Mutations in an atypical TIR-NB-LRR-LIM resistance protein confer autoimmunity

Dongling Bi1†, Kaeli C. M. Johnson2,3‡, Zhaohai Zhu1, Yan Huang2,3, Fang Chen1, Yuelin Zhang1 and Xin Li2,3*

1 National Institute of Biological Sciences, Beijing, China
2 Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada
3 Department of Botany, University of British Columbia, Vancouver, BC, Canada

INTRODUCTION

In order to defend against microbial infection, plants employ a complex immune system that relies partly on resistance (R) proteins that initiate intricate signaling cascades upon pathogen detection. The resistance signaling network utilized by plants is only partially characterized. A genetic screen conducted to identify novel defense regulators involved in this network resulted in the isolation of the snc6-1D mutant. Positional cloning revealed that this mutant contained a molecular lesion in the chilling sensitive 3 (CHS3) gene, thus the allele was renamed chs3-2D. CHS3 encodes a TIR-NB-LRR R protein that contains a C-terminal zinc-binding LIM (Lin-11, Isl-1, Mec-3) domain. Although this protein has been previously implicated in cold stress and defense response, the role of the LIM domain in modulating protein activity is unclear. The chs3-2D allele contains a G to A point mutation causing a C1340 to Y1340 substitution close to the LIM domain. It encodes a dominant gain-of-function mutation. The chs3-2D mutant is severely stunted and displays curled leaf morphology. Additionally, it constitutively expresses PATHOGENESIS-RELATED (PR) genes, accumulates salicylic acid, and shows enhanced resistance to the virulent oomycete isolate Hyaloperonospora arabidopsidis (H.a.) Noco2. Subcellular localization assays using GFP fusion constructs indicate that both CHS3 and chs3-2D localize to the nucleus. A third chs3 mutant allele, chs3-3D, was identified in an unrelated genetic screen in our lab. This allele contains a C to T point mutation resulting in an M1017 to V1017 substitution close to the LIM domain. Additionally, a chs3-2D suppressor screen identified two revertant alleles containing secondary mutations that abolish the mutant morphology. Analysis of the locations of these molecular lesions provides support for the hypothesis that the LIM domain represses CHS3 R-like protein activity. This repression may occur through either autoinhibition or binding of a negative defense regulator.

Keywords: innate immunity, Arabidopsis, resistance protein, CHS3, LIM domain

The majority of plant R proteins possess a central nucleotide-binding (NB) site, a number of leucine-rich repeats (LRRs) at the C terminus, and either a coiled-coil (CC) or Toll/interleukin1-receptor-like (TIR) domain at the N terminus (Dangl and Jones, 2001). There are roughly 150 R genes encoding NB-LRR proteins distributed throughout the Arabidopsis genome (Meyers et al., 2003). Signaling through many CC-NB-LRR R proteins requires NON-SPECIFIC DISEASE RESISTANCE 1 (NDR1) as an intermediate (Aarts et al., 1998), while signaling through most TIR-NB-LRR R proteins typically involves ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), PHYTOALEXIN-DEFICIENT 4 (PAD4), and SENESCEENCE-ASSOCIATED GENE 101 (SAG101; Feyes et al., 2005). These pathways appear to later converge and result in the deployment of similar defense mechanisms.

One key step in resistance signaling is the accumulation of the defense hormone SA. Downstream of SA synthesis, NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) functions as an essential positive regulator of resistance (Cao et al., 1994; Rairdan and Delaney, 2002). Through its interactions with the TGA family of basic leucine zipper transcription factors,
Factors, NPR1 modulates the expression of PR genes (Dong, 2004). The npr1-1 mutation abolishes SA-induced PR gene expression and pathogen resistance, and does not induce the expression of the pPR2-GUS reporter gene (Gao et al., 1994). Thus, a suppressor screen to identify negative regulatory components involved in resistance signaling can be carried out in the npr1 genetic background quite simply by using pPR2-GUS reporter gene expression for mutant isolation. Early screens resulted in the identification of SUPPRESSOR OF NPR1, INDELCIBLE 1 (SNI1), SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNI1), SUPPRESSOR OF SALICYLIC ACID INSENSITIVE (SSI) genes, and SUPPRESSOR OF NIM1-1 (SON1; Li et al., 1999, 2001; Shah et al., 1999, 2001; Kim and Delaney, 2002; Shirano et al., 2002). Recent independent screens have led to the identification of SNC2, SNC3, SNC4, and SNC5 (renamed SUPPRESSOR OF RPS4-RLD; SRFR1) as intermediates in plant pathogen resistance (Bi et al., 2010; Li et al., 2010; Zhang et al., 2010).

In this study, we report the identification and characterization of a snc6-1D mutant isolated from an npr1-1 suppressor screen. SNC6 encodes CHS3, a TIR-NB-LRR protein with an additional LIM (Lin-11, Isl-1, Mec-3; Freyd et al., 1990) domain at the C terminus. Thus, snc6-1D was renamed chs3-2D. The chs3-2D mutant is dwarfed and displays curled leaf morphology. It constitutively induces the expression of PR genes, shows enhanced oomycete resistance, and accumulates SA. Two revertant mutants were isolated through a chs3-2D suppressor screen. Another gain-of-function mutant allele, chs3-3D, has also been identified in our lab as part of an independent mos4 (modifier of sncl, 4) suppressor screen. Analysis of the locations of these mutations provides further insight into the structural and functional details of the different domains of this unique R-like protein.

RESULTS
IDENTIFICATION AND GENETIC CHARACTERIZATION OF THE snc6-1D npr1-1 MUTANT

In plants, NPR1 is an essential regulatory component that functions downstream of salicylic acid (SA) induction in the systemic acquired resistance (SAR) pathway (Dong, 2004). In order to identify novel negative defense regulators independent of NPR1, a suppressor screen (described previously by Gao et al., 2008) was carried out in the npr1-1 background. The snc6-1D npr1-1 mutant was identified by its constitutive expression of pPR2-GUS (Figure 1A). When the snc6-1D npr1-1 mutant (with pPR2-GUS) was backcrossed with SNCG npr1-1 (with pPR2-GUS), the F1 progeny displayed the mutant morphology and constitutively expressed the pPR2-GUS reporter gene (data not shown), indicating that snc6-1D is a dominant mutation. Additionally, the snc6-1D npr1-1 mutant displays dwarfed morphology and curled leaves (Figure 1B). These morphological traits are similar but much more severe than those observed in the autoimmune R gene mutant sncl (Li et al., 2001).

PR GENE EXPRESSION AND PATHOGEN RESISTANCE IN snc6-1D npr1-1

In snc6-1D npr1-1, constitutive pPR2-GUS reporter gene expression was observed primarily in the leaves and petioles (Figure 1A). The induction of pPR2-GUS likely occurs via an NPR1-independent pathway in this mutant. When the expression levels of endogenous PR1 and PR2 were examined using RT-PCR analysis, both PR genes showed constitutively enhanced expression in snc6-1D npr1-1 (Figures 1C,D).

The biotrophic oomycete Hyaloperonospora arabidopsis H.a. is virulent on wild type Arabidopsis plants. Upon infection with H.a. isolate Noco2, the snc6-1D npr1-1 mutant supported ~50 times less oomycete growth than wild type and npr1-1 plants (Figure 1E). Therefore, in keeping with the observed constitutive PR gene expression, the snc6-1D mutation activates constitutive disease resistance in the npr1-1 background.

SA ACCUMULATION IN snc6-1D npr1-1

The dwarfed size and curled leaf phenotypes observed in the snc6-1D npr1-1 mutant, similar to those displayed in sncl plants, are typical of plants with elevated SA levels (Bowling et al., 1997). High-pressure liquid chromatography was used to measure the endogenous levels of both free SA and salicylic acid β-glucoside (SAG), the storage form of the defense hormone that enables the release of free SA as required by the plant. Levels of free SA were ~5 times higher in snc6-1D npr1-1 than in wild type or npr1-1, and levels of total SA (SA + SAG) were ~25 times higher than in wild type and ~5 times higher than in npr1-1 (Figure 1F). SA accumulation observed under uninduced conditions in snc6-1D npr1-1 is likely partly responsible for the constitutive PR gene expression and disease resistance observed in the mutant.

MAP-BASED CLONING OF THE snc6-1D LOCUS

To map the snc6-1D locus, snc6-1D npr1-1 (Col-0 ecotype) was crossed with wild type (SNC6 NPR1) Ler ecotype. Rough mapping showed the snc6-1D mutation is located on the top of chromosome 5 between markers T24H18 and F17K4. Further fine mapping narrowed the location of the mutation between markers MPI7 and MRG7, an interval of approximately 120 kb (Figure 2A).

Sequencing of PCR fragments covering this region revealed a single G to A point mutation in the fifth exon of At5g17890 (Figure 2B). A mutation in this gene had previously been identified and characterized in a screen for temperature sensitive mutants, and the gene was named CHILLING SENSITIVE 3 (CHS3; Yang et al., 2010). Thus, the snc6-1D allele was renamed chs3-2D. CHS3 is predicted to encode a protein of 1613 amino acids containing TIR, NB, LRR, and LIM domains (Figure 5B). The chs3-2D mutation results in a C1340 to Y1340 substitution close to the LIM domain. Since chs3-2D is dominant, we hypothesized this might be a gain-of-function mutation.

ch3-2D IS A GAIN-OF-FUNCTION MUTATION

To determine the genetic nature of the chs3-2D mutation, wild type plants were transformed with either CHS3-GFP or chs3-2D-GFP fusion constructs under the control of its endogenous promoter. When transformed with CHS3-GFP, the transformants displayed wild type morphology, PR gene expression, and H.a. Noco2 resistance (two independent representative lines are shown in Figures 3A–D). In contrast, lines transformed with chs3-2D-GFP displayed mutant morphology, constitutive PR gene expression and enhanced resistance to H.a. Noco2. This indicates that chs3-2D is a gain-of-function mutation resulting in constitutive disease resistance. Although chs3-2D-GFP is able to confer autoimmunity, we were not able to observe the GFP signal of the fusion
protein in transgenic plants, probably due to the very low protein levels.

**CHS3 AND chs3-2D LOCALIZE TO THE NUCLEUS**

Nuclear localization of CHS3 is suggested by WoLFpSORT, although the PredictProtein program indicates that the protein does not contain a known nuclear localization signal (Rost et al., 2003; Horton et al., 2007). To experimentally examine the subcellular localization of CHS3, *Arabidopsis* mesophyll protoplasts were transformed with either CHS3-GFP or chs3-2D-GFP fusion constructs. GFP fluorescence was detected exclusively in the nucleus of protoplasts for both constructs (Figure 4). No obvious differences in GFP fluorescence subcellular localization were observed.

**IDENTIFICATION OF ADDITIONAL chs3 ALLELES**

A chs3-2D suppressor screen was undertaken to find mutant alleles causing a reversion to wild type morphology. Two revertant chs3 alleles were identified (Figure 5A). Sequence analysis revealed that the first allele, chs3-2D-r1, contains a C to T point mutation in the LRR domain resulting in an L716 to F716 substitution while the second allele, chs3-2D-r1, has a G to A point mutation in the LRR-LIM linker region that causes an E1007 to G1007 substitution. An additional chs3 gain-of-function allele, chs3-3D, was identified from a separate mos4 suppressor screen undertaken in our lab (the mos4 suppressor screen will be described in future publications). Similar to chs3-2D, the chs3-3D mutant displays a dwarf, curled leaf phenotype and constitutively expresses the pPR2-GUS reporter gene, present in wild type (WT) and npr1-1 mutant background, in WT, npr1-1, and snc6-1D npr1-1 plants. Seedlings were grown on MS media for 14 days prior to staining for GUS activity. The three photographs shown were taken at different magnifications in order to effectively capture GUS staining, thus seedling size should not be compared between panels. (B) Morphology of wild type (WT), npr1-1, and snc6-1D npr1-1 plants. Representative 4-week-old soil-grown plants were photographed. (C,D) Endogenous expression of (C) PR1 and (D) PR2 relative to Actin 1 in WT, npr1-1, and snc6-1D npr1-1. Values presented are averages of three replicates ± SD. (E) Growth of *H.a. Noco2* on WT, npr1-1, and snc6-1D npr1-1 mutants 7 days post-infection. Values presented are averages of three replicates ± SD. (F) Endogenous free and total SA levels in WT, npr1-1, and snc6-1D npr1-1. Plants were grown on soil for 4 weeks and leaf tissue was collected for SA extraction. SA levels were analyzed using high-pressure liquid chromatography. Values presented are averages of four replicates ± SD.

**FIGURE 1 | Phenotypic characterization of snc6-1D npr1-1.** (A) Expression of the pPR2-GUS reporter gene, present in wild type (WT) and npr1-1 mutant background, in WT, npr1-1, and snc6-1D npr1-1 plants. Seedlings were grown on MS media for 14 days prior to staining for GUS activity. The three photographs shown were taken at different magnifications in order to effectively capture GUS staining, thus seedling size should not be compared between panels. (B) Morphology of wild type (WT), npr1-1, and snc6-1D npr1-1 plants. Representative 4-week-old soil-grown plants were photographed. (C,D) Endogenous expression of (C) PR1 and (D) PR2 relative to Actin 1 in WT, npr1-1, and snc6-1D npr1-1. Values presented are averages of three replicates ± SD. (E) Growth of *H.a. Noco2* on WT, npr1-1, and snc6-1D npr1-1 mutants 7 days post-infection. Values presented are averages of three replicates ± SD. (F) Endogenous free and total SA levels in WT, npr1-1, and snc6-1D npr1-1. Plants were grown on soil for 4 weeks and leaf tissue was collected for SA extraction. SA levels were analyzed using high-pressure liquid chromatography. Values presented are averages of four replicates ± SD.
reporter gene (Figure A1 in Appendix). This allele contains a C to T point mutation in the LRR–LIM linker region that causes an M1017 to V1017 substitution. The locations of these mutations are presented along with the locations of the chs3-1 (Yang et al., 2010) and chs3-2D mutations (Figure 5B).

**DISCUSSION**

The chs3-2D mutation characterized in this study was shown to suppress the enhanced disease susceptibility phenotypes of npr1-1 through the activation of constitutive defense responses. The chs3-2D npr1-1 double mutant displayed dwarfed, curled leaf morphology similar to that of snc1, a well characterized autoimmune mutant. Additionally, chs3-2D npr1-1 constitutively expressed the pPR2-GUS reporter construct as well as endogenous PR1 and PR2, and accumulated the defense hormone SA. The molecular lesion responsible for these mutant phenotypes is located close to the C-terminal LIM domain of the CHS3 R-like protein, and was shown to be a gain-of-function mutation. The mutation does not seem to alter the nuclear localization of the protein. Two revertant alleles were isolated and an additional gain-of-function mutant allele (chs3-3D) was identified in separate genetic screens. The mutation sites in these gain-of-function and loss-of-function alleles provide further understanding as to the potential function of the enigmatic LIM domain of CHS3.

**chs3-2D MUTANT PHENOTYPES SUBTLY DIFFER FROM THOSE OF chs3-1, chs2/rpp4, AND ssi4**

The chs3-1 mutant exhibits enhanced freezing tolerance and constitutive resistance against *Pseudomonas syringae* pv. *tomato* (Pto DC3000 when grown at 16°C, yet when grown at 22°C these phenotypes are mitigated (Yang et al., 2010). Another chilling sensitive R protein mutant, chs2, contains a dominant point mutation in the NB–ARC1 domain of RPP4 (Huang et al., 2010). Like chs3-1, the gain-of-function resistance phenotypes of this mutant are only observable at temperatures below 16°C. Similarly, the chs3-2D npr1-1 morphological phenotypes are temperature sensitive, with mutant plants displaying acute stunting when grown at 16°C (data
**FIGURE 4** | **CHS3-GFP and chs3-2D-GFP localize to the nucleus.** 
CHS3-GFP and chs3-2D-GFP fluorescence as observed by confocal microscopy in Arabidopsis mesophyll protoplast cells (I: GFP, II: Autofluorescence, III: Bright field, IV: Merged). Experiments were repeated with multiple cells for each construct.

**FIGURE 5** | **Identification of chs3-2D revertants and location of a number of chs3 mutations.** (A) Morphology of WT, chs3-2D npr1-1, chs3-2D-1, and chs3-2D-2 soil-grown plants. Representative plants were photographed 4 weeks after germination. (B) Predicted CHS3 protein structure and locations of chs3-1 (Yang et al., 2010), chs3-2D, chs3-3D, chs3-2D-1, and chs3-2D-2 mutations. TIR, Toll/Interleukin-1-receptor-like; NB, nucleotide-binding site; LRR, leucine-rich repeat; LIM, Lin-11, Isl-1, and Mac-3 domain. Arrows indicate the locations of the point mutations corresponding to chs3 mutations.

not shown). However, constitutive disease resistance is maintained in chs3-2D npr1-1 mutant plants grown at 22˚C (Figure 1). As well, the chs3-1 mutation is recessive in the Col-0 background while the chs3-2D mutation is dominant, which is more typical of gain-of-function mutations. These data indicate that chs3-2D is a more severe gain-of-function allele of CHS3. The chs3-2D allele may be of use in future genetic studies of resistance signaling as its constitutive defense response phenotypes are more stable, exhibited at both 16 and 22˚C (data not shown).

The ss14 mutant identified in an earlier npr1 suppressor screen displays an autoimmune phenotype similar to that of chs3-2D, caused by a semi-dominant point mutation in the NB domain of a TIR-NB-LRR R protein (Shirano et al., 2002). However, rather than displaying chilling sensitivity, this mutant is sensitive to humidity; at a relative humidity of 95%, the dwarfed size and constitutive resistance associated with this mutation are abolished (Zhou et al., 2004). Taken together with the chilling sensitive mutants, this indicates that disease resistance and environmental stress response pathways are intricately tied in plants, although the degree of overlap and the precise signaling events required for each pathway remain unclear.

**CHS3 IS A NUCLEAR PROTEIN**

Wild type CHS3 fused to GFP localizes to the nucleus, and this subcellular localization is unaltered by the chs3-2D mutation (Figure 4). This is not a surprising finding as a number of R proteins, including barley Mla10, tobacco N, and Arabidopsis RPS4 and SNC1, have been shown to localize to the nucleus, although in some cases nuclear localization requires the presence of effector proteins (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009). As well, a number of LIM proteins localize to the nucleus (reviewed in Kadrmas and Beckerle, 2004). The expression of the CHS3-GFP fusion construct in the Col-0 wild type background confers wild type resistance, whereas chs3-2D-GFP expressed in the wild type background confers constitutive defense response induction (Figure 3). The constitutive resistance phenotypes observed in the chs3-2D-GFP transgenic plants provide further evidence that this protein is correctly localized. The lack of observable GFP fluorescence in transgenic plants indicates that CHS3-2D is of very low abundance but likely has high defense activation activity. We did not observe differences in localization in the protoplast system between wild type and chs3-2D-GFP. However, that does not exclude the possibility that in plants quantitative localization differences contribute to differences in defense response activation.

**INSIGHTS INTO LIM DOMAIN FUNCTION**

CHS3 belongs to the TIR-NB-LRR subclass of R proteins. In terms of R protein functionality, the presence of the N-terminal TIR domain determines which downstream intermediates will be involved in resistance signaling (Feys et al., 2005). Studies of the flax L6 R protein show that the TIR domain self-associates, likely forming a homodimer, and is required for resistance signaling (Bernoux et al., 2011). The TIR domain has also been implicated in the induction of the cell death response (Frost et al., 2004; Weaver et al., 2006; Swiderski et al., 2009). Likewise, the NB domain appears to play a critical role in resistance signaling and the provocation of cell death (Rairdan et al., 2008). It acts in concert with the ARC1 and ARC2 subdomains to bind nucleotides, and by this means regulates R protein activity (Tameling et al., 2006).
The N terminus and LRR domain are thought to be involved in the regulation of localized cell death, as suggested by domain swap experiments between tomato R protein homologs Mi-1.1 and Mi-1.2 (Hwang et al., 2000). Studies on Rx, a *Solamun tuberosum* R protein required for resistance to Potato Virus X, found that autoactivation was conferred upon deletion of the LRR domain or by specific point mutations in the LRR or NB domains (Bendahmane et al., 2002). Domain swap experiments between Rx and the homologous R protein Gpa2 indicate that interactions between the LRR and ARC2 domains may be required for autoinhibition of the R protein in the absence of a pathogen (Rairdan and Moffett, 2006). These experiments set a precedent for the repression of R protein activity via intramolecular interactions. While the detailed functional roles of the above domains are still being elucidated, comparatively little is known about the additional C-terminal LIM domain possessed by CHS3.

Genes encoding LIM domain-containing proteins have been identified in all well-studied eukaryotic genomes, yet most domain characterization studies have been done in animals. While early experiments demonstrated LIM domain-specific DNA-binding activity in *vitro* (Baltz et al., 1996; Nishiya et al., 1998), LIM domains are thought to serve primarily as protein–protein interaction interfaces and are involved in dimerization (Feuerstein et al., 1994; Schmeichel and Beckerle, 1994; Arber and Caroni, 1996). Animals possess a large number of diverse LIM proteins that have been implicated in a variety of biological processes, including the regulation of actin organization, axon guidance, cell-fate determination, and cell signaling (reviewed in Kadrmas and Beckerle, 2004). Plants contain fewer LIM proteins, and in *Arabidopsis* LIM proteins are encoded by genes from two distinct subfamilies. The first consists of six genes encoding proteins with two LIM domains that are homologous to animal CRP proteins (Eliasson et al., 2000). These proteins bind actin filaments and regulate actin cytoskeleton organization (Papuga et al., 2010). The genes in the second subfamily encode DA1 and seven DA1-related (DAR) proteins, including CHS3 (DAR4), which are plant-specific proteins each containing a single conserved LIM domain (Li et al., 2008). While DA1 is involved in seed and organ size regulation and CHS3 is associated with resistance signaling and cold response (Yang et al., 2010), the role of the LIM domain in modulating these activities remains ambiguous.

The locations of the constitutive resistance-inducing *chs3* mutations provide some insight. The *chs3-1* allele carries a point mutation in an intron–exon junction in a domain of unknown function close to the LIM domain, possibly resulting in a truncated protein due to splicing discrepancies (Yang et al., 2010). The *chs3-2D* mutation is caused by a C1340 to Y1340 substitution close to the LIM domain. Additionally, a *chs3-3D* mutation identified in an unrelated *mos4* suppressor screen results from an M1017 to V1017 substitution in the LRR–LIM linker region (*Figure 5*). The distribution of these mutations throughout a number of domains highlights the importance of the C terminus in regulating R-like activity in CHS3. The entire C terminus may be required for autoinhibition, with the regions surrounding the LIM domain playing an essential role in maintaining correct folding of the LIM to confer proper repression. On the other hand, repression may be a result of binding to a negative regulator. The mutation sites may all be located in close proximity in the three-dimensional protein, perhaps as part of a binding site for an interacting repressor protein.

The mutation sites resulting in a reversion to wild type morphology in *chs3-2D* mutant plants give pause for thought. One revertant contains an L716 to F716 substitution in the LRR domain. Due to the complex intramolecular interactions between the TIR, NB, and LRR domains required for R protein activation, this mutation may result in loss of R protein activity. The other revertant allele contains a G to A point mutation that causes an E1007 to G1007 substitution in the LRR–LIM linker region, in close proximity to the *chs3-3D* molecular lesion. Plants heterozygous for either *chs3-2D-r1* or *chs3-2D-r2* in the *chs3-2D* background exhibit an intermediate morphological phenotype (data not shown), indicating that these mutations are semi-dominant. This is similar to what was observed for the *CHS3* loss-of-function T-DNA insertion alleles *chs3-2*, *chs3-3*, *chs3-4* (*Figure 5B*; Yang et al., 2010), thus providing support for *chs3-2D-r1* and *chs3-2D-r2* being loss-of-function mutations as well. A similar *R* gene dosage effect has been observed in plants heterozygous for the *snc1* mutant, as the stunted morphological trait is recessive while the constitutive *PR* gene expression trait is semi-dominant (Li et al., 2001).

It is possible that the gain-of-function mutations surrounding the LIM release the self-inhibition of the LIM motif while certain loss-of-function mutations enhance its repressive activity through their different impacts on protein structure and conformation. Alternatively, these mutations may have opposing effects on protein-binding sites. The gain-of-function mutations may weaken binding platforms for negative regulators, while certain loss-of-function mutations may stabilize these interaction sites. It is also possible that the reversion mutations result in a loss of constitutive defense response activation simply by destabilizing the CHS3 protein or disrupting the whole protein structure leading to complete loss-of-function of the protein.

**A PUTATIVE MODEL FOR LIM FUNCTIONALITY**

Yang et al. (2010) previously hypothesized that the LIM domain acts to repress R protein activity in CHS3 in the absence of pathogenic effector molecules through intramolecular interactions. Indeed, intramolecular autoinhibitory activity of the LIM domain has been observed with a number of animal LIM proteins (Nagata et al., 1999; Garvalov et al., 2003), and as stated above autoinhibition of R protein activity via intramolecular interactions has been observed in Rx (Rairdan and Moffett, 2006). The unique LIM domain of CHS3 may add another layer of complexity to this self-regulation. Thus, in one proposed model for LIM domain regulation of CHS3 R protein activity, the LIM domain binds directly to the N terminus of CHS3 in the absence of a pathogen, preventing R protein activation. Upon the detection of Avirulence (*Avr*) proteins by the LRR domain a conformational change may take place, alleviating inhibition imposed by the LIM domain. However,
Arabidopsis thaliana (ecotype Col-0) plants were grown under a line were tested for a gain of approximately 26,000 M2 plants. Half of the seedlings from a given approximately 1300 M1 plants were grown on MS media, yielding by GUS staining. Seeds were collected from the remaining plants of the reporter gene were treated with EMS. The seeds from pPR2-GUS H.a. (50,000 spores/mL) and scored 7 days post-treatment.

The npr1-1 suppressor screen was previously described by Gao et al. (2008). Briefly, seeds from npr1-1 plants containing the pPR2-GUS reporter gene were treated with EMS. The seeds from approximately 1300 M1 plants were grown on MS media, yielding approximately 26,000 M2 plants. Half of the seedlings from a given line were tested for a gain of pPR2-GUS reporter gene expression by GUS staining. Seeds were collected from the remaining plants of those lines displaying constitutive pPR2-GUS expression. The M2 plants of these lines were sprayed with an H. a. Noco2 suspension (50,000 spores/mL) and scored 7 days post-treatment.

For PR gene expression analysis was extracted from 14-day-old seedlings grown on MS medium using the RNAiso reagent (Takara). Reverse transcription was done using M-MLV reverse transcriptase (Takara). Real-time PCR was undertaken for wild type-like progeny, PCR amplified with CHS3 primers and sequenced using the BigDye® Direct Cycle Sequencing kit (Applied Biosystems). The obtained sequence was compared with the sequence to search for mutations. The primer sequences used were 5′-CTGGACTCA-3′ (At5g17890-R1), 5′-CTGTTAGTATGTGGC-3′ (At5g17890-R2), 5′-GCATACTGAGCTTAACAAGG-3′ (17890-R3), and 5′-CTGAGGTGCTCTTCTTGCTC-3′ (17890-R5), 5′-GCAAACACTTGTTTATCAACG-3′ (17890-F1), 5′-CAGAGTCCAGGACCATAACTCC-3′ and 5′-CGATGAAATCTGCTTC-3′, the primers used to amplify pPR2-GUS reporter gene expression by GUS staining. Seeds were collected from the remaining plants of those lines displaying constitutive pPR2-GUS expression. The M2 plants of these lines were sprayed with an H. a. Noco2 suspension (50,000 spores/mL) and scored 7 days post-treatment.

For PR gene expression analysis was extracted from 14-day-old seedlings grown on MS medium using the RNAiso reagent (Takara). Reverse transcription was done using M-MLV reverse transcriptase (Takara). Real-time PCR was undertaken for wild type-like progeny, PCR amplified with CHS3 primers and sequenced using the BigDye® Direct Cycle Sequencing kit (Applied Biosystems). The obtained sequence was compared with the sequence to search for mutations. The primer sequences used were 5′-attctaatttaattgcaaaac-3′ (At5g17890-F1), 5′-CACACTGATTGATGCTCTTG-3′ (At5g17890-F2), 5′-aatggcttgattactgcaac-3′ (At5g17890-F3), 5′-TCAGTATGTGGCC TCATGAAG-3′ (At5g17890-F4), 5′-TTTCATGATGGTGATG-GGA-3′ (At5g17890-F5), 5′-GCAAACACTTGTTTATCAACG-3′ (At5g17890-R1), 5′-CTGTTAGTATGTGGC-3′ (At5g17890-R2), 5′-TCAGTATGTGGCC TCATGAAG-3′ (At5g17890-F4), 5′-TTTCATGATGGTGATGGGA-3′ (At5g17890-F5), 5′-GCAAACACTTGTTTATCAACG-3′ (At5g17890-R1), 5′-CCCTGAGAAACATTTGATC-3′ (At5g17890-R3), 5′-ATCTAATCTTTAGGCTGACTC-3′ (At5g17890-R4), and 5′-atcataacaacatttgcgtcg-3′ (At5g17890-R5).

MOLECULAR CLONING
The full-length CHS3 gene including the promoter region but lacking the stop codon was PCR amplified from Col-0 genomic DNA and cloned into a pCAMBIA1305-GFP vector in-frame to the N terminus of GFP using KpnI and BamHI restriction sites. The CHS3-GFP expression clone was sequenced to confirm in-frame fusion. Transformation was done using the floral dip method (Clough and Bent, 1998), and transformants were selected on MS medium supplemented with 30 μg/mL hygromycin. One-week-old seedlings were used to prepare protoplasts and confocal microscopy was used to analyze GFP fluorescence as previously described (Yoo et al., 2007). The same procedure was carried out for chs3-2D.

REVERTANT SCREEN
To identify revertant mutations in chs3-2D, EMS was used to mutagenize seeds of chs3-2D. The M1 progeny were allowed to self-fertilize and the M2 seeds were collected from these plants. These seeds were divided into 216 pools each representing approximately 10 M1 plants. The M2 progeny were screened for wild type morphology. Genomic DNA was extracted from wild type-like progeny, PCR amplified with CHS3 primers and sequenced using the BigDye® Direct Cycle Sequencing kit (Applied Biosystems). The obtained sequence was compared with the wild type CHS3 sequence to search for mutations. The primer sequences used were 5′-atcataatttaattgcaaaac-3′ (At5g17890-F1), 5′-CACACTGATTGATGCTCTTG-3′ (At5g17890-F2), 5′-aatggcttgattactgcaac-3′ (At5g17890-F3), 5′-TCAGTATGTGGCC TCATGAAG-3′ (At5g17890-F4), 5′-TTTCATGATGGTGATG-GGA-3′ (At5g17890-F5), 5′-GCAAACACTTGTTTATCAACG-3′ (At5g17890-R1), 5′-CCCTGAGAAACATTTGATC-3′ (At5g17890-R3), 5′-ATCTAATCTTTAGGCTGACTC-3′ (At5g17890-R4), and 5′-atcataacaacatttgcgtcg-3′ (At5g17890-R5).

ACKNOWLEDGMENTS
We would like to thank Oliver Dong and Virginia Woloshen for careful reading of the manuscript. This work was supported by grants from the Chinese Ministry of Science and Technology to Yuelin Zhang and from the National Science and Engineering Research Council (NSERC) to Xin Li. Kaeli C. M. Johnson is supported by an NSERC Alexander Graham Bell Canada Graduate Scholarship (M).
REFERENCES
Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. (1998). Differences in sensitivity to EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 95, 10306–10311.

Arber, S., and Caroni, P. (1996). Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ. Genes Dev. 10, 289–300.

Baltz, R., Evvard, J. L., Bourdon, V., and Steinmetz, A. (1996). The pollen-specific LIM protein PLIM-1 from sunflower binds nucleic acids in vitro. Sex. Plant Reprod. 9, 264–268.

Bendahanane, A., Farnham, G., Moffett, P., and Baulcombe, D. (2012). Consitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. Plant J. 32, 195–204.

Bernoux, M., Ve, T., Williams, S., Warthesen, C., Hatters, D., Valkov, E., Zhang, Y., and Li, X. (2009). Analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. Cell Host Microbe 9, 200–211.

Bi, D., Cheng, Y. T., Li, X., and Zhang, Y. (2010). Activation of plant immune responses by a gain-of-function mutation in an atypical receptor-like kinase. Plant Physiol. 153, 1771–1779.

Browning, S. A., Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1997). The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 9, 1915–1924.

Burch-Smith, T. M., Schiff, M., Caplan, J. L., Tsao, J., Czymbek, K., and Dinesh-Kumar, S. P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. PLoS Biol. 5, e68. doi:10.1371/journal.pbio.0050068

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6, 1583–1592.

Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., Garcia, A. V., Wirthmueller, L., Després, C., Parker, J. E., Zhang, Y., and Li, X. (2009). Nuclear pore complex component MOS7/Sup88 is required for innate immunity and nuclear accumulation of defense regulators in Arabidopsis. Plant Cell 21, 2503–2516.

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformati- gion of Arabidopsis thaliana. Plant J. 16, 735–743.

Dangl, J. L., and Jones, J. D. G. (2001). Plant pathogens and inte- grated defense responses to infection. Nature 411, 826–833.

Dodd, P. N., Lawrence, G. J., and Ellis, J. G. (2001). Six amino acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the P and P2 rust resistance specificities in flax. Plant Cell 13, 163–178.

Dong, X. (2004). NPR1, all things considered. Curr. Opin. Plant Biol. 7, 547–552.

Eliasson, A., Gass, N., Mundel, C., Baltz, R., K., and Steinmetz,A. (2000). Molecular and expression analysis of a LIM protein gene family from flowering plants. Mol. Gen. Genet. 264, 257–267.

Ellis, J. G., Lawrence, G. J., Luck, J. E., and Dodd, P. N. (1999). Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. Plant Cell 11, 495–506.

Feuerstein, R., Wang, X., Song, D., Cooke, N. E., and Liebhaber, S. A. (1994). The LIM/double zinc-finger motif functions as a protein dimerization domain. Proc. Natl. Acad. Sci. U.S.A. 91, 10565–10569.

Feys, B. J., Wiermer, M., Bhut, R. A., Moisan, L. J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J. E. (2005). Arabidopsis SEQUENCE-ASSOCIATED GENE1 Stabilizes and Signals within an ENHANCED DISEASE SUSCEPTIBILITY1 Complex in Plant Immune Innate. Plant Physiol. 171, 2601–2613.

Frey, G., Kim, S. K., and Horvitz, H. R. (1990). Novel cytochrome-rich motif and homeodomain in the product of the Caenorhabditis elegans cell lineage gene lin-11. Nature 344, 876–879.

Frost, D., Way, H., Howles, P., Luck, J., Manners, J., Hardham, A., Finnegan, E., and Ellis, J. (2004). Tobacco transgenic for the flax rust resistance gene L expresses both NPR1-dependent and NPR1-independent resistance genes. Plant Cell 17, 2082–2093.

Garvalov, B. K., Higgins, T. E., and Delaney, T. P. (2002). Six amino acid changes in vitro. Arabidopsis thaliana beta-strand/beta-turn motif determine differences in gene-for-gene specificity. Mol. Gen. Genet. 264, 257–267.

Garvalov, B. K., Higgins, T. E., and Delaney, T. P. (2002). The PredictProtein server. Nucleic Acids Res. 30, W321–W326.

Kim, H. S., and Delaney, T. P. (2002). Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. Plant Cell 14, 1469–1482.

Li, X., Clarke, J. D., Zhang, Y., and Dong, X. (2001). Activation of an EDS1-mediated R gene pathway in the ncl1 mutant leads to constitutive, NPR1-independent pathogen resistance. Mol. Plant Microbe Interact. 14, 1131–1139.

Li, X., Zhang, Y., Clarke, J. D., Li, Y., and Dong, X. (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SN1, through a screen for suppressors of npr1-1. Cell 98, 329–339.

Li, Y., Li, S., Bi, D., Cheng, Y. T., Li, X., and Zhang, Y. (2010). SRFR1 negatively regulates plant NR-LRR resistance protein accumulation to prevent autoimmunity. PLoS Pathog. 6, e1001111. doi:10.1371/journal.ppat.1001111

Li, Y., Zheng, L., Corke, F., Smith, C., and Bevan, M. W. (2008). Tolerance of final seed and organ size by the DAI gene family in Arabidopsis thaliana. Genetics Dev. 22, 1331–1336.

Meyers, B. C., Kosik, A., Griego, A., Kuhl, H., and Michelmore, R. W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell 15, 809–834.

Nagata, K., Ohashi, K., Yang, N., and Mizuno, K. (1999). The N-terminal LIM domain negatively regulates the kinase activity of LIM-kinase 1. Biochem. J. 341, 99–105.

Nishiya, N., Sabe, H., Nose, K., and Shibamanu, M. (1998). The LIM domains of hic-5 protein recognize specific DNA fragments in a zinc-dependent manner in vitro. Nucleic Acids Res. 26, 4267–4273.

Papini, P., Hofmann, D., Sterle, M., Moes, D., Moreau, E., Tholl, S., Steinmetz, A., and Thomas, C. (2010). Arabidopsis LIM proteins: a family of actin binders with distinct expression patterns and modes of regulation. Plant Cell 22, 3034–3052.

Raidan, G. J., Collier, S. M., Sacco, M. C., Baldwin, T. B., Boertien, T., and Moffett, P. (2008). The coiled-coil and nucleotide binding domains of the potato Rx disease resistance protein function in pathogen recognition and signaling. Plant Cell 20, 739–751.

Raidan, G. J., and Delaney, T. P. (2002). Role of salicylic acid and NMI/NPR1 in race-specific resistance in Arabidopsis. Genetics 161, 803–811.

Raidan, G. J., and Moffett, P. (2006). Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. Plant Cell 17, 2082–2093.

Rost, B., Yachdav, G., and Liu, J. (2003). The PredictProtein server. Nucleic Acids Res. 32, W321–W326.

Schmeichel, K. L., and Beckerle, M. C. (1994). The LIM domain is a modular protein-binding interface. Cell 79, 211–219.

Shaj, J., Kachroo, P., and Klessig, D. F. (1999). The Arabidopsis npr1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. Plant Cell 11, 191–206.

Shaj, J., Kachroo, P., Nandi, A., and Klessig, D. F. (2001). A recessive mutation in the Arabidopsis SS12 gene confers SA- and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. Plant J. 25, 563–574.
Shen, Q. H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I. E., and Schulze-Lefert, P. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science 315, 1098–1103.

Shen, Q. H., Zhou, F., Bieri, S., Haizel, T., Shirasu, K., and Schulze-Lefert, P. (2003). Recognition specificity and RAR1/SGT1 dependence in barley Mla disease resistance genes to the powdery mildew fungus. Plant Cell 15, 732–744.

Shirano, Y., Kachroo, P., Shah, J., and Klessig, D. (2002). A gain-of-function mutation in an Arabidopsis Toll Interleukin 1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell 14, 3149–3162.

Swiderski, M. R., Birker, D., and Jones, J. D. G. (2009). The TIR Domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. Mol. Plant Microbe Interact. 22, 157–165.

Tameling, W. I. L., Vossen, J. H., Albrecht, M., Lengauer, T., Berden, J. A., Haring, M. A., Cornelissen, B. J., and Takken, F. L. (2006). Mutations in the NB-ARC domain of I-2 that impair ATP hydrolysis cause autoactivation. Plant Physiol. 140, 1233–1245.

Weaver, L. M., Swiderski, M. R., Li, Y., and Jones, J. D. (2006). The Arabidopsis thaliana TIR-NB-LRR R-protein, RPP1A; protein localization and constitutive activation of defence by truncated alleles in tobacco and Arabidopsis. Plant J. 47, 829–840.

Wirthmueller, L., Zhang, Y., Jones, J. D., and Paker, J. E. (2007). Nuclear accumulation of the Arabidopsis immune receptor RPS4 is necessary for triggering ED51-dependent defense. Curr. Biol. 17, 2023–2029.

Yang, H., Shi, Y., Liu, J., Guo, L., Zhang, X., and Yang, S. (2010). A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in Arabidopsis. Plant J. 63, 283–296.

Yoo, S. D., Cho, Y. H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 July 2011; accepted: 14 October 2011; published online: 31 October 2011.

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2011 Bi, Johnson, Zhu, Huang, Chen, Zhang and Li. This is an open-access article subject to a non-exclusive license between the authors and Frontiers Media SA, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and other Frontiers conditions are complied with.
FIGURE A1 | Characterization of chs3-3D  (A) Morphology of WT, snc1, snc1 mos4, and chs3-3D soil-grown plants. Representative plants were photographed 4 weeks after germination.  (B) Expression of the pPR2-GUS reporter gene, present in WT and snc1 mutant background, in the above-mentioned genotypes. Seedlings were grown on MS media for 14 days prior to staining for GUS activity. The three photographs shown were taken at different magnifications in order to effectively capture GUS staining; thus seedling size should not be compared between panels.