An E4 Ligase Facilitates Polyubiquitination of Plant Immune Receptor Resistance Proteins in Arabidopsis

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INTRODUCTION

Plants have evolved complex immune systems to fight invading microbial pathogens that threaten normal growth and development. Resistance (R) protein-mediated defense is an effective mechanism that senses the molecular activities of secreted pathogen effector molecules, such as AvrB and AvrRpt2, through either direct or indirect recognition (Staskawicz et al., 1995; Bent and Mackey, 2007). Most R proteins share homology with animal nucleotide binding oligomerization domain receptors, with common structural features including a central nucleotide binding domain and C-terminal Leu-rich repeats (Maekawa et al., 2011). Plant nucleotide binding and leucine-rich repeat domains (NLRs) can be further divided into two subclasses based on their N-terminal coiled-coil or Toll/Interleukin1 receptor domains (Aarts et al., 1998). Upon effector recognition, NLR R proteins activate diverse downstream responses that usually culminate in a hypersensitive response to effectively restrict further pathogen spread (Jones and Dangl, 2006). NLR R protein levels have to be tightly regulated to enable appropriate defense output and to avoid autoimmunity caused by R protein overaccumulation.

Autoimmune mutants, such as suppressor of npr1-1, constitutive 1 (snc1) and suppressor of salicylic acid insensitivity of npr1-5, 4 (ssi4), in which NLR R protein activity or stability is enhanced, exhibit constitutive defense phenotypes, including elevated defense marker gene expression, accumulation of the defense hormone salicylic acid, and enhanced pathogen resistance, all of which contribute to reduced plant growth (Li et al., 2001; Shirano et al., 2002). In order to balance plant immunity with proper growth and development, NLR R protein activity and turnover need to be tightly controlled (Shirasu, 2009). However, the molecular mechanism by which NLR R protein levels are controlled is unclear. There is growing evidence suggesting that ubiquitination plays an important role in NLR R protein-mediated immunity (Liu et al., 2002; Zeng et al., 2004; Trujillo and Shirasu, 2010; Cheng and Li, 2012). Recent research revealed that the F-box protein Constitutive expressor of PR genes 1 (CPR1), a subunit of the E3 ubiquitin (Ub) ligase complex SKP1-CULLIN1-F-box (SCF), targets R proteins, including SNC1 and Resistance to Pseudomonas syringae 2 (RPS2), for degradation to prevent R protein overaccumulation and resultant autoimmunity (Cheng et al., 2011; Gou et al., 2012). Thus, Ubp/proteasome-mediated degradation may function as a crucial mechanism for regulating the turnover of plant R proteins.

In eukaryotes, degradation of selective target proteins such as transcription factors, cell cycle regulators, signal transducers, and misfolded proteins often occurs through the Ub/proteasome pathway (Hershko and Ciechanover, 1998). Ubiquitination is achieved by a series of well-characterized reactions catalyzed by enzymes, including Ub-activating enzyme E1, Ub-conjugating enzyme E2, and Ub ligase E3, which add Ub moieties to their substrates, typically marking them for subsequent degradation (Hochstrasser, 1996; Pickart, 2001). In vitro ubiquitination assays have demonstrated that the combined activities of E1, E2, and E3 enzymes are necessary and sufficient for the recognition and ubiquitination of many target substrates. Although polyubiquitinated
proteins are the preferred substrates of the 26S proteasome, only a few Ub moieties can be efficiently added to the substrate by E1, E2, and E3 enzymes alone. Efficient polyubiquitination beyond two or three Ubs often relies on E4 ligase activity (Koegl et al., 1999). In yeast, Ub-conjugating E4 factor Ubiquitin Fusion Degradation Protein2 (UFD2) facilitates polyubiquitin chain assembly in conjunction with E1, E2, and E3s. The current model for E4 function in animals and yeast suggests that it may form E3-E4 or E4-substrate complexes to coordinate the transfer of Ub from E2 to the substrate or sequentially add Ub to the substrate after E3 initially ubiquitinates the target (Koegl et al., 1999; Hoppe et al., 2004).

Yeast E4 UFD2 is an evolutionarily conserved protein with a C-terminal U-box domain. A number of E4 orthologs have been functionally studied so far: the yeast UFD2, the human or mouse Ubiquitin factor E4A/E4B (UBE4A/UBE4B), the Caenorhabditis elegans UFD2, and NOSA from Dictyostelium (Johnson et al., 1995; Pukatzki et al., 1998; Koegl et al., 1999; Hoppe et al., 2004; Matsumoto et al., 2004). Here, we report the isolation, characterization, positional cloning, and functional analysis of Arabidopsis thaliana MUSE3 (for Mutant, snc1-enhancing 3), which encodes a Ub-conjugating E4 factor. Our study reveals that MUSE3 functions as an E4 factor and works downstream of the E3 ligase SCFCPR1 to facilitate the polyubiquitination and degradation of R proteins, including SNC1 and RPS2.

RESULTS

The muse3-1 Mutation Enhances snc1-Mediated Autoimmunity in the mos4 snc1 Background

In Arabidopsis, the dominant mutant snc1 exhibits constitutive activation of plant defense responses, including elevated defense marker gene expression and enhanced resistance against both bacterial and oomycete pathogens (Li et al., 2001; Zhang et al., 2003). The mutant modifier of snc1, 4 (mos4-1) allele, which was originally identified as a genetic suppressor of snc1, reverts snc1 to wild-type-like phenotypes in the snc1 background (Palma et al., 2007). The triple mutant muse3-1 mos4 snc1 was isolated from the recently described muse forward genetic screen in the mos4-1 snc1 background that was designed to isolate negative regulators of R protein-mediated immunity (Huang et al., 2013). When backcrossed to mos4 snc1, wild-type-like morphology was observed in all F1 progeny, suggesting that the muse3-1 mutation is recessive. As shown in Figure 1A, this triple mutant reverts to an snc1-like morphology despite the presence of the mos4 allele, exhibiting more severe dwarfism than snc1.

To confirm that other defense-related phenotypes are enhanced in the muse3-1 mos4 snc1 mutant, the expression levels of defense marker Pathogenesis Related (PR) genes PR-1 and PR-2 were examined by RT-PCR. As shown in Figure 1B,
expression of both PR-1 and PR-2 was increased to snc1-like levels in the triple mutant. Consistently, the snc1-like enhanced resistance against the virulent pathogen Hyaloperonospora arabidopsis Noco2 was restored in the muse3-1 mos4 snc1 seedlings (Figure 1C). As snc1 exhibits higher SNC1 protein accumulation compared with the wild type (Cheng et al., 2011), which can be partially suppressed by mos4, the SNC1 level was also examined in the triple mutant. As shown in Figure 1D, the SNC1 protein level in the triple mutant is similar to that in snc1. Taken together, muse3-1 enhances all snc1-associated defense phenotypes in the mos4 snc1 background.

Positional Cloning of muse3-1

To identify muse3-1, a positional cloning approach was utilized. Triple mutant muse3-1 mos4 snc1 (in the Arabidopsis ecotype Columbia background) was crossed with Arabidopsis ecotype Landsberg erecta. F1 plants were allowed to self-fertilize to generate a segregating F2 population. Using 24 F2 plants with snc1-enhancing morphology as a crude mapping population, the muse3-1 mutation was mapped to the top arm of chromosome 5 through linkage analysis. The mutation was further flanked by markers T31P16 and MPI7 using an additional 96 snc1-enhancing F2 plants (Figure 2A). To confirm the initial mapping and further localize the mutation region, a larger fine-mapping population was generated using progeny from several F2 lines that are heterozygous at both NCR1 (snc1) and MOS4 (mos4), aiming to avoid interference from those two loci. With 549 F3 plants from the fine-mapping population, 69 recombinants were found between T31P16 and MPI7. As shown in Figure 2A, the muse3-1 mutation was eventually found to lie between markers T9L3 and MQK4 and was located on BAC clone T20K14.

To identify potential molecular lesions in muse3-1, Illumina whole-genome sequencing was performed on nuclear DNA isolated from muse3-1 mos4 snc1 plants. After comparison between mutant and the Arabidopsis wild-type reference genome sequences, several candidate genes with mutations were identified. Some of the mutations could be excluded as candidates after detailed sequence analysis, because they were either false positive or silent mutations that did not cause amino acid changes. Direct Sanger sequencing confirmed the most likely candidate gene mutation using genomic DNA from muse3-1 mos4 snc1. A C-to-T transition occurred in At5g15400 in the 10th exon (Figures 2B and 2C), changing Gln-789 to an early stop codon and truncating the predicted U-box domain of the protein (Figure 2D).

BLAST analysis revealed that MUSE3 is a single-copy gene encoding a putative Ub-conjugating E4 factor with a conserved central Ub-elaborating factor core domain (also called the UFD2p core domain) and a C-terminal U-box domain (Figure 2D). Phylogenetic analysis of MUSE3 and its homologs showed that MUSE3 is a highly conserved protein in all sequenced eukaryotes (Supplemental Figure 1). The best studied MUSE3 homolog is the yeast E4 Ub ligase UFD2, which promotes Ub chain elongation of various target substrates (Hoppe, 2005). MUSE3 shares 35% and 30% amino acid similarity with UFD2 and UBE4, respectively. Protein alignment of the predicted UFD2p core domains and the U-boxes between MUSE3 and UFD2 further confirmed that these domains share high sequence homology and are highly conserved (Supplemental Figure 2).

The SAIL_713_A12 T-DNA insertion allele of muse3, renamed muse3-2, was obtained from the ABRC. muse3-2 carries a T-DNA insertion in the 11th exon of At5g15400 (Figure 2B). To test whether muse3-2 is allelic to muse3-1, a homozygous muse3-2 plant was crossed with homozygous muse3-1 mos4 snc1. In the F1 generation, all the plants exhibit a similar dwarfism to muse3-1 mos4 snc1 (Figure 2E), indicating that muse3-2 fails to complement muse3-1 and that muse3-2 is allelic to muse3-1.

MUSE3 Encodes a Putative Ub-Conjugating E4 Factor That Localizes to Both Nucleus and Cytoplasm

To confirm that the mutation found in At5g15400 is responsible for the muse3 mutant phenotypes and to better understand the function of MUSE3, full-length genomic clones of At5g15400 under the control of its native promoter with or without a C-terminal Green Fluorescent Protein (GFP) gene were transformed into muse3-1 mos4 snc1 plants. As shown in Figures 3A and 3B and Supplemental Figures 3A and 3B, both transgenes exhibit growth defects including sensitivity to ethanol or hydroxyurea. By confocal fluorescence microscopy and subcellular fractionation followed by immunoblot analysis, MUSE3-GFP fluorescence was observed in both nuclei and cytoplasm of leaf epidermal and root cells (Figure 3C). Subcellular fractionation confirmed that MUSE3-GFP is indeed present in both the nuclei-depleted and nuclei-enriched fractions (Supplemental Figure 4). Thus, MUSE3 seems ubiquitous, having both nuclear and cytoplasmic localizations.

Arabidopsis MUSE3 Complements the Yeast ufd2Δ Mutant Phenotypes

Previously, the yeast knockout strain ufd2Δ was shown to exhibit growth defects including sensitivity to ethanol or hydroxyurea at 37°C (Koege et al., 1999). Since UFD2 is a close homolog of MUSE3, we tested whether MUSE3 is orthologous to UFD2 using a yeast complementation experiment. As shown in Figure 4, the ufd2Δ mutant strain exhibits no growth defect on SD-Leu plates. However, compared with the wild-type strain, ufd2Δ cells did not grow well and exhibited obvious sensitivity to either 10% ethanol or 100 mM hydroxyurea. By contrast, ufd2Δ cells expressing MUSE3 grew much better on plates containing 10% ethanol than did the ufd2Δ strain, although not as well as wild-type cells. Furthermore, ufd2Δ strains expressing MUSE3 grew as well as the wild type on plates containing 100 mM hydroxyurea. These data demonstrate that Arabidopsis MUSE3 is able to complement the ufd2Δ knockout phenotypes, suggesting that MUSE3 is a functional ortholog of the yeast UFD2 E4 ligase that is involved in Ub chain elongation.
Muse3 Mutants Exhibit Enhanced Disease Resistance Phenotypes

Our study of the muse3-1 mos4 snc1 triple mutant indicates that MUSE3 functions as a negative regulator of snc1-mediated immunity. To determine its general role in plant immunity, we generated the muse3-1 single mutant by crossing muse3-1 mos4 snc1 with wild-type plants, followed by allele-specific genotyping in the F2 generation. We also utilized the muse3-2 allele for single mutant analyses. As shown in Figure 5A, both muse3-1 and muse3-2 plants resemble wild-type plants, having a slightly smaller size as quantified by fresh weight analysis of

Figure 2. Positional Cloning of muse3-1.

(A) Map position of muse3-1 on chromosome 5. BAC clones and numbers of recombinants from the mapping population (in parentheses) are indicated. (B) Gene structure of MUSE3 (At5g15400). There is only one known splice form of MUSE3. Boxes indicate exons and lines indicate introns. Gray regions represent untranslated regions, and black regions represent coding regions. The asterisk indicates the site of the C-to-T mutation in muse3-1. The arrow indicates the position of the T-DNA insertion site in muse3-2. (C) Genomic DNA sequence comparison between MUSE3 and muse3-1. In muse3-1, a C-to-T point mutation occurs in the 10th exon and is indicated as a lowercase letter. (D) The predicted protein domains of MUSE3. One UFD2p core domain is indicated as a long black rectangle. The short rectangle represents the U-box domain. The asterisk indicates where the protein truncation occurs due to the mutation in muse3-1. Bar = 100 amino acids. (E) Morphology of 3-week-old plants of the wild type, muse3-2, and muse3-1 mos4 snc1 and F1 of muse3-2 crossed with muse3-1 mos4 snc1. The genotypes of the F1 plants were confirmed by PCR using mutation-specific primers. Because snc1 morphology is semidominant and mos4 is recessive, severely dwarfed F1 progeny suggest failed complementation between muse3-1 and muse3-2, both of which are recessive. Bar = 1 cm.

Figure 3. MUSE3-GFP Complements the Molecular Lesion in muse3-1 and Localizes to Both Nuclei and Cytoplasm.

(A) Morphology of the wild type, snc1, mos4 snc1, muse3-1 mos4 snc1, and two representative transgenic lines expressing MUSE3-GFP under the control of its native promoter in the muse3-1 mos4 snc1 triple mutant background. Bar = 1 cm. (B) Quantification of H. arabidopsidis (H.a.) Noco2 sporulation for the indicated genotypes. Data are mean values of four replicates ± so. (C) Confocal images of MUSE3-GFP fluorescence in leaf epidermis and root cells of plate-grown muse3-1 mos4 snc1 transgenic plants expressing MUSE3-GFP. Cell walls were stained with propidium iodide to visualize the cell outlines. C, cytoplasm; CS, cytoplasmic strands; N, nucleus. Bars = 10 μm.
The top panels were used as equal inoculum controls. Hydroxyurea (HU), incubated for 3 d at 37°C for several days as indicated: synthetic de PR-2 and Leu + 10% ethanol (ETOH), incubated for 6 d in aerial tissues (Figure 5B). The relative expression levels of PR-1 and PR-2 were increased in both mutant alleles (Figure 5C). To determine whether muse3 also altered resistance against virulent pathogens, muse3-1, muse3-2, and wild-type control plants were challenged with the virulent oomycete H. arabi-dopsis Noco2 or the bacterial pathogen Pseudomonas syringae pv maculicola ES4326. As shown in Figures 5D and 5E, resistance against both pathogens was enhanced by both muse3 mutations. Because SNC1 accumulates in the muse3-1 mos4 snc1 triple mutant, the SNC1 level was also examined in muse3 single mutants. As shown in Figure 5F, both muse3-1 and muse3-2 exhibited higher SNC1 protein levels, although wild-type-like SNC1 transcription levels were detected (Figure 5C). These data indicate that mutations in MUSE3 cause enhanced disease resistance and stabilize SNC1.

Constitutive Defense Responses in muse3-2 Are Partially Suppressed by Knocking Out SNC1

To test whether the enhanced resistance responses in the muse3 mutants are due to increased accumulation of SNC1 protein, we introduced snc1-r1, a loss-of-function deletion allele of SNC1 (Zhang et al., 2003), into muse3-2. Analysis of PR gene expression showed that constitutive expression of both PR-1 and PR-2 was only partially reduced in muse3-2 snc1-r1 (Figure 6A). In addition, enhanced resistance against the virulent pathogen H. arabidopsidis Noco2 in muse3-2 was partially suppressed by the snc1-r1 mutation (Figure 6B). However, knocking out SNC1 did not significantly affect the muse3-2–associated resistance against Pseudomonas ES4326 (Figure 6C). These data suggest that the overaccumulation of SNC1 in muse3-2 contributes only partially to the enhanced resistance phenotypes of muse3, indicating that MUSE3 may target additional NLRs other than SNC1 for degradation.

To test whether MUSE3 affects the stability of the coiled-coil type NLR RPS2, we crossed the previously reported RPS2-HA transgene into the muse3-2 background (Axtell and Staskawicz, 2003). As shown in Figure 6D, RPS2-HA accumulates more in muse3-2, indicating that MUSE3 also contributes to the turnover of RPS2. Without MUSE3, RPS2 becomes more stable.

MUSE3 Facilitates the Degradation of SNC1 and RPS2 Mediated by CPR1

The phenotypic analysis of muse3 mutants suggests that MUSE3 may negatively regulate the stability of SNC1 and RPS2. As the F-box protein CPR1, a component of the SCF E3 complex, has been shown to target SNC1 and RPS2 for degradation (Cheng et al., 2011), we hypothesized that MUSE3 may function together with CPR1 to regulate SNC1 and RPS2 degradation through the Ub/proteasome pathway. To test this hypothesis, we employed a transient expression system in Nicotiana benthamiana by coexpressing SNC1-FLAG or RPS2-FLAG with either CPR1-FLAG or MUSE3-HA alone or CPR1-FLAG and MUSE3-HA together to reveal their biochemical relationships (Gou et al., 2012). As shown in Figures 7A and 7B, when coexpressed with CPR1, SNC1 and RPS2 exhibited decreased accumulation compared with expressing SNC1 or RPS2 alone, which is consistent with CPR1 targeting SNC1 and RPS2 for degradation (Cheng et al., 2011; Gou et al., 2012). When coexpressed with MUSE3, both SNC1 and RPS2 expression levels were unaffected, indicating that, unlike CPR1, MUSE3 does not function as an E3 by itself that directly targets these substrates for degradation. However, when coexpressed with both CPR1 and MUSE3, the protein levels of SNC1 and RPS2 were much lower than when coexpressed with CPR1 alone, demonstrating that MUSE3 facilitates the degradation of SNC1 and RPS2 mediated by the E3 SCFCPR1. A similar trend in SNC1 levels was observed in snc1 overexpressing both CPR1 and MUSE3-GFP in Arabidopsis. Overexpression of CPR1 reduces the level of SNC1 and suppresses snc1-mediated dwarfism (Figures 7C and 7D), whereas overexpression of MUSE3-GFP alone did not alter either the SNC1 level or the morphological phenotypes of snc1. However, the SNC1 level was further reduced when MUSE3-GFP was expressed together with CPR1 (Figures 7C and 7D). Interestingly, we sometimes were able to detect polyubiquitinated RPS2 when it was coexpressed with both CPR1 and MUSE3 in N. benthamiana, supporting the role of MUSE3 as an E4 in polyubiquitination (Supplemental Figure 5). However, we never
observed polyubiquitinated forms of SNC1, even from proteasome inhibitor MG132-pretreated leaf samples (Supplemental Figure 6), possibly due to transience or instability of the polyubiquitinated forms of the substrate protein.

The genetic and biochemical associations between MUSE3 and SNC1, RPS2, and CPR1 prompted us to test whether MUSE3 interacts with SNC1, RPS2, or CPR1 in planta. Coimmunoprecipitation (Co-IP) using coexpressed MUSE3-HA with either SNC1-FLAG, RPS2-FLAG, or CPR1-FLAG fusion protein in N. benthamiana with anti-FLAG agarose beads revealed that MUSE3-HA coimmunoprecipitated only with SNC1, but not with RPS2 or CPR1, in the elution fractions (Figures 8A to 8C). These results were confirmed by reciprocal Co-IP experiments using anti-HA agarose beads (Supplemental Figures 7A to 7C). Taken together, MUSE3 seems to function downstream of CPR1 to facilitate the polyubiquitination and degradation of SNC1 and RPS2. MUSE3 appears to function with SNC1 through direct protein–protein interaction, while its activity with RPS2 seems indirect (Supplemental Figure 8).

**DISCUSSION**

In eukaryotes, selective protein degradation by the Ub/proteasome system primarily requires E1, E2, and E3 enzymes. However, E4 activity is often needed for polyubiquitination of many target proteins to ensure efficient downstream degradation (Koegl et al., 1999; Hoppe, 2005). E4 factors often catalyze polyubiquitin chain assembly in conjunction with E1, E2, and E3 enzymes. The first and best-studied example of E4 function is UFD2, encoded by a single-copy gene in yeast. In the work of Koegl et al. (1999), UFD2 efficiently facilitates the polyubiquitination of Ub-ProtA, a model UFD substrate, only together with E1, E2, and E3 enzymes. The first and best-studied example of E4 function is UFD2, encoded by a single-copy gene in yeast. In the work of Koegl et al. (1999), UFD2 efficiently facilitates the polyubiquitination of Ub-ProtA, a model UFD substrate, only together with E1, E2, and E3 but not with just E1 and E2, suggesting the requirement of E3 for E4 function on certain substrates. Subsequent studies demonstrated that Ufd2p can also act as an E3 ligase, as it is able to bind to E2 and its U-box domain is structurally similar to the E3 RING domains (Tu et al., 2007). The two proposed possible working models of UFD2 and its corresponding E3 Ufd4p are (1) a sequential model, where UFD2 functions downstream of Ufd4p to add further Ub is to the substrate, and (2) a cooperative model, in which UFD2 works in conjunction with E3 Ufd4p for polyubiquitin chain assembly (Tu et al., 2007). Although UFD2 is an evolutionarily conserved protein present in all eukaryotes, its biological function in plants has never been studied.

![Figure 5. Characterization of Two muse3 Single Mutant Alleles.](image)

(A) Morphology of 4-week-old soil-grown plants of the wild type, muse3-1, and muse3-2. Bar = 1 cm. (B) Aerial fresh weight (FW) of 3-week-old plants of the indicated genotypes. Bars represent means of 12 replicates ± so. The experiments were repeated three times with similar results. (C) Relative expression of PR-1, PR-2, and SNC1 in the wild type, muse3-1, and muse3-2 as determined by real-time RT-PCR. Total RNA was extracted from 2-week-old plants grown on half-strength MS plates and reverse transcribed to cDNA. The PR-1, PR-2, and SNC1 expression levels were normalized using ACTIN1. Bars represent means of three replicates ± so. The experiment was repeated three times with similar results. (D) Quantification of H. arabidopsis (H.a.) Noco2 sporulation on wild-type, muse3-1, and muse3-2 seedlings. Data are mean values of four replicates ± so.

(E) Bacterial growth of Pseudomonas (P.s.m.) ES4326 on wild-type, muse3-1, and muse3-2 plants. Leaves of 4-week-old plants were infiltrated with a bacterial suspension at OD600 = 0.0005. Leaf discs within the infected area were taken right after infiltration (day 0) and 3 d after infiltration (day 3) to quantify bacterial colony-forming units (cfu). Bars represent means of five replicates ± so. One-way ANOVA was used to calculate statistical significance between genotypes as indicated by different letters (P < 0.0005). (F) SNC1 protein levels in the wild type, muse3-1, and muse3-2. Signals from nonspecific proteins (NSPs) served as internal loading controls.
From this investigation, the putative *Arabidopsis* E4 factor MUSE3 complemented the yeast *ufd2* mutant strain phenotypes (Figure 4), suggesting that MUSE3 has E4 activity and is an ortholog of UFD2. Since *muse3* mutant plants exhibited enhanced disease resistance and elevated levels of NLR proteins, including SNC1 and RPS2 (Figures 5 and 6), MUSE3 seems to play negative roles in plant immunity and turnover control of NLR receptors. Since the enhanced resistance in *muse3* is only partly due to increased SNC1 accumulation (Figure 6), we speculate that MUSE3 probably has multiple NLR targets. Previously, it was shown that the F-box protein CPR1, a component of an SCF E3 complex, targets SNC1 and RPS2 for degradation (Cheng et al., 2011; Gou et al., 2012). However, overexpression of MUSE3 in the snc1 background did not suppress snc1-associated morphological phenotypes or affect SNC1 accumulation (Figures 7C and 7D), suggesting that MUSE3 alone is not sufficient to affect SNC1 level. Transient coexpression of SNC1 with MUSE3 in *N. benthamiana* also demonstrated that MUSE3 alone is not sufficient to affect SNC1 or RPS2 accumulation (Figures 7A and 7B). However, when expressed with CPR1, MUSE3 can enhance SNC1 and RPS2 degradation in *N. benthamiana* (Figures 7A and 7B). This is also true when both CPR1 and MUSE3 are stably transformed in *Arabidopsis* (Figure 7D). These biochemical data thus support the sequential model where MUSE3 seems to act downstream of CPR1 to facilitate SNC1 and RPS2 degradation (Supplemental Figure 8). It is likely that Ub is first transferred via the E3 SCFCPR1 complex to substrates including SNC1 and RPS2. After adding one to three Ub moieties, the SCFCPR1 E3 complex may become inactive and dissociate from the substrates. The ubiquitinated substrates may recruit MUSE3 and an E2 in order to transfer additional Ub from the E2 to the substrates. The polyubiquitinated substrates thus become efficient substrates for proteasome degradation. This sequential model is further supported by our Co-IP data showing that MUSE3 associates with SNC1 but not with CPR1 (Figures 8A and 8C; Supplemental Figures 7A and 7C).

Although both SNC1 and RPS2 seem to be substrates of MUSE3, we believe that the modes of action of MUSE3 on these targets may be different. SNC1 appears to directly interact with MUSE3 (Figure 8; Supplemental Figure 7), whereas the MUSE3–RPS2 interaction may require an unknown adaptor or facilitator protein. Unlike with SNC1, for which polyubiquitinated forms of the protein were never observed on immunoblots, in half of our coexpression experiments with RPS2 in *N. benthamiana*, we were able to detect laddering signals that are presumed to represent a variety of polyubiquitinated forms of RPS2 (Supplemental Figure 5). The polyubiquitinated RPS2 signals appear to be slightly stronger when coexpressing CPR1 and MUSE3 compared with expressing CPR1 alone (Supplemental Figure 5), suggesting that MUSE3 has E4 activity that is involved in efficient polyubiquitin chain assembly. We are not sure why laddering was not observed consistently in all experiments. It may be due to subtle differences in tissue collection and growth conditions. In addition, higher molecular weight signals above RPS2–FLAG were detected in the beads fraction of our Co-IP experiment, and these were confirmed to correspond to polyubiquitinated forms of RPS2 by immunoblotting with anti-Ub antibody (Figure 8B). The polyubiquitinated laddering pattern
was never observed for SNC1 samples, indicating that the turnover rate of polyubiquitinated RPS2 and SNC1 is probably different. Even with the application of the proteasome inhibitor MG132, we only observed higher SNC1 accumulation but never polyubiquitinated forms of the protein (Supplemental Figure 6), indicating that they may be too transient or unstable to be detected.

An important avenue of further investigation will be to identify other NLRs being targeted by MUSE3. We do not expect all R proteins to be targeted by MUSE3, since the enhanced immunity of muse3 mutants is not severe (Figure 5). How MUSE3 achieves its substrate specificity will also be an interesting question to address in future studies.

We are likewise intrigued by the very mild growth defects observed in the muse3 mutants. Although MUSE3/UFD2 family genes are highly conserved in eukaryotes, the phenotypes of the mutants suggest that they are not needed for most other biological processes, including general plant growth and development under laboratory conditions. In yeast and humans, UFD2 was found to target many substrates involved in different processes; thus, it is unlikely that MUSE3 is solely dedicated to immune response regulation in the plant kingdom. In the Arabidopsis genome, there are two E1s, more than 37 E2s, and over

![Figure 7. MUSE3 Facilitates CPR1-Mediated Degradation of SNC1 and RPS2.](image)

(A) SNC1-FLAG, MUSE3-HA, and CPR1-FLAG levels in N. benthamiana leaves expressing the indicated proteins. Signals from nonspecific proteins (NSPs) served as internal loading controls.

(B) RPS2-FLAG, MUSE3-HA, and CPR1-FLAG levels in N. benthamiana leaves expressing the indicated proteins. Signals from nonspecific proteins (NSPs) served as internal loading controls.

(C) Morphology of 4-week-old soil-grown plants of the indicated genotypes. #1 and #2 are two independent F3 lines homozygous for snc1. 35S:CPR1, or pMUSE3:MUSE3-GFP that were identified from a cross between 35S:CPR1 in snc1 (T1-5; Cheng et al., 2011) and pMUSE3: MUSE3-GFP in snc1 (T1-10). Bar = 1 cm.

(D) Immunoblot analysis of SNC1 protein levels in the indicated genotypes. Total protein was extracted from 4-week-old soil-grown plants. Rubisco levels served as the internal loading control.

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![Figure 8. MUSE3 Directly Associates with SNC1 but Not with RPS2 and CPR1.](image)

(A) In planta Co-IP of MUSE3-HA using SNC1-FLAG.

(B) In planta Co-IP of MUSE3-HA using RPS2-FLAG. The polyubiquitinated forms of RPS2 detected (indicated with asterisks) were confirmed when the same samples were probed with anti-Ub antibody in immunoblot analyses.

(C) In planta Co-IP of MUSE3-HA using CPR1-FLAG. N. benthamiana leaves were infiltrated with Agrobacterium cells containing constructs expressing the indicated proteins. Total protein extracts were subjected to immunoprecipitation (IP) with anti-FLAG agarose beads. Input, Elute, and Beads fractions were probed with anti-FLAG or anti-HA antibodies in immunoblot analyses.
1300 potential E3s (Hatfield et al., 1997; Bachmair et al., 2001; Vierstra, 2003). As the most numerous and diverse components of ubiquitination pathways, these E3s determine the specificity of substrates in various biological processes (Smalle and Vierstra, 2004; Liu et al., 2012). MUSE3, as the only E4 identified in Arabidopsis, most likely helps a variety of E3 ligases for efficient degradation of their substrates. Therefore, it will be important in the future to perform a variety of detailed phenotypic analyses of muse3 mutants in the plant research community in order to reveal other important E3 partners and protein targets of MUSE3 in additional biological processes other than immunity.

METHODS

Plant Growth Conditions and Mutant Screen

All Arabidopsis thaliana plants were grown in climate-controlled chambers at 22°C under a 16-h-light (120 µmol m⁻² s⁻¹)/8-h-dark cycle. The muse screen using ethyl methanesulfonate was performed as described previously (Huang et al., 2013). muse3-1 was identified from the screen in the mos4-1 snc1 background. The primers for mutant genotyping are as follows: mos4-F, 5'-CTGCTTGGTGAACATCCAC-3', and mos4-R, 5'-GATCTGTCTCCAGAAGTGCC-3'; snc1_Xba-FL, 5'-ATGAGTGAGGTCCCATTACTG3', and snc1_Xba-NR, 5'-GATCCATTGTTTGCTGGAAGA-3'; MUSE3_WT-F, 5'-AACAAGACTCTTCCATTG3', and MUSE3_R, 5'-GTGTCGACCTCAGTCTATG-3'.

Gene Expression Analysis

About 70 mg of total plant tissue was collected from 2-week-old seedlings grown on half-strength Murashige and Skoog (MS) medium. RNA was extracted using the Totally RNA kit (Ambion, now part of Invitrogen). Superscript II reverse transcriptase (Invitrogen) was used to reverse transcribe 0.4 µg of total RNA to obtain cDNA. Levels of cDNAs were initially normalized with ACTIN1 by real-time PCR using the QuantIFAST SYBR Green PCR kit (Qiagen). For RT-PCR, the cDNA was amplified by PCR using 94°C for 2 min and 28 cycles of 94°C for 20 s, 58°C for 30 s, and 68°C for 1 min. The products were subsequently analyzed by agarose gel electrophoresis and ethidium bromide staining. ACTIN1 expression was used to standardize transcript levels in each sample. The primers for RT-PCR analyses used in this study are as follows: PR-1-F, 5'-GTTGTCAGTCTTGGTGGG-3'; PR-1-R, 5'-CACTATCTGACGAGG-3'; PR-2-F, 5'-GCTTCTCTCTCAACCACACAGC-3'; PR-2-R, 5'-CCCTAGTGTACCAGGAATCTG-3', and ACTIN1-F, 5'-GAGTATACTTCACGAGACG-3', and ACTIN1-R, 5'-CAGAGTCACGACATACCG-3'.

Pathogenic Infections

Infection experiments with the oomycete Hyaloperonospora arabidopsidis Noco2 and Pseudomonas syringae pv maculicola ES4326 were performed as described (Li et al., 2001). Briefly, H. arabidopsidis Noco2 infection was performed on 2-week-old seedlings by spraying H. arabidopsidis Noco2 spore suspension at the concentrations indicated in the figure legends. Plants were maintained at 18°C under a 12-h-light/12-h-dark cycle with 80% humidity, and the infection level was quantified 7 d after inoculation by counting the number of conidiophores per gram of tissue using a hemocytometer.

Total Protein Extraction

Fifty milligrams of leaf tissue from 4-week-old soil-grown plants was harvested and ground into powder using liquid nitrogen. The samples were homogenized in extraction buffer (100 mM Tris-HCl, pH 8, 0.1% SDS, and 2% β-mercaptoethanol). After centrifuging for 5 min at 13,200 rpm in a standard microfuge, the supernatant of each sample was transferred to a new microtube with 4× Laemmli loading buffer (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and boiled for 5 min at 95°C. The sample was diluted to a final concentration of 1× loading buffer. Immunoblot analysis was performed afterward to analyze samples using specific antibodies. The anti-FLAG antibody and anti-Ub antibodies were from Sigma-Aldrich. The anti-HA antibody was from Roche.

Positional Cloning and Illumina Whole-Genome Sequencing

Positional cloning of muse3-1 was performed using a previously described strategy (Huang et al., 2013). The markers used for mapping were designed according to the insertion/deletion or single nucleotide polymorphisms between the genomic sequences of Columbia and Landsberg erecta ecotypes that are available on TAIR (http://www.Arabidopsis.org). Once the site of the mutation was localized to a small region of ~1 Mb, the genomic DNAs of mutant seedlings from the mapping population were sequenced with Illumina whole-genome sequencing following the instruction manual from New England Biolabs. Briefly, the purified genomic DNA was sonicated into fragments of ~300 bp, which were set to end repair, adapt ligation, and adaptor ligation. After removal of unligated adapters, the ligated DNA was enriched by PCR to create a genomic DNA library. The Illumina sequencing library was then sequenced using an Illumina genome analyzer. After comparison with wild-type genomic sequence, the potential mutations within the flanking area were further analyzed by Sanger sequencing.

Construction of Plasmids

The MUSE3 genomic sequence plus 1.5-kb regions both upstream of the start codon and downstream of the stop codon was amplified by primers 5'-CGCGGATCCGCAATGCTTTAGTCTACATTACCG-3' and 5'-ACGGG_TCTACTACATTACCATGCC-3'. The amplification fragment was then digested with XbaI and SalI and cloned into pCAMBIA1305-GFP to generate pCAMBIA1305-MUSE3. For the construction of pCAMBIA1305-pMUSE3: MUSE3-GFP, the fragment containing 1.5-kb sequence upstream of the start codon of MUSE3 and the MUSE3 genomic sequence without the stop codon was amplified by 5'-GCGGATCCGCAATGCTTTAGTCTACATTACCATGCC-3' and 5'-GTCACGACATAACATCAC-3'. After BamHI and SalI digestion, the fragment was ligated into pCAMBIA1305-GFP. These constructs were electroporated into Agrobacterium tumefaciens strain GV3101 and subsequently transformed into muse3-1 mos4 snc1 by a floral dipping method (Clough and Bent, 1998). For transient expression experiments in Nicotiana benthamiana, the coding sequence of MUSE3 was PCR amplified from wild-type cDNA using primers 5'-CGGGTACCATGCACGAGAATAATGACACACG-3' and 5'-CGGGGTACCATGCACGAGAATAATGACACACG-3'. The amplified fragment was then digested with KpnI and BamHI and cloned into pCAMBIA1300-3SS-MUSE3-3'XHA. The plasmid was electrotransfected into Agrobacterium tumefaciens and used for transient expression and Co-IP in N. benthamiana. For yeast complementation, full-length MUSE3 cDNA was PCR cloned into yeast expression vector p426-VPD with primers 5'-CGGGTACCATGCACGAGAATAATGACACACG-3' and 5'-ACGGGATCCGCAATGCTTTAGTCTACATTACCATGCC-3'.
empty vector control plasmids were introduced into ufd2Δ and wild-type yeast strains using a standard polyethylene glycol/lithium acetate yeast transformation protocol (http://labs.fhrc.org/gottschi/yeast%20protocols/transform.html). Yeast transformants were grown overnight, serially diluted, and plated onto the appropriate media to assay for growth.

Subcellular Fractionation

Subcellular fractionation of MUSE3-GFP was performed as described previously (Cheng et al., 2009). In brief, 2-week-old plate-grown seedlings (1 g) were harvested and ground to a fine powder in liquid nitrogen and mixed with 2 mL of cold lysis buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl₂, 250 mM Suc, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was filtered through 95- and 40-μm nylon mesh sequentially. The flow-through was spun at 1500g for 10 min, and the supernatant consisting of the cytosolic fraction was collected and mixed with 4× Laemmli loading buffer and heated at 95°C for 5 min. The pellet was washed four times with 3 mL of nuclear re-suspension buffer consisting of 20 mM Tris-HCl, pH 7.4, 25% glycerol, 2.5 mM MgCl₂, and 0.2% Triton X-100. The final pellet was mixed with 50 μL of 1× Laemmli loading buffer and heated at 95°C for 5 min. Then, two fractions were loaded on a 10% SDS-PAGE gel for protein separation. Antibodies used for immunoblot analyses were as described: anti-histone H3 (Feyes et al., 2005), anti-GFP (Wirthmueller et al., 2007), and anti-PEPC (Noël et al., 2007)

Transient Expression and Co-IP in N. benthamiana

Transient expression in N. benthamiana was performed as described previously (Van den Ackerveken et al., 1996). The Co-IP experiment was performed following the protocol from Moffett et al. (2002), with minor modifications. Agrobacterium strains expressing the target genes with specific tags under the control of the 35S promoter were first inoculated in Luria-Bertani medium with kanamycin selection and grown at 28°C for 16 to 18 h. Then, the bacterial cells were transferred into the new culture medium [10.5 g/L K₂HPO₄, 4.5 g/L KH₂PO₄, 1.0 g/L (NH₄)₂SO₄, 0.5 g/L sodium citrate, 1 mM MgSO₄, 0.2% Glc, 0.5% glycerol, 50 μM aceto-syringone, 10 mM MES, pH 5.6, and 50 μg/mL kanamycin] by 1:50 dilution and incubated for 8 to 12 h. The bacteria were then pelleted at 3000g for 10 min and resuspended in MS buffer (4.4 g/L MS medium, 10 mM MES, and 150 μM aceto-syringone), and two strains were mixed to a final concentration of OD₆₀₀ = 0.3 for infiltration on 4-week-old N. benthamiana leaves.

For protein immunoprecipitation, ~4 g of N. benthamiana leaf tissue was harvested 38 h after infiltration and ground to a fine powder in liquid nitrogen. Two milliliters of extraction buffer per 1 g of tissue (10% glycerol, 25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM DTT, 2% [w/v] polyvinylpyrrolidone, and 1% protease inhibitor cocktail [Roche]) was added to the powder and homogenized by further grinding. The sample was spun at 15,000g for 10 min at 4°C. Ninety microliters of the supernatant was saved as input. Thirty microliters of prewashed protein A or protein G beads was subsequently added to the rest of the supernatant containing 0.15% Nonidet P-40 (Nonidet P-40 substitute). After 30 min of incubation at 4°C, the mixture was spun at 3000g for 1 min to remove the beads. The supernatant was transferred to a tube with 30 μL of anti-FLAG M2 beads (Sigma-Aldrich) and incubated with agitation for 3 h at 4°C. The beads were then spun down at 8000 rpm in a microfuge for 1 min at 4°C and washed eight times with 1 mL of extraction buffer containing 0.15% Nonidet P-40 before immunoprecipitated proteins were eluted with 100 μL of 250 μg/mL 3× FLAG peptides (Sigma- Aldrich). SDS loading buffer was added to each sample and boiled for 5 min. The samples were run on SDS-PAGE gels for further immunoblot analysis.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database or the Arabidopsis Genome Initiative database under the following accession numbers: At5g15400, NP_586313 (MUSE3); At4g16890, NP_193422 (SNC1); At3g18165, NP_566599 (MOS4); At2g14610, NP_179068 (PR-1); At3g57260, NP_191285 (PR-2); At2g37620, NP_566988 (ACTIN1). The accession numbers for all the sequences used in phylogenetic analysis include the following: XP_002873722 (Arabidopsis lyrata); NP_010091 (Saccharomyces cerevisiae UFD2); NP_004779 (Homo sapiens UBE4A); NP_006039 (H. sapiens UBE4B); XP_001777615 (Physcomitrella patens); XP_002328979 (Ricinus communis); XP_002324089 (Populus trichocarpa); XP_003633847 (Vitis vinifera); BAK06841 (Hordeum vulgare); EAZ7404 (Oryza sativa japonica); EAY9039 (Oryza sativa indica); XP_002467679 (Sorghum bicolor); XP_002964116 (Selaginella moellendorfii); XP_001422027 (Ostreococcus lucimarinius); XP_003064107 (Micromonas pusilla); NP_071305 (Mus musculus). The T-DNA insertion allele of muse3 (renamed muse3-2) is designated SAIL_713_A12.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phylogenetic Relationship between MUSE3 and Its Homologs.

Supplemental Figure 2. Multiple Alignments of MUSE3, Yeast UFD2, and Human UBE4.

Supplemental Figure 3. MUSE3 Complements the Molecular Lesion in muse3-1 mos4 snc1.

Supplemental Figure 4. MUSE3-GFP Localizes to Both Nuclei and Cytosplasm.

Supplemental Figure 5. MUSE3 Facilitates CPR1-Mediated Degradation of RPS2.

Supplemental Figure 6. MG132 Treatment Enhances SNC1 Accumulation, although No Polyubiquitinated Form of SNC1 Was Detected.

Supplemental Figure 7. MUSE3 Associates with SNC1 but Not with RPS2 and CPR1 Directly.

Supplemental Figure 8. A Sequential Model of How MUSE3 Facilitates CPR1-Mediated SNC1 and RPS2 Degradation.

Supplemental Data Set 1. Text File of the Sequences and Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 1.

Supplemental References

ACKNOWLEDGMENTS

We thank Yan Li and Yueelin Zhang (University of British Columbia and NIBS, China) for Illumina sequencing of muse3-1 and mutation analysis, Kaeli Johnson for critical reading of the manuscript, and Brian Staskawitz (University of California, Berkeley) for seeds of the RPS2-4A transgenic line. This article was supported by the Natural Sciences and Engineering Research Council of Canada (to X.L.), by the Chinese Scholarship Council (Ph.D. scholarship to Y.H.), and by the Deutsche Forschungsgemeinschaft (to C.R., V.L., and M.W.).

AUTHOR CONTRIBUTIONS

Y.H. performed most of the experiments under the supervision of X.L. The confocal images of MUSE3-GFP were produced by C.R., V.L., and M.W. The yeast ufd2 knockout strain for the yeast complementation test was generated by S.M. S.H. carried out the phylogenetic analysis. Y.H.
REFERENCES


An E4 Ligase Facilitates Polyubiquitination of Plant Immune Receptor Resistance Proteins in *Arabidopsis*

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*Plant Cell* 2014;26:485-496; originally published online January 21, 2014; DOI 10.1105/tpc.113.119057

This information is current as of July 9, 2017