Autoimmunity conferred by chs3-2D relies on CSA1, its adjacent TNL-encoding neighbour

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Plant innate immunity depends on the function of a large number of intracellular immune receptor proteins, the majority of which are structurally similar to mammalian nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins. CHILLING SENSITIVE 3 (CHS3) encodes an atypical Toll/Interleukin 1 Receptor (TIR)-type NLR protein with an additional Lin-11, Isl-1 and Mec-3 (LIM) domain at its C-terminus. The gain-of-function mutant allele chs3-2D exhibits severe dwarfism and constitutively activated defense responses, including enhanced resistance to virulent pathogens, high defence marker gene expression, and salicylic acid accumulation. To search for novel regulators involved in CHS3-mediated immune signaling, we conducted suppressor screens in the chs3-2D and chs3-2D pad4-1 genetic backgrounds. Alleles of sag101 and eds1-90 were isolated as complete suppressors of chs3-2D, and alleles of sgt1b were isolated as partial suppressors of chs3-2D pad4-1. These mutants suggest that SAG101, EDS1-90, and SGT1b are all positive regulators of CHS3-mediated defense signaling. Additionally, the TIR-type NLR-encoding CSA1 locus located genomically adjacent to CHS3 was found to be fully required for chs3-2D-mediated autoimmunity. CSA1 is located 3.9 kb upstream of CHS3 and is transcribed in the opposite direction. Altogether, these data illustrate the distinct genetic requirements for CHS3-mediated defense signaling.

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Plants have evolved a multi-layered immune system to protect them from various pathogen infections. The first layer of the defense surveillance mechanism relies on the recognition of pathogen-associated molecular patterns (PAMPs) via cell-surface localized pattern-recognition receptors (PRRs)1. PAMPs consist of conserved molecular features of pathogens, such as bacterial flagellin and fungal chitin. Recognition by PRRs leads to the induction of PAMP-triggered immunity, which results in mitogen-activated protein kinase cascade activation, generation of reactive oxygen species, callose deposition, and accumulation of the plant defense hormone salicylic acid1. Successful pathogens are able to deliver effectors into plant cells to perturb these defense responses, leading to Effector-Triggered Susceptibility. In order to counteract these effectors plants have evolved resistance (R) proteins, which constitute the second layer of the plant immune system. Upon specific recognition of effectors, R protein activation leads to the activation of effector-triggered immunity (ETI). ETI is rapid and robust, often culminating in a hypersensitive response (HR), which is a specific type of programmed cell death2.

Most plant R proteins resemble mammalian nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins. Genome-wide analysis reveals that in Arabidopsis there are about 150 typical NLR proteins3, which can be further divided into two subgroups based on their N-termini: those with a Toll/Interleukin 1 Receptor (TIR) domain are termed TNLs, and those with a coiled-coil motif are termed CNLs4. Through previous genetic studies, it seems that TNLs and CNLs have different requirements for downstream signaling. TNL-mediated signaling relies upon the nucleo-cytoplasmic ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/PHYTOALEXIN DEFICIENT 4 (PAD4)/SENCESCENCE-ASSOCIATED GENE101 (SAG101) complex2–7. However, CNL signaling likely depends on the membrane-bound NONRACE SPECIFIC DISEASE RESISTANCE 1 (NDR1)8,9.
Arabidopsis CHILLING SENSITIVE 3 (CHS3) encodes an atypical TNL protein with a Lin-11, Isl-1 and Mec-3 (LIM) domain at its C terminus. LIM domain-containing proteins are found across eukaryotes and have been implicated as regulators of a variety of biological processes, including but not limited to gene expression and signal transduction. It is hypothesized that the LIM domain may act as a repressor domain in CHS3. Whether this non-canonical TNL transduction is to be explored.

**Methods**

**Plant growth.** Seeds were sterilized by soaking them in a solution of 15% bleach and 0.1% Tween 20 followed by rinsing twice with sterile water. Seeds were cold treated in the dark at 4°C for three days. Plate-grown plants were grown on 1/2 MS media at 22°C and exposed to a 16 h light and 8 h dark regime.

**Oomycete infection assay.** Two-week-old seedlings were spray-inoculated with H. arabidopsis sporulation on the indicated genotypes following inoculation with pathogen isolate H.a. Noco2. Two-week-old soil-grown seedlings were sprayed with a spore suspension of H.a. Noco2 at a concentration of 100,000 spores/mL of water. The plants were then covered and incubated for seven days in a high humidity growth chamber. Spores were counted in water suspension using a hemocytometer (bars represent means of n replicates ± 3 or 4 with 4 plants each).

**Gene expression analysis.** Total RNA was extracted from 13-day-old seedlings grown on 1/2 MS media using the RNA Mini-preps Kit (Bio Basic Inc.). Total RNA was then reverse transcribed using Superscript II reverse transcriptase (Applied Biological Materials). The resulting cDNA was used as template for PCR. Both PR1 and ACTIN7 were amplified with 28 cycles while PR2 was amplified with 30 cycles. PCR products were then run on 1% agarose gel containing ethidium bromide and imaged using an Alphalnager HG (Alphalnnotech). The primers used to amplify ACTIN7 were 5’-CAGATTCTGAGCAGCACATACCC-3’ and 5’-GGTTGATAGGCTGTTGGATGC-3’, the primers used to amplify PR1 were 5’-GTTAACCTGCCTGTTGGTTCT-3’ and 5’-CACATAATGCCACGAGGATG-3’, and the primers used to amplify PR2 were 5’-GCTCTTCTTCTCAACACACAGC-3’ and 5’-CGTTGATACCCGGAATCCG-3’.

For CSA1 gene expression analysis, the cDNAs of wild type, chs3-2D, chs3-2D pad4-1, chs3-2D eds1-90-10, and chs3-2D pad4-1 mutants in the Col-0 ecotype of Arabidopsis thaliana were crossed with the Landsberg erecta (Ler) ecotype. Among the F2 population, the chs3-2D locus was genotyped using insertion/deletion marker MIP7 (5’-GTGAAATCTAATTAGCCGCA-3’ and 5’-CTCTTGTATACCCGACCGTG-3’), Plants homozygous for chs3-2D were used for further linkage analysis based on plant size and morphology.

**Results**

**Identification and characterization of suppressors of chs3-2D.** The chs3-2D autoimmune mutant exhibits severe dwarfism. In order to search for regulatory and signaling components required for CHS3-mediated defense response, we screened for mutants that can suppress the autoimmunity of chs3-2D, using suppression of stunted growth as a proxy during the primary screen. chs3-2D seeds were first mutagenized by ethyl methanesulfonate (EMS). The M1 plants were grown at 28°C to harvest the M2 seeds as chs3-2D autoimmunity and concomitant dwarfism are temperature sensitive; the mutant is seedling lethal at 23°C, but fertile at 28°C. The M2 population from approximately 2000 M1 plants were initially screened for individuals that were significantly larger than the original mutant. Mutants exhibiting a morphological suppression of chs3-2D-associated phenotypes were then subjected to a secondary screen, in which resistance to the virulent oomycete H. arabidopsis sporulation on the indicated genotypes following inoculation with pathogen isolate H.a. Noco2. Two-week-old soil-grown seedlings were sprayed with a spore suspension of H.a. Noco2 at a concentration of 100,000 spores/mL of water. The plants were then covered and incubated for seven days in a high humidity growth chamber. Spores were counted in water suspension using a hemocytometer (bars represent means of n replicates ± 3 or 4 with 4 plants each).
strain H. a. Noco2 was examined. Mutants that displayed enhanced susceptibility to H. a. Noco2 as compared to chs3-2D were selected for further characterization. The genetic background of all mutants was verified by directly sequencing the CHS3 locus. Mutants carrying intragenic mutations in CHS3 as revealed by the sequencing were classified as intragenic mutants and eliminated from further analysis. In summary, eight independent soc (suppressor of chs3-2D) lines with second-site mutations were isolated.

As shown in Figure 1A, all eight soc chs3-2D mutants can completely suppress the morphology of chs3-2D. Consistent with the morphological suppression, all of them showed significantly enhanced susceptibility to H. a. Noco2 (Figure 1B). In addition, the expression of PR genes was significantly reduced in all mutants as compared to chs3-2D (Figure 1C). Taken together, these data suggest that all of the eight soc chs3-2D mutants contain mutations that suppress the autoimmune phenotypes of chs3-2D.

chs3-2D is only marginally suppressed by pad4-1. In addition to chs3-2D, another gain-of-function autoimmune mutant allele, chs3-1, was isolated from a forward genetic screen searching for chilling sensitive mutants. Epistasis analysis revealed that the constitutive activation of defense response in chs3-1 fully depends on EDS1 and partially relies on PAD4, suggesting that a PAD4-independent pathway might play an important function in CHS3-mediated defense response. When we created the chs3-2D pad4-1 double mutant, the presence of the PAD4-independent pathway in chs3-2D-mediated defense response was further confirmed as pad4-1 only marginally suppressed the chs3-2D autoimmune phenotypes. chs3-2D pad4-1 only showed a slight morphological suppression of the chs3-2D-associated dwarfism (Figure 2A) and still exhibited constitutive resistance to H. a. Noco2 (Figure 2B).

Identification and characterization of suppressors of chs3-2D pad4-1. In order to identify PAD4-independent regulators involved in the chs3-2D-mediated signaling pathway, we conducted a suppressor screen in the chs3-2D pad4-1 background. A similar screening strategy was used as described above for the chs3-2D suppressor screen. Two socp (suppressor of chs3-2D pad4-1) mutants were isolated from the screen. As shown in Figure 3A, socp1 chs3-2D pad4-1 and socp2 chs3-2D pad4-1 exhibit significant morphological suppression of chs3-2D pad4-1. When the two mutants were challenged with H. a. Noco2, they exhibited considerable susceptibility compared to chs3-2D pad4-1 (Figure 3B). In addition, the constitutive expression of PR genes in chs3-2D pad4-1 was suppressed in the socp1 chs3-2D pad4-1 and socp2 chs3-2D pad4-1.
mutants to some extent (Figure 3C). Therefore, socp1 and socp2 were able to partially suppress the autoimmune of chs3-2D pad4-1.

**CHS3-mediated defense responses are completely dependent on EDS1-90 and SAG101.** To map the SOC loci, the soc chs3-2D mutants (which were generated in the Columbia (Col-0) ecotype) were crossed with wild type Landsberg erecta plants. Crude mapping using insertion/deletion markers specific to the two ecotypes revealed that soc1 is linked to SAG101 on chromosome 5. Since SAG101 is a known downstream component of TNL-mediated immunity, we hypothesized that soc1 might contain a mutation in SAG101. Indeed, direct Sanger sequencing revealed that soc1 carried a G to A mutation in the second exon of SAG101 (At5g14930) resulting in a G1458 to A1458 substitution (Figure 4A). By using similar mapping strategies, we mapped soc2, soc3 and soc4 to chromosome 3, close to EDS1 (At3g48090). Sanger sequencing revealed that all three mutants carried mutations in EDS1-90. One eds1-90 allele had a G to A mutation leading to a G483 to R483 substitution, and the other two alleles contained mutations at intron-exon junctions, which result in splice pattern changes (Figure 4A). Complementation test further confirmed that those three mutants were allelic to each other as they failed to complement each other (Figure 4B).

**PAD4-independent CHS3-mediated defense signaling is partially dependent on SGT1b.** Crude mapping of the two socp mutants isolated in the chs3-2D pad4-1 suppressor screen indicated that they both displayed linkage at the top of chromosome 4, a region that contains the known defense regulator SGT1b. Sanger sequencing uncovered that socp1 had a G to A splice site mutation in the fifth intron of SGT1b, and socp2 contained a point mutation leading to a G328 to E328 substitution (Figure 4A). These mutations in SGT1b are able to partially suppress the morphological and resistance phenotypes of chs3-2D pad4-1, suggesting that SGT1b positively regulates CHS3-mediated defense responses, which might function independently of PAD4.

**Immune signaling mediated by CHS3 requires its neighbour TNL protein CSA1.** Mapping of soc5, soc6, soc7 and soc8 revealed that these suppressor loci were all closely linked with chs3-2D. Genomic DNA from soc6 chs3-2D was then sequenced using Illumina next generation sequencing. Upon comparison with the Col-0 reference sequence, a point mutation causing a G233 to E233 amino acid substitution was identified in CSA1, a gene adjacent to CHS3. Direct Sanger sequencing of the remaining soc alleles revealed that they contain independent mutations in CSA1. This indicates that CSA1 is required for the autoimmunity responses of chs3-2D. Complementation was not observed from pair-wise crosses among these mutants, confirming that they contain mutations in the same gene (Figure 5A). CSA1 is adjacent to and divergently transcribed from CHS3, with an approximate 3.9 kb genomic region between their start codons (Figure 5B). It encodes a typical TNL.

When the CSA1 expression was examined in chs3-2D background, we observed over two-fold higher expression of the TNL gene in both chs3-2D and chs3-2D pad4-1 (Figure 5C). However, eds1-90 alleles can completely abolish the up-regulation of CSA1 in chs3-2D, indicating that the induced expression of CSA1 in chs3-2D is mediated through EDS1.

**Discussion**

CHS3 is an atypical TNL protein with an additional LIM domain at its C terminus. The exact function of the LIM domain is still unclear. It has been proposed that it inhibits the NLR protein in its native state. The gain-of-function chs3-2D allele results in extreme dwarfism and enhanced resistance to virulent pathogens. Epistasis analysis indicates that PAD4, which is thought to be a critical regulator downstream of many TNL immune receptors, is only partially required for the chs3-2D phenotypes. The distinctive features of chs3-2D provide us with an excellent background in which to conduct genetic suppressor screens in order to identify downstream components involved in CHS3-mediated defense pathway, which seems to differ from those involved in canonical TNL-mediated signaling.

In this study, we determined that CHS3-mediated signaling relies differently on defense-related lipase-like proteins (EDS1/PAD4/ SAG101) than signaling pathways downstream of other typical TNLs. Mutations in SAG101 can completely suppress the autoimmune responses of chs3-2D, whereas mutations in PAD4 fail to do so. This suggests that CHS3-mediated signaling is independent of PAD4.
munity of chs3-2D (Figure 1 and Figure 4A), while the suppression by pad4-1 is marginal (Figure 2), suggesting that CHS3-mediated signaling relies more strongly on SAG101. Genetic redundancy between PAD4 and SAG101 was previously suggested. However, previous research provided evidence that EDS1 forms distinct complexes with PAD4 and SAG101 with non-redundant signaling roles. Our findings potentially support this model. Although genetic redundancy between the two Col-0 EDS1 genes (EDS1-80 and EDS1-90) was demonstrated for immune signaling mediated by the CNL HYPERSENSITIVE RESPONSE TO TCV, CHS3-mediated defense responses seem to rely more on EDS1-90, as three mutations in EDS1-90, while none in EDS1-80, were found to completely abolish the autoimmunity in chs3-2D. It is therefore possible that CHS3 preferentially utilizes EDS1-90 and SAG101 for its defense activation, while EDS1-80 and PAD4 are marginally used.

Figure 5 | Complementation test of four csal mutations and gene arrangements of CHS3 and CSA1. (A) Morphology of three-week-old soil-grown plants of the indicated genotypes from the pair-wise allelism test. Two representative F1 plants were shown for each cross. (B) Boxes indicate exons while lines indicate introns. The encoded protein domains are denoted below the corresponding genomic regions. The directions of gene transcription are indicated as arrows. (C) CSA1 gene expression in plants of wild type, chs3-2D, chs3-2D pad4-1, chs3-2D eds1-90-10, and chs3-2D eds1-90-11. Total RNA was extracted from two-week-old plate-grown seedlings. Relative CSA1 expression levels were determined by real-time PCR. Values were normalized to the expression of ACTIN7. Error bars represent SD from three replicates.
Previous studies have shown that SGT1b negatively regulates defense responses mediated by the TNL SNC1 (SUPPRESSOR OF NPR1, CONSTITUTIVE 1) by altering its accumulation, as mutations in SGT1b lead to higher SNC1 levels. However, SGT1b appears to positively regulate CHS3-mediated defense responses, as mutations in SGT1b can partially suppress the phenotypes of chs3-2D pad4-1 (Ref. 10 and current study). Together, these data further highlight the differential roles SGT1b plays in NLR-mediated immunity. In the case of CHS3, SGT1b may serve as a molecular chaperon for proper assembly of the TNL complex for defense activation. In contrast, for SNC1, SGT1b is likely more involved in the SCF complex formation for ubiquitination and further degradation of this TNL.

The results of our suppressor screens have shown that the autoimmunity of chs3-2D requires the TNL CSA1, as loss-of-function mutations in CSA1 can completely suppress the chs3-2D phenotypes. CSA1 is adjacent to and divergently transcribed from CHS3, sharing an approximate 3.9 kb genomic region upstream of their start codons. This genomic arrangement is reminiscent of the R gene pair RPS4 and RRS1, whose hetero-dimerization is required for effector recognition. They are in a head-in-head arrangement with a 264 bp intragenic region, and are transcribed in opposite directions. The promoter regions probably overlap and this gene pair is likely under transcriptional co-regulation. RRS1, a TNL immune receptor with an extra WRKY domain at the C-terminus, confers recognition of the non-pathogen Pseudomonas syringae pv. tomato. Moreover, CHS3 needs its TNL protein neighbour, CSA1 to confer defense responses. The interaction between these two TNL proteins in planta will be investigated in the future. Instead of relying on the EDS1/PAD4/SAG101 module, CHS3 signaling seems to preferentially employ the EDS1-90/SAG101 complex (Figure 6).

Proposed working model for CHS3-mediated defense pathway. From our suppressor screens, we isolated several downstream regulators of CHS3-mediated signaling. Based on our current genetic data, we propose a working model for the CHS3-mediated pathway. SGT1b may form a chaperone complex with RAR1 and HSP90 to properly assemble the CHS3 activation complex. The C-terminal LI M domain has been hypothesized to inhibit CHS3 protein activation in the absence of pathogens. Moreover, CHS3 needs its TNL protein neighbour, CSA1 to confer defense responses. The interaction between these two TNL proteins in planta will be investigated in the future. Instead of relying on the EDS1/PAD4/SAG101 module, CHS3 signaling seems to preferentially employ the EDS1-90/SAG101 complex (Figure 6).
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Author contributions
F.X., C.Z. and X.L. designed the experiments and wrote the manuscript; F.X. and C.Z. performed all the experiments described in the figures; K.J. and Y.L. performed primary screens. V.C., J.D.J. and E.B.H. contributed to the cloning of CSA1. All authors reviewed the manuscript.

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