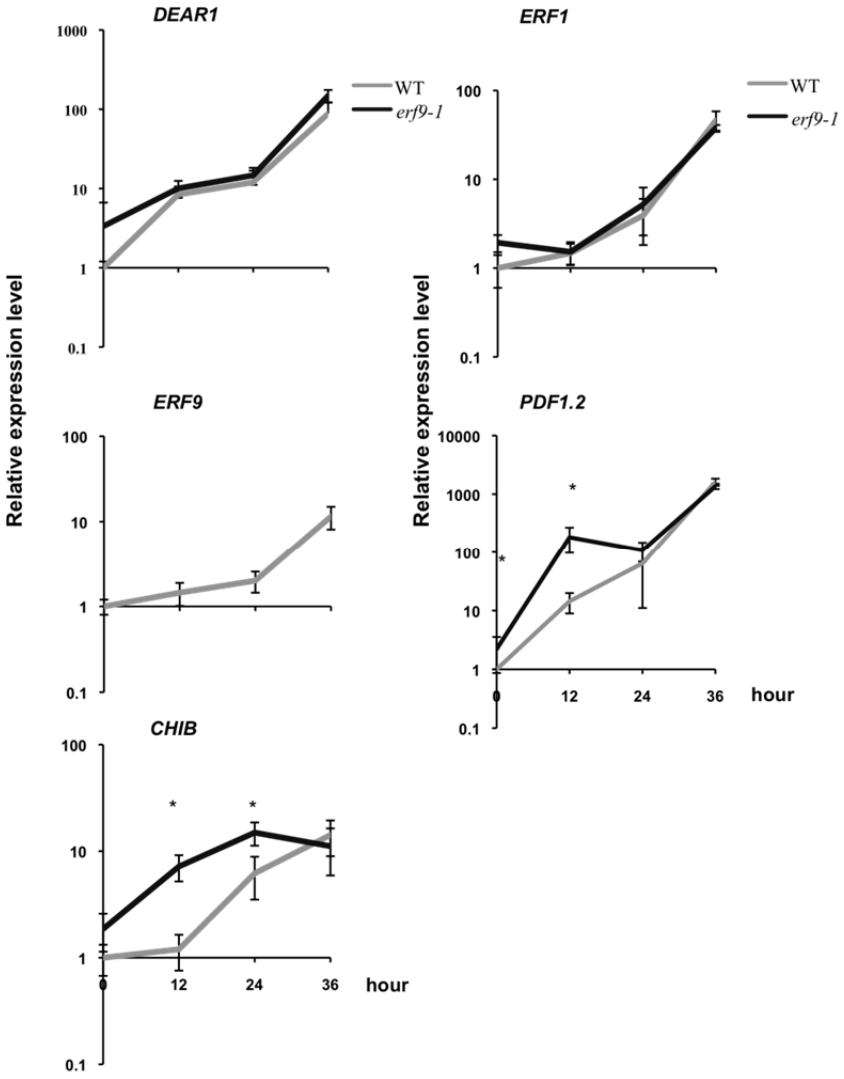
DEAR1
ERF1
ERF9
PDF1.2
CHIB
WT
erf9-1
Relative expression level
hour
0
12
24
36
0.1
1
10
100
1000
10000
*

Figure 2-5.

Figure 2-5. Gene expression analysis after B.cinerea infection.

Analysis of DEAR1, ERF1, ERF9 PDF1.2, and CHIB expression in WT and erf9-1 mutant using qPCR. Arabidopsis leaf samples were collected 0, 12, 24, and 36 hours post-inoculation with B. cinerea. Transcript levels for all genes were normalized to the expression of ACT2 measured in the same samples and expressed logarithmically. An asterisk (*) for PDF1.2 and CHIB genes indicates that erf9-1 mutant is significantly different from WT ($P < 0.05$). Results are mean values ± standard deviation; $n = 3$.

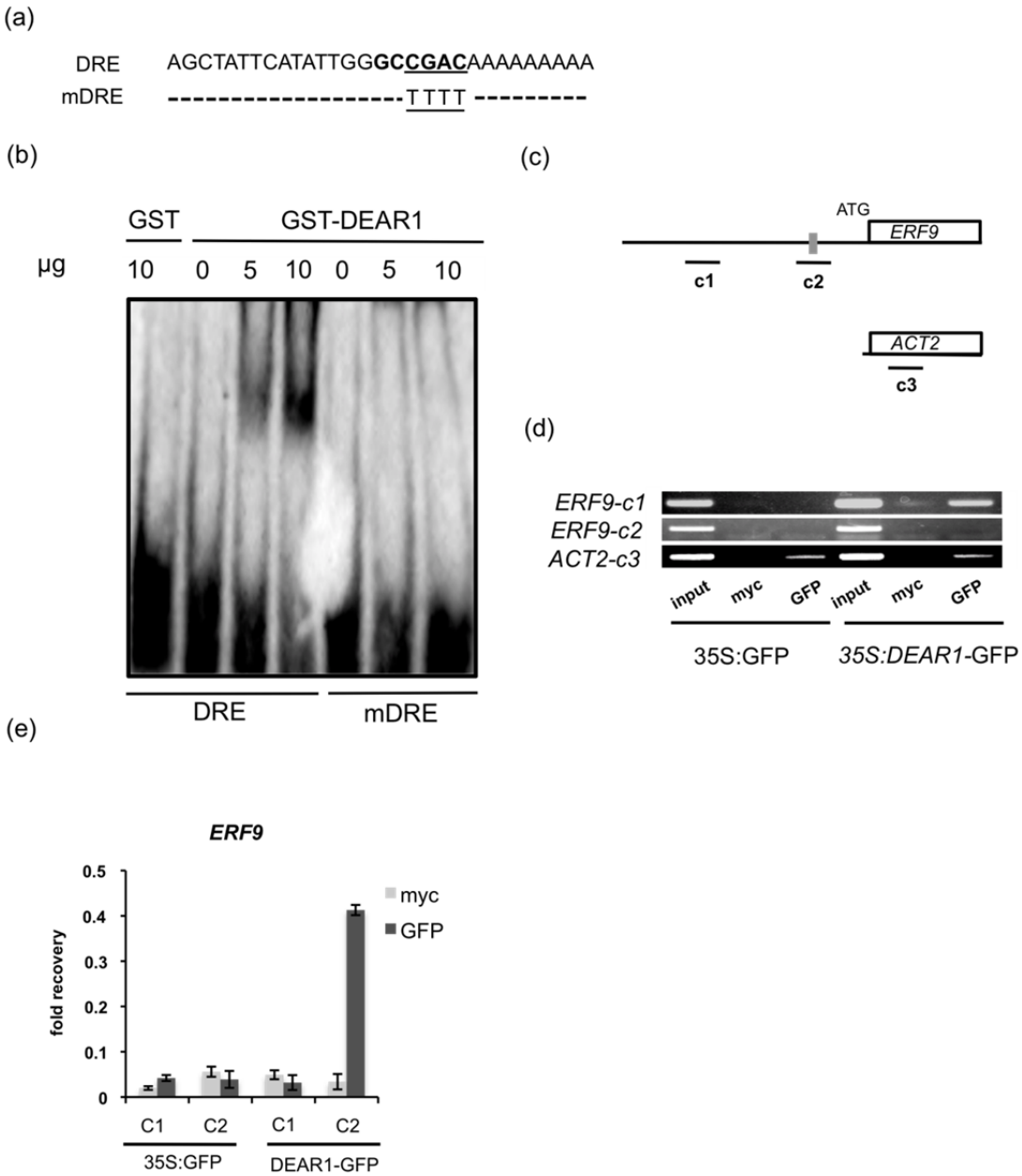
(a)
DRE AGCTATTCATATTGGGCCGACAAAAAAAAA
mDRE ----------TTTT ---------
(b)
GST
GST-DEAR1
μg
10 0 5 10 0 5 10
DRE
mDRE
(c)
ATG
ERF9
c1
c2
ACT2
c3
(d)
ERF9-c1
ERF9-c2
ACT2-c3
input myc GFP input myc GFP
35S:GFP
35S:DEAR1-GFP
(e)
ERF9
fold recovery
0.5
0.4
0.3
0.2
0.1
0
myc
GFP
C1 C2 C1 C2
35S:GFP
DEAR1-GFP

Figure 2-6.

Figure.2-6 *In vivo* and *in vitro* analyses of DEAR1 binding to ERF9 promoter. (a) Nucleotide sequences of probes used in EMSA. **(**b) DEAR1 binding to the DRE element. The oligo-nucleotide probes of wild type DRE (DRE) and mutated DRE (mDRE) used in gel shift assay are listed in (a). DNA probe (4 ng) alone or incubated with 5 μg or 10 μg of recombinant protein (GST-DEAR1) were assayed. Recombinant GST protein was used as control. (c) Schematic model of primer positions at *ERF9* locus. c1 and c2 indicate promoter region of *ERF9* and c3 coding region of *ACT2*. Gray box indicates DRE *cis*-element. Semi-qPCR (d) and qPCR (e) from ChIP of samples showed specificity of DEAR1 binding to *ERF9* promoter. We amplified three regions indicated in (c) to samples before (in put) and after immunoprecipitation (anti-myc and anti-GFP beads) from 35S:GFP (control) and 35S:DEAR1-GFP plants. (e) The fold recovery was normalized against input samples from the same experiment. Error bars indicate SD of technical triplicates.

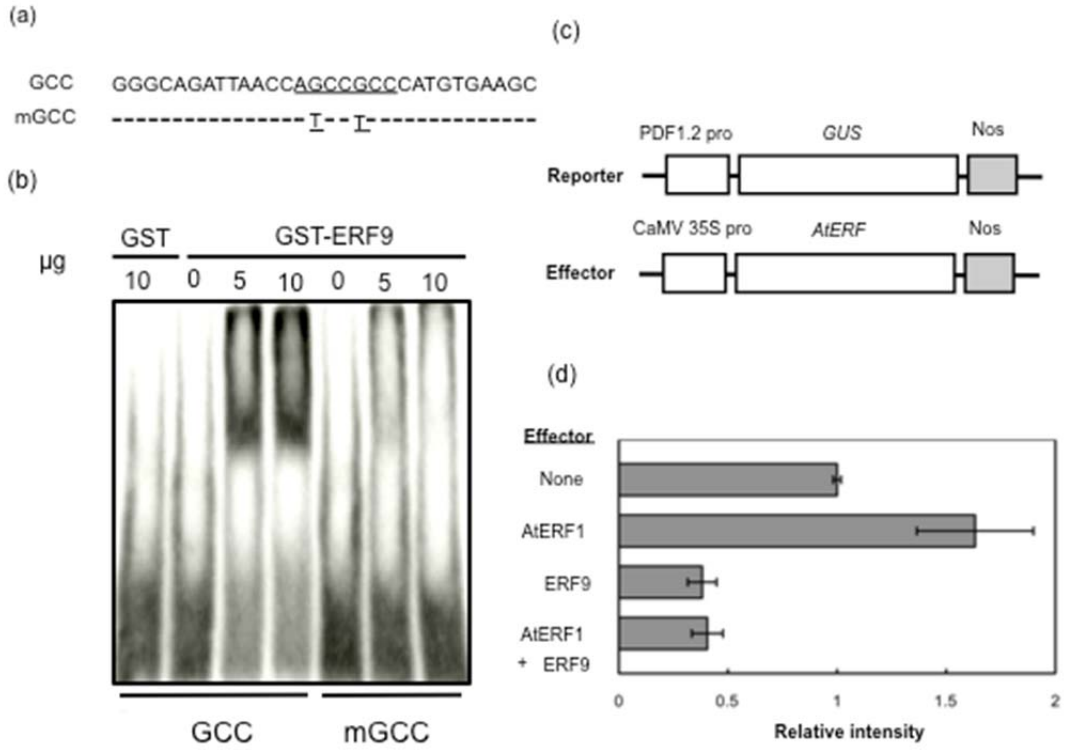
(a)
GCC GGGCAGATTAACCAGCCGCCCATGTGAAGC
mGCC ----------I--I----------
(b)
μg
GST
GST-ERF9
10 0 5 10 0 5 10
GCC
mGCC
(c)
PDF1.2 pro
GUS
Nos
Reporter
CaMV 35S pro
AtERF
Nos
Effector
(d)
Effector
None
AtERF1
ERF9
AtERF1 + ERF9
0 0.5 1 1.5 2
Relative intensity

Figure 2-7.

Figure 2-7. ERF9 binds to *PDF1.2* promoter and trans-regulates *PDF1.2* gene with AtERF1.

(a) Nucleotide sequences of probes used in EMSA. (b) ERF9 binding to the GCC element at the *PDF1.2* promoter site. The oligo-nucleotide probes of wild type GCC (GCC) and mutated GCC (mGCC) indicated in (a) used in gel shift assay are listed. DNA probe (4 ng) alone and incubated with 5 μg or 10 μg of recombinant protein (GST-ERF9) were assayed. Recombinant GST protein was used as control. (c) Schematic representation of reporter and effecter plasmids. To generate the reporter gene construct, a 500-bp region containing the GCC box sequence from the Arabidopsis PDF1.2 gene was fused transcriptionally to the GUS gene. For effector plasmids, the coding region of each AtERF cDNA (AtERF1 or ERF9) was overexpressed under control of the CaMV 35S promoter. (d) Transient assays were performed using the PEG method. The reporter gene was transfected with each effecter plasmid or empty vector as a control. To normalize for transfection efficiency, Renilla LUC gene plasmid under the control of CaMV promoter was cotransfected in each experiment. Bars indicate standard error of three replicates. All LUC activities are expressed relative to reporter construct alone (value set at 1).

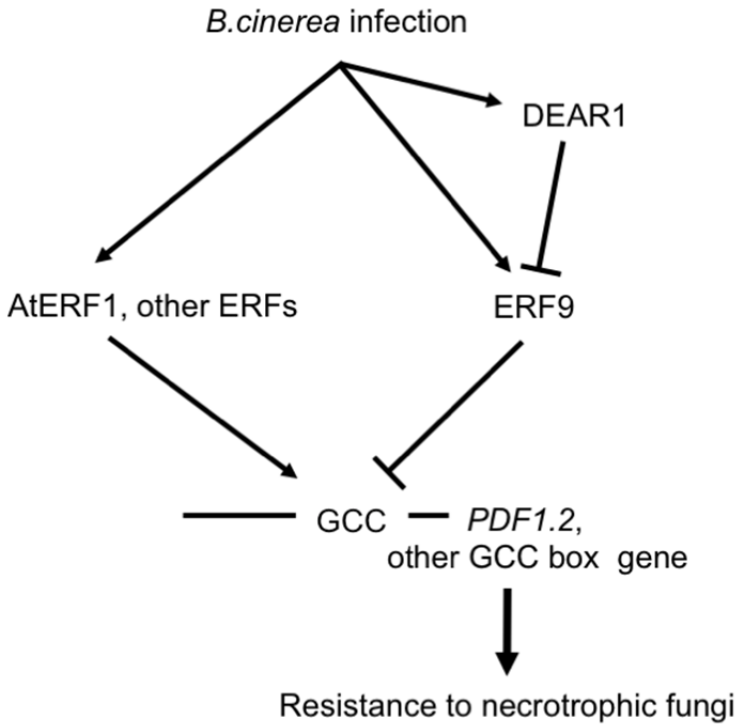
B.cinerea infection
DEAR1
AtERF1, other ERFs
ERF9
GCC
PDF1.2,
other GCC box gene
Resistance to necrotrophic fungi

Figure 2-8.

Figure 2-8. Proposed model of DEAR1 dependent AtERF9-mediated resistance to *B.cinerea*.

(→) and (┤) indicate promotion and repression, respectively.

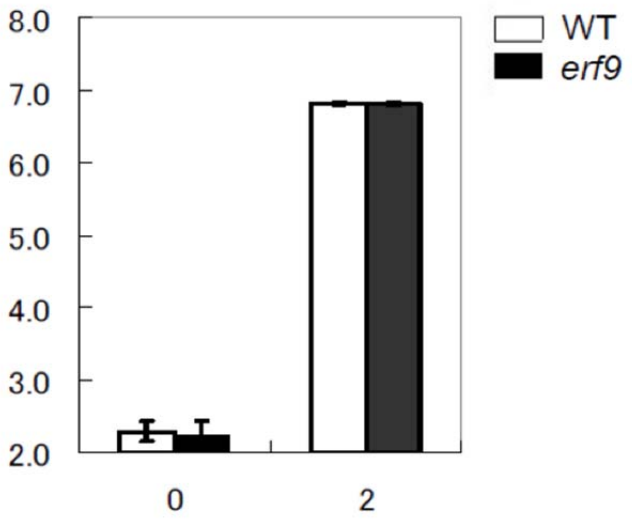

Figure2-S1.

Figure 2-S1.Analysis of bacterial growth (*P. syringae* DC3000) on wild-type (WT) and *erf9-1* mutant plants.

Error bars represent the standard error of three replicates. Each experiment was repeated twice with similar results.

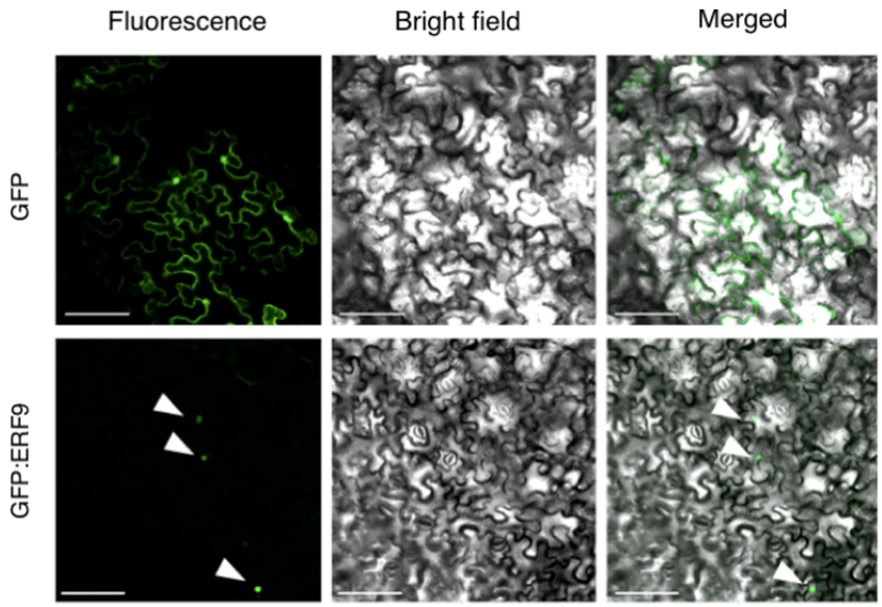

Figure2-S2.

Figure 2-S2. Subcellular localization of GFP-ERF9 fusion protein in *N. benthamiana* leaf epidermal cells.

Images in the top column show the control plasmid expressing only GFP. Those in the bottom column show the GFP-ERF9 fusion protein expressed in *N. benthamiana* leaf epidermal cells. Cells were examined under fluorescence (left), bright field (middle), and as a merged image (right), showing either diffused (control plasmid) or nuclear localization of the proteins (ERF9 fusion). Scale bar: 100 μm.

	Gene Identification	Locus ID	FI	DRE motif	Position	St
ERF Family	ERF1	At3g23240	2.17	non		
	ERF2	At5g47220	2.26	none		
	ERF3	At1g50640	0.77	ACCGAC	-340	-
	ERF4	At3g15210	0.99	none		
	ERF5	At5g47230	1.58	none		
	ERF6	At4g17490	83	none		
	ERF7	At3g20310	0.76	ACCGAC	-308	-
	ERF8	At1g53170	0.79	none		
	ERF9	At5g44210	0.24	GCCGAC	-298	+
	ERF10	At1g03800	0.8	none		
	ERF11	At1g28370	1.64	none		
	ERF12	At1g28360	1.37	none		

FI: Fold Induction in 35S::DEAR1 in microarray data

St: Strand +sence - antisence

DER motif shows in the 1000bp upstream of the translation start site of genes

Table 2-S1.

Table 2-S1. Expression and cis-elements of ERF family genes in the DEAR1 overexpressor. Data was cited from part of Supplementary Table S1 of Tsutsui *et al*.

Table S2
Oligonucleotide primers used for RT-PCR and qPCR

Primer name	Primer Sequence
DEAR1-1	GACGCCGGAGTAGCAGTAAA
DEAR1-2	AAAAGACAGCCGTGTCGTATG
ERF9-1	CAAGACAGGCGAACGGTAGA
ERF9-2	TCCGGGAAGAGGGAAATTAG
PDF1.2-1	CACCCTTATCTTCGCTGCTC
PDF1.2-2	GCACAACTTCTGTGCTTCCA
ERF1-1	CAATCTTGTGTAACCGGTCAGAGC
ERF1-2	CACCGTCAATCCCTTATCCATTCC
PDF1.2 pro-1	ATCAAAGCTTTGCTGCTCTTGAGATCAACC
PDF1.2 pro-2	AAACTTGGATCCGATGATTATTACTATTTTGTTTT
ACT2-1	AAACCTCAAAGACCAGCTCTTC
ACT2-2	AACGATTCCTGGACCTGCCT
CHI-B1-1	AGTTCGGCTGGTGCGGTAAC
CHI-B1-2	TGGCGCTCGGTTCACAGTAGT
ERF9-c1F	TGCGTCCATAGCATCCTCTA
ERF9-c1R	TCAACTTTATTGATGAACATTGTTTT
ERF9-c2F	CATAGAAGTATAGAACCGTGGCTATT
ERF9-c2R	GCTCTCTAACGATTAAATGAGTTTTAC
ACT2-c1F	TTGACTACGAGCAGGAGATGG
ACT2-c1R	CAAACGAGGGCTGGAACAAG

Table 2-S2.

Table 2-S2. Oligonucleotide primers used for qPCR

Chapter 3

Arabidopsis NSL2 regulated cell death were modulated by chloroplast enzyme activity and protein degradation

Summary

Cell death is a common feature of living cells. Previously, we isolated necrotic spotted lesion 2 (*nsl2*) mutant, it showed constitutive cell death, dwarf and resistance for pathogen phenotypes as well as NSL1. NSL2 and NSL1 contain membrane attack complex/perforin (MACPF) domain, that are conserved in bacteria, fungi, mammals and plants. However, the mechanism of the cell death was not clear other than the deficit of the genes. We found that the mechanism of nsl2 induced cell death is regulated by chloroplast enzyme activity and accumulation of chloroplast protein. The *nsl2* chloroplast size and shape were different from wild type plant (WT). Moreover *nsl2* was more accelerated senescence during dark induction than WT. With the accelerating senescence, more reducing the pheophorbide *a* oxygenase and accumulating pheophorbide *a* in *nsl2*. It had been reported as protein accumulation in dependent of light regulation cell death. These data suggested NSL2 induced cell death was regulated by chloroplast protein regulation.

Introduction

Plants are exposed to multiple environment, they often experience wide variations both in the biotic and abiotic stress, as well as pathogen and virus infection, insect attack, drought, cold, wounding, and senescence.

Cell death is one of the adaptation mechanisms to their environment, growth and development to proceed harmoniously, the plants have to adjust to these changes, these process, plant cell death related in many case (Jones *et al* 2001). Plant cell death Morphology is classified two types, Vacuolar and Necrosis. Vacuolar type of cell death is often manifested by a gradual decrease in the volume of the cytoplasm and a concomitant increase in the volume occupied by lytic vacuoles (van Doorn et al 2011). Necrosis type of cell death is characterized by early rupture of the plasma membrane, shrinkage of the protoplast and absence of vacuolar cell death features (vanDoorn *et al* 2011). When inducing cell death, it is known that active oxygen make important work. Reactive oxygen species (ROS) included free radicals such as superoxide anion, hydroxyl radical, hydrogen peroxide, singlet oxygen are related inducing cell death. Reduction of molecular oxygen by high-energy exposure or electron-transfer reactions leads to make the highly reactive ROS (Sharma *et al* 2012). In planta, ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments (Sharma *et al* 2012). ROS are made as a product of cell metabolism; their levels are activated by exposure to environment stresses. It has been demonstrated that ROS, including H_2O_2, are a important factor in the sequence of events taking place on the onset of infection, leading in many cases to hypersensitive response (HR) and the vitalization of the resistance genes, as well as in other mechanism associated with response to pathogen infection (Bolwell *et al* 1995).

Plant defense responses that associated with HR are activated by salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Corné *et al* 1998). The SA signal pathway is primarily activated by and effective in mediating defense against

biotrophic pathogens and the ET/JA pathway is primarily induced and effective in mediating resistance against necrotrophic pathogens (Dong *et al* 2009). The resistance related hormones, JA, SA and others produced from chloroplast. (Hyun *et al* 2008). Hence, the variation of the defected genes which has influence on a chloroplast, changes appeared in phenomena, such as a disease response and aging and so on, for example, the lack of accelerated cell death 2 (ACD2). *ACD2* encodes a red chlorophyll catabolite reductase. The mutant lack of ACD2 showed cell death at long light condition not so short light (Mach et al 2001).

Membrane-attack complex/perforin (MACPF) proteins are transmembrane pore forming proteins that are important in both human immunity and pathogens (Gilbert *et al* 2012). In the Arabidopsis *thaliana*, there are conserved four MACPF domain containing proteins. The NSL1 and NSL2 were reported as a negative regulator of the SA and ET/JA mediated pathway with programmed cell death in plant immunity (Morita-Yamamuro *et al* 2005; Noutoshi *et al* 2006). Previously, we previously isolated NSL2 mutant, *nsl2* shows lesion mimic cell death. We have shown *nsl2* had a bacterial resistance and *NSL2* induced biotic and abiotic stress response (Morita-Yamamuro *et al* 2005; Tsutsui *et al* 2006) as well as *NSL1* (Noutoshi *et al* 2006). Additionally, whole plant resistance, the systemic acquired resistance was induced by *NSL2* degradation (Asada *et al* 2010). However, NSL2 induced cell death mechanism is little known in molecular levels. In this chapter, we reported the cell death mechanism in *nsl2*. At the dark treatment, *nsl2* was more accelerated the senescence than normal condition. At the same time, the cleavage products of chloroplast proteins were more accumulated.

Materials and Methods

Plant material and experimental conditions

Arabidopsis thaliana ecotype Columbia (WT) and *nsl2* mutant and NSL2 transgenic homozygous line (G1, M1) were used in this study, G1 and M1 line were transformed GFP(pGWB5) and myc(pGWB17) tag fusion in C-terminal NSL2 CDS in WT plant back ground. The *nsl2* mutant (SALK_002259) was obtained from Arabidopsis Biological Resource Center (Ohio State University, OH, USA). The seeds were sterilized in 10% v/v bleach for 10 min. After five washes in sterilized water, the seeds were grown on square petri dishes containing seeds were germinated on plates containing 1/2 MS medium. After sowing, the dishes were kept in darkness at 4°C for 3–4 days, and then transferred to a growth chamber under 18h light and 6h dark at 22°C as described previously (Morita-Yamamuro *et al* 2005). The plant dark treatment, 17 days old plate grown plants in the plate were placed in dark boxes in the same chambers in which they had initially been grown. In western blot, gene expression analysis, each sample were collected each time point after dark treatment.

Molecular cloning

Full-length cDNA fragments of NSL1 gene (AT1G28380), NSL2 gene (At1G29690), NSL3 (AT1G14780), NSL4 gene (AT4G24690) were amplified by PCR using the NSLs entry1 and NSLs entry2 and introduced into the pENTR/D-TOPO vector (Invitrogen) to generate the plasmid pENTR. The full-length NSLs was then recombined into the pGWB T-DNA binary vector (Curtis and Grossniklaus 2003) according to the Gateway instruction manual (Invitrogen), placing the full-length NSLs gene under control of the CaMV 35S promoter (35S::NSLs). All PCR products and inserts were verified by DNA sequencing. All primer sequences are listed in Supplemental Table3-1.

Gene expression analysis

Total RNAs were isolated from about 50 mg samples using Trizol reagent (Invitrogen). After RNase-free DNase treatment, the first-strand cDNA syntheses of RNA samples were performed using 1μg of total RNA and ReverTra Ace® (TRT-101 Toyobo). Semi RT PCR was used to measure the gene expression using specific primers (Supplemental Table 3-1) with *Actin* as the internal control (Sato *et al* 2011).

Westernblot analysis

Total plant protein for western-blot analysis was extracted from each terms of plant. In 4-week-old plants of wild-type Arabidopsis, *nsl2* mutant, and 35S::NSL2-GFP transgenic lines. Protein concentration was determined by the method of Bradford. Fifteen micrograms of total protein was loaded per well for SDS-PAGE. The proteins were electrophoretically transferred to Hybond-C membranes (Amersham) using the Trans-Blot Cell (Bio-Rad). Anti-GFP, NYC, PAO, C1 antibodies were used for western-blot analyses.

Measurements of photosystem II efficiency

Photosystem II electron transport rates were determined with PAM-2000 fluorometer (H. Waltz, Effeltrich, Germany) equipped with a leaf clip holder. Rosette leaves grown for 3 for 4 weeks were used for fluorescence measurement (Morita-Yamamuro et al 2004).

Results and Discussion

Phenotypic characterization of the NSL2 overexpressor

The *nsl2* mutant showed necrotrophic like cell death and dwarf phenotypes (Morita-Yamamuro *et al* 2004). However, NSL2 role in inducing cell death is not yet known. So we made NSL2 overexpressor and observed phenotypes. The overexpression of *NSL*2 increases mRNA and protein levels (Figure S3-1 A, B), and showed cell death phenotypes but not whole plant (Figure 3-1A). The other overexpress line shows almost same, data not shown. The morphological analysis of cell death structure in *nsl2* mutant, over expressor and WT, the *nsl2* cell size was smaller than WT and over expressor (Figure 3-1B). Moreover the chloroplasts were decreasing and small as compared with WT (Figure 3-1B), about chloroplast, could not see any deference in WT and overexpressor (Figure 3-1B). The chloroplast development in cotyledons has been shown to be different in several ways from true leaves (Albrecht *et al* 2008). These data indicated that NSL2 affected cell death, cell size and chloroplast formation, and defect of NSL2 had a most part to these phenotypes.

The expression pattern of NSL2 and other NSLs were expressed in different organs

Since *nsl1* and *nsl2* leads to a phenotypic change in leaves, root, cauline. The expression pattern of these genes was determined in different organs of the plant. Total RNA was isolated from seedlings and from different parts of 6 week-old plants grown on soil that include roots, rosette leaves, cauline leaves, stem, flower, and siliques. Reverse transcribed RNA was monitored for the presence of *NSL2* and other *NSLs* transcripts, using *ACT2* as a loading control. In all tissues and to a lower extent also in roots of light-grown plants but not in etiolated seedlings and in senescing tissues the *NSL2* and other *NSLs* transcript could be detected (Figure 3-2A). In order to the histological localization of *NSL2*, *NSL2* promoter-GUS transgenic plants were generated (Morita-Yamamuro *et al* 2005). GUS activity

was detected in all tissues examined in 2 weeks old plant (Morita-Yamamuro *et al* 2005). About 5-6-week-old plant, the GUS activity was little at the rosette leaves (Figure 3-2B), but in flower, siliques, and cauline were high level (Figure 3-2B). From these results, *NSL2* expression differed by growth of a plant and there was tissue specificity expression of *NSL2* and other *NSLs* in mature plant.

Localization of NSL2 and other NSLs

The styryl dye FM-4-64 is commonly used as an endocytotic marker (Fishov and Woldringh 1999), in this study, conducted in plants. NSL2 have MACPF domain. The MACPF domain containing proteins were localized in membrane by attached to their C-terminal domain (Rosado *et al* 2008). We try to make clear NSL2 localization that is plant MACPF containing protein. We used a *N. benthamiana* leaves for agroinfiltration assay (yang *et al* 2000). The NSL2 and other NSLs localized in plasma membranes and cytosol (Figure 3-3A), and other NSLs co-localization with NSL2 (Figure 3-3B). These result suggested that MACPF containing proteins localized in cytosol and membrane in plant cell as same as animal cell.

***nsl2* and NSL2 over expressor accumulate ROS around cell death site**

H_2O_2 accumulation is critical role for plant cell death, against plant pathogen, virus, herbivories and many invaders (Breusegem *et al* 2006). To visualize H_2O_2 generation in situ, WT, *nsl2* and NSL2 over expressor leaves were transferred to a DAB solution. The DAB assays, reddish-brown precipitates were deposited at the sites of H_2O_2 accumulation (Thordal-Christensen *et al* 1997). The *nsl2* and NSL2 over expressor showed accumulation of H_2O_2 at around leaves but not WT (Figure3-4A). For cell death measurement, conductance / ion leakages was used as index (Mackey *et al* 2003). We measure the electro ion leakage for progression of cell death. The *nsl2* enhanced electrolyte leakage than WT and NSL2 ox in rosette leaves (Figure3-4B). However, there was no difference at the cotyledon in WT and NSL2 ox (Figure3-4C). According to the Asada et al, cell death was

guided shortly after suppression of *NSL2* (Asada *et al* 2010). In addition, *NSL2* was strong induced *P. syringae* infection and plant hormone treatment (Thuthui *et al* 2009). These data indicated that *NSL2* suppression or degradation is induced cell death directory or indirectory, but *NSL2* activation or accumulation is not so strong relation with cell death.

NSL2 induced H_2O_2 and b.cinerea infection

ROS-specific *cis* regulatory elements were known as AAGTCAAA in *NSL2* promoter region but not other *NSLs* genes (Figure 3-3A) (Veselin *et al* 2012). The expression of *NSL2* and other *NSLs* were examined in 4weeks old plant treated with 50mM H_2O_2 at 0, 0.5, 1, 3, 6 hours (Figure 3-5B,). *NSL2* and other *NSLs* were induced at different time intervals after H_2O_2 treatment. *NSL2*, *NSL3* and *NSL1*, *NSL4* showed same expression pattern (Figure 3-5B). In this assay, we used H_2O_2 treatment maker gene as *OXI1*. The expression of *OXI1* was singled out for the inducibility in response to ROS sources such as H_2O_2. To define the spatial-temporal expression pattern of NSL2 under wounding and bacterial infection, we examined the pattern of GUS expression in NSL2::GUS transgenic plants (Morita-Yamamuro *et al* 2005). As shown in Fig. 3-5C and D, wounding stress and *B.cinerea* infection triggers a strong increase NSL2 expression in leaves. In the previous study, NSL2 was induced many biotic and abiotic stress (Morita-Yamamuro *et al* 2005; Asada *et al* 2010; Thuthui *et al* 2009). These data indicated NSL2 induced many stress and expressed in the around site which received stress.

The nsl2 accelerated senescence and chlorophyll degradation at the dark condition

Senescence is able to induce by dark treatment (Nooden et al 1988). Chlorophyll degradation and cell death are observed with senescence. At the normal condition, *nsl2* showed early senescence phenotypes than WT (Figure 3-6A), and NSL2 ox (data not shown). When occurred in the senescence,

photosynthetic activities and chlorophyll amount were more reduced in *nsl2* than WT (Figure 3-6 B, C). From these results, we analyzed protein metabolism as a further marker of senescence progression. We tested the PSII-associated light-harvesting complex II (LHC II), PSII core subunits (D1) and large subunit of Rubisco (RBCL). The western blot analysis, these proteins were low levels in *nsl2* than WT and NSL2 ox at the dark induction (Figure 3-S2). From the immunoblot result, *nsl2* had seen clear difference than WT and NSL2 ox. So we further analyzed focus the *nsl2*. W*e measured photosynthetic activities* by fluorescence emitted. The *nsl2* mutant old leaves and young leaves dropped in the photosynthetic activities than the WT plant (Figure 3-5B, C). Chlorophyll and chloroplast protein were used as markers for the senescence since both were degraded during the senescence process (Hortensteiner *et al* 2006). Moreover, we analysed chloroplast protein abundant at the dark condition in *nsl2*. The results showed that *nsl2* have reduced total chlorophyll pigments and D1 and RBCL amount at the normal condition and dark treatment, suggesting that the *nsl2* accelerated chlorophyll breakdown normal condition and more accelerated dark induction.

Analysis of senescence related gene expression and protein abundant.

The chlorophyll catabolism pathway, during leaf senescence was known in a biochemical and cell biological detail (Ougham *et al* 2008). The chlorophyllase 1 (*CLH1*), Accelerated Cell Death 2*ACD2* (*ACD2*) and pheophytinase (*PPH*) were involved in the chlorophyll break down processes (Hörtensteiner *et al* 2011; Kariola *et al* 2005,). At the normal condition, the *CHL1* and *PPH* were activated for chlorophyll catabolism (Figure 3-7A) (Ougham et al 2008). For the gene expression analysis, the *nsl2* activated the *CHL1* and *PPH* than WT (Figure 3-7B). On the other hand, *CHL1* and *ACD2* were not expressed in each plant at the dark treatment. At the dark condition, *PPH* was more induced in each plant than the normal condition. About the protein abundance, pheophorbide *a* oxygenase (PAO) and Non-Yellow Coloring1 (NYC1) were reduced in normal condition in *nsl2* than

WT, moreover these protein more reduced in dark induction in each plants. These data suggested that *nsl2* activated chlorophyll catabolism in dark condition. We measured chlorophyllase activity such as chlorophyllide *a* (Chlide a), pheophytin a (Phy a) and Pheophorbide (pheide *a*). pheide *a* is reported to control Mg-chelatase activity (Figure 3-7A) (Pöpperl et al. 1997) and related cell death (Hirashima et al 2009). The *nsl2* accumulated pheide *a* and reduced PAO (Table 3-1 Figure 3-7B). These data shows *nsl2* related cell death was induced chlorophyll degradation, and more activated these processes in dark treatment.

Acknowledgments

We are very thanks to Dr Ayumi Tanaka and Ryoichi Tanaka for helpful advice. Dr Yutaka Asada, great full comment for our experiment. We are grateful to Mr Kathumata Kuniaki and Ms Rikako Tanaka for helpful advices and experimental support.

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Figure and Legend

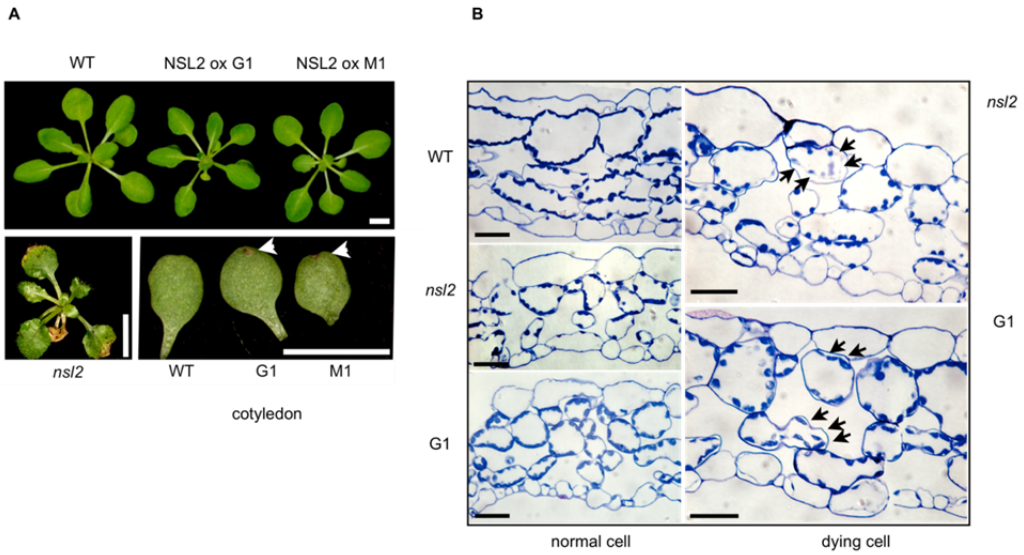
A
WT
NSL2 ox G1
NSL2 ox M1
nsl2
WT
G1
M1
cotyledon
B
WT
nsl2
G1
nsl2
G1
normal cell
dying cell

Figure 3-1.

Figure 3-1. Phenotypes of NSL2 overexpressor.

(A) Whole plant phenotype. White arrowhead shows cell death site in cotyledon. 4-week-old plant. Scale bar = 5 mm. (B) Cross section of upper epidermis and palisade cells of leaves. Scale bars in (a)-(e) = 100um

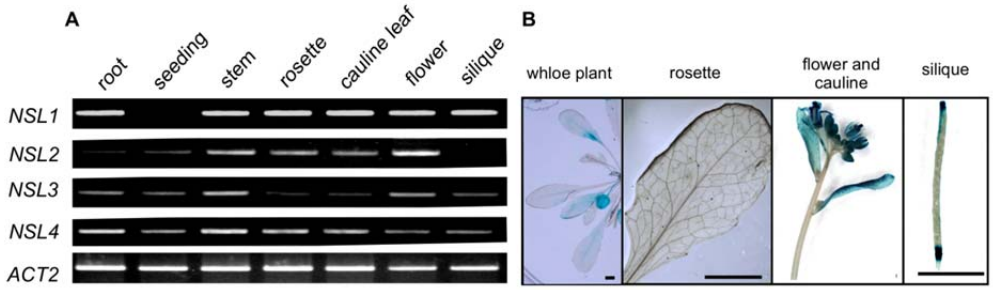
A
root
seeding
stem
rosette
cauline leaf
flower
silique
NSL1
NSL2
NSL3
NSL4
ACT2
B
whloe plant
rosette
flower and cauline
silique

Figure 3-2.

Figure 3-2. Expression Pattern of *NSL2* and other *NSLs*.

Total RNA was isolated from various tissues. *Actin* amplification served as the control. (A) The expression of NSL2 and other NSLs in Arabidopsis organs and seedling. Root, stem, cauline, flower and siliques was isolated from 5 weeks old plant, seedling was collected from 5 days after germination. (B) The transgenic pNSL2:GUS expression in 5-6-week-old plant.

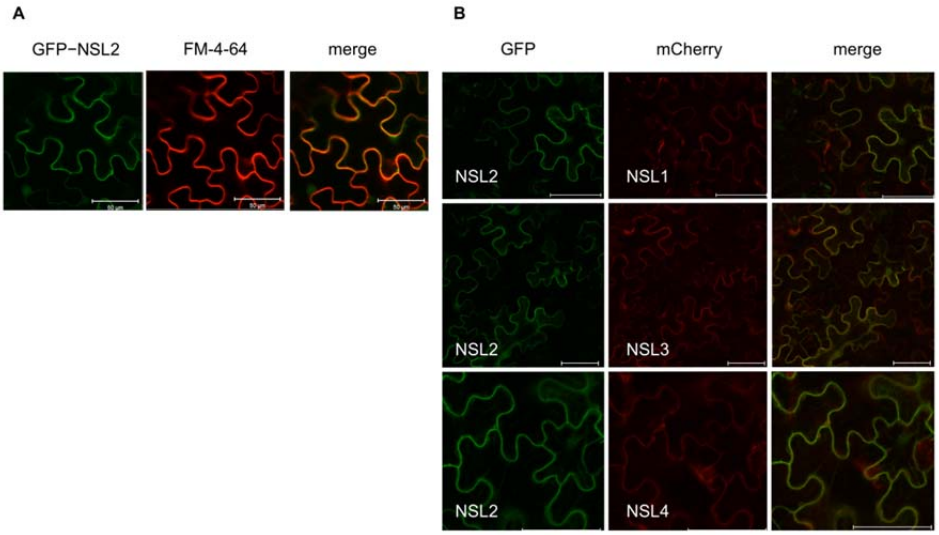
A
GFP-NSL2
FM-4-64
merge
B
GFP
mCherry
merge
NSL2
NSL1
NSL2
NSL3
NSL2
NSL4

Figure 3-3.

Figure. 3-3 NSL2 and other NSLs localization

The constructs were agroinfiltrated into N. benthamiana leaves. (A) GFP-NSL2 fluorescence, FM4-64 fluorescence, merges. (Left to right). (B) Co-localization of NSL2 and mCherry-NSL1, NSL3 and NSL4 localization. Scale bars, 50 μm (A, B).

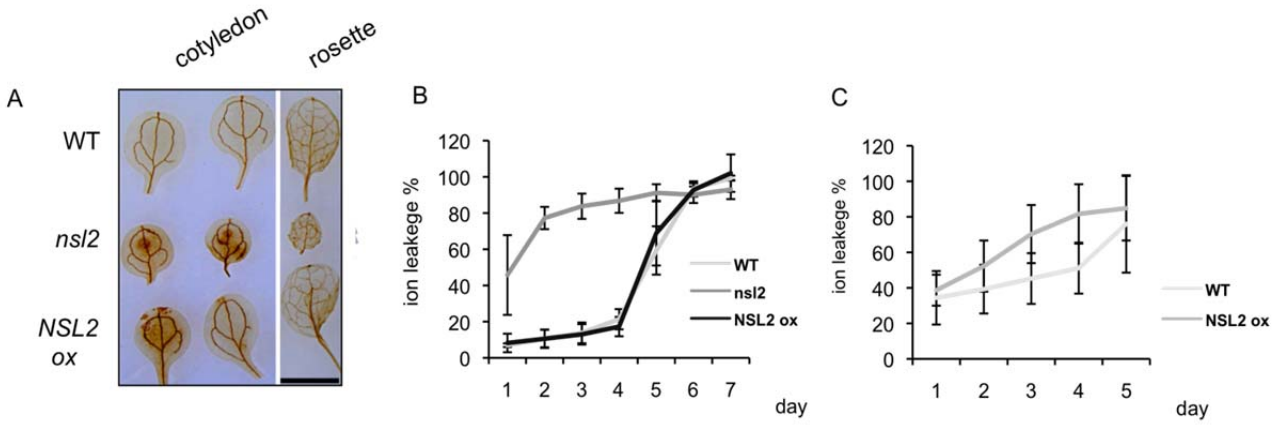
A
cotyledon
rosette
WT
nsl2
NSL2
ox
B
ion leakege %
120
100
80
60
40
20
0
1 2 3 4 5 6 7
day
WT
nsl2
NSL2 ox
C
ion leakege %
120
100
80
60
40
20
0
1 2 3 4 5
day
WT
NSL2 ox

Figure 3-4.

Figure 3-4.NSL2 related ROS accumulation and cell death

(A) DAB staining of WT, nsl2 and NSL2 ox. Scale bar = 5mm. Black arrow head shows the site of ROS accumulation. (B, C) Electrolyte leakage of WT, nsl2 and NSL2 ox plants. The plants were 2-week-old rosette leaves and cotyledon (A, B, C).

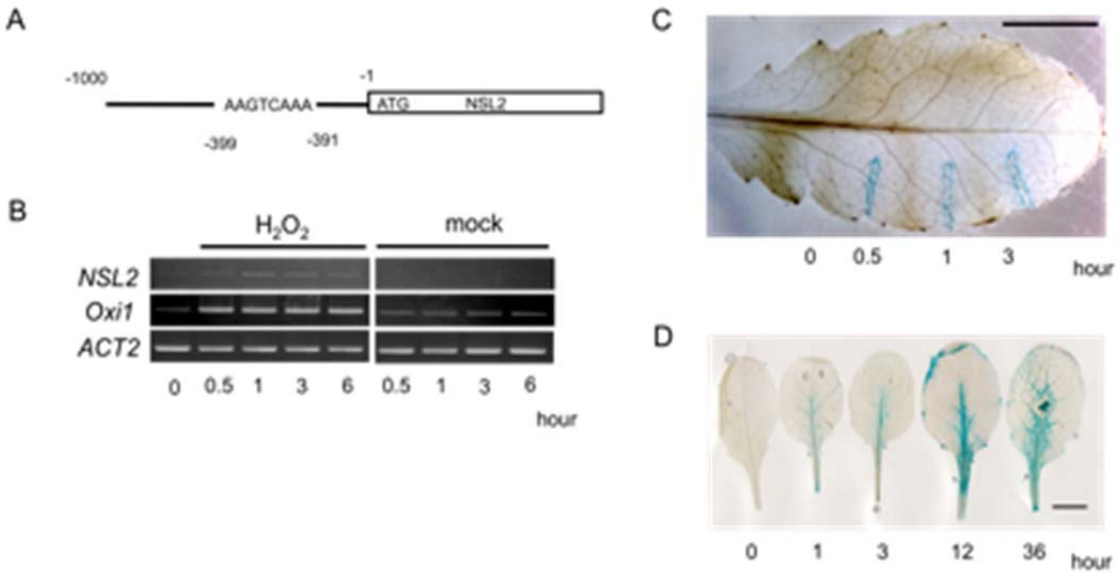
A
-1000
-1
AAGTCAAA
ATG
NSL2
-399
-391
B
H2O2
mock
NSL2
Oxi1
ACT2
0 0.5 1 3 6 0.5 1 3 6
hour
C
0 0.5 1 3
hour
D
0 1 3 12 36
hour

Figure 3-5.

Figure 3-5. NSL2 induced H_2O_2, wounding and bacterial infection.

(A) Schematic structure of the NSL2 promoter. The *cis*-element sites 391 bp up stream form first ATG. (B) Expression analysis of NSL2 after H2O2 treatment. *Oxi1* used as a H_2O_2 treatment control, mock treatment used as water. (C) The expression analysis of pNSL2-GUS by wounding and *B.cinerea* infection. Wounding was performed 0 to 3 hours, *B.cinerea* infection was performed 0 to 36 hours after infection. The plant was 5-6 week-old plant.

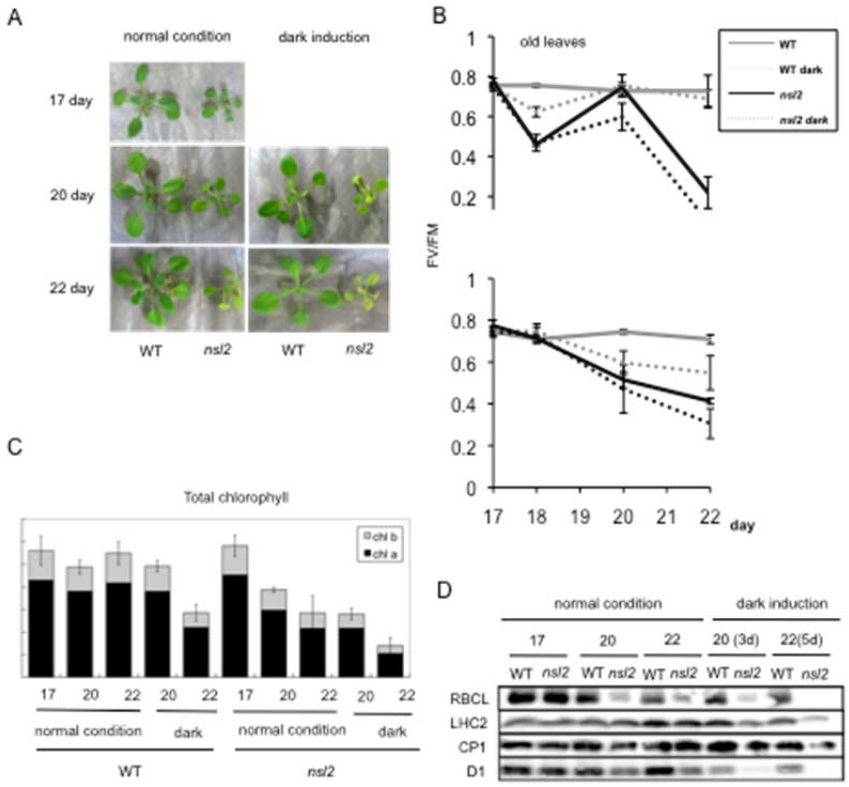
A
normal condition
dark induction
17 day
20 day
22 day
WT
nsl2
WT
nsl2
B
old leaves
WT
WT dark
nsl2
nsl2 dark
FV/FM
1
0.8
0.6
0.4
0.2
0
17
18
19
20
21
22
day
C
Total chlorophyll
chl b
chl a
17
20
22
20
22
17
20
22
20
22
normal condition
dark
normal condition
dark
WT
nsl2
D
normal condition
dark induction
17
20
22
20 (3d)
22(5d)
WT
nsl2
WT
nsl2
WT
nsl2
WT
nsl2
WT
nsl2
RBCL
LHC2
CP1
D1

Figure 3-6.

Figure 3-6. The *nsl2* accelerated senescence at the dark condition.

(A) Dark-induced senescence in WT and nsl2. (B) The potential photochemical efficiency (Fv/Fm) at young leaves (first and second leaves) and old leaves (5th and 6th leaves) in dark and normal condition. (C) Measurement of chlorophyll amount during normal condition and dark-induced senescence. (D) Immunoblot analysis of photosynthesis-related protein abundance in *nsl2* and WT plant during dark-induced senescence. Antibodies against the following proteins were employed. The RBCL, LHCII, CP1 and D1.

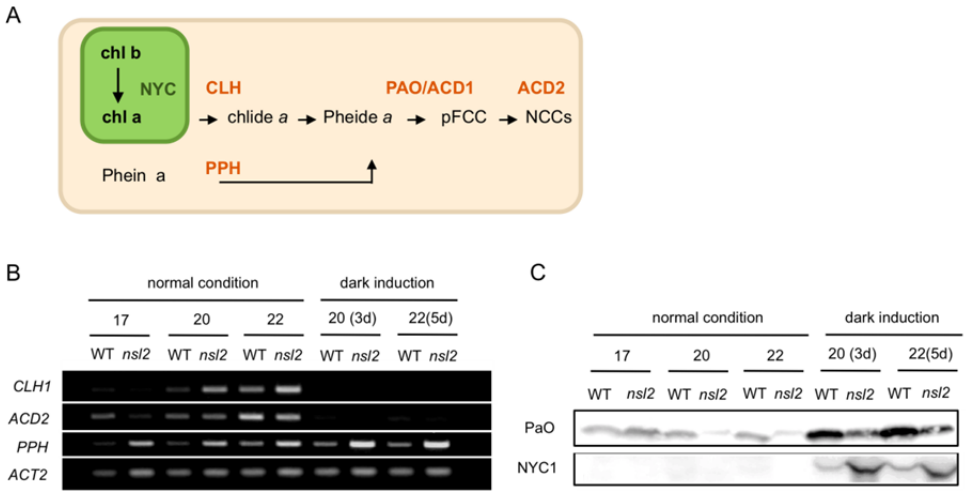
A
chl b
NYC
chl a
CLH
chlide a
Pheide a
PAO/ACD1
pFCC
ACD2
NCCs
Phein a
PPH
B
normal condition
dark induction
17
20
22
20 (3d)
22(5d)
WT nsl2 WT nsl2 WT nsl2 WT nsl2 WT nsl2
CLH1
ACD2
PPH
ACT2
C
normal condition
dark induction
17
20
22
20 (3d)
22(5d)
WT nsl2 WT nsl2 WT nsl2 WT nsl2 WT nsl2
PaO
NYC1

Figure 3-7.

Figure. 3-7 The chlorophyllase related gene expression and protein accumulation.

(A) A suggestive model for chlorophyll breakdown pathway with integrating the recent findings. (B) Gene expression analysis of chlorophyllase related gene at the normal and dark condition. RNA was extracted from whole plant. (C) Immunoblot analysis of PaO and NYC1.

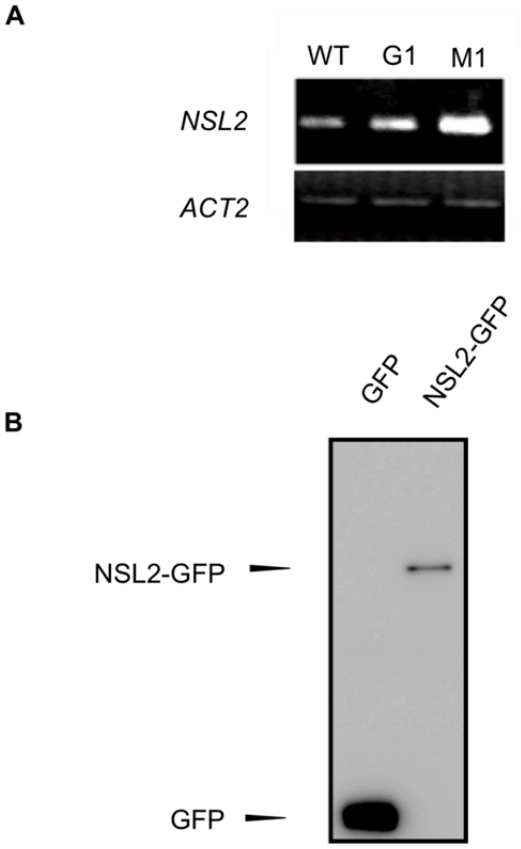

Figure. S3-1

Figure. S3-1 Overexpression of NSL2 in WT plant

(A) Gene expression analysis of NSL2, compared with WT and overexpression lines. (B) Western blot analysis of NSL2-GFP. The sample was immunoprecipitated with anti-GFP antibody. The plant used about 2-3 week-old plant. 35S::GFP transgenic plant was used as control.

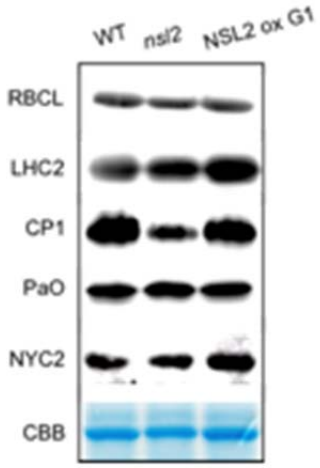

Figure. S3-2

Figure S3-2. Western blot analysis of WT, nsl2 and NSL2 overexpressor.

WB assay was perfumed with anti-RBCL, LHC2, CP1, PaO and NYC2 antibody. The plant was about 2-3 week-old. The sample was extracted whole plant. Loading control was visualized by CBB staining.

	normal condition		Dark induction	
	WT	*nsl2*	WT	*nsl2*
chlorophyllide a	12.1±1.0	13.1±1.0	6.3±0.6	8.67±0.6
pheophytin a	2406.3±96.9	1714.7±1.0	1703±367.8	991.7±524.2
pheophorbide a	N.D	N.D	N.D	53.7±23.2

Table 3-1

Table 1. The accumulation of chlorophyll catabolites in WT and *nsl2*