

Cytosolic activation of cell death and stem rust resistance by cereal MLA-family CC–NLR proteins

Stella Cesari^a, John Moore^a, Chunhong Chen^a, Daryl Webb^b, Sambasivam Periyannan^a, Rohit Mago^a, Maud Bernoux^a, Evans S. Lagudah^a, and Peter N. Dodds^{a,1}

^aCommonwealth Scientific and Industrial Research Organization Agriculture, Canberra, ACT 2601, Australia; and ^bCentre for Advanced Microscopy, Australian National University, Canberra, ACT 0200, Australia

Edited by Detlef Weigel, Max Planck Institute for Developmental Biology, Tubingen, Germany, and approved July 12, 2016 (received for review April 5, 2016)

Plants possess intracellular immune receptors designated “nucleotide-binding domain and leucine-rich repeat” (NLR) proteins that translate pathogen-specific recognition into disease-resistance signaling. The wheat immune receptors Sr33 and Sr50 belong to the class of coiled-coil (CC) NLRs. They confer resistance against a broad spectrum of field isolates of *Puccinia graminis* f. sp. *tritici*, including the Ug99 lineage, and are homologs of the barley powdery mildew-resistance protein MLA10. Here, we show that, similarly to MLA10, the Sr33 and Sr50 CC domains are sufficient to induce cell death in *Nicotiana benthamiana*. Autoactive CC domains and full-length Sr33 and Sr50 proteins self-associate *in planta*. In contrast, truncated CC domains equivalent in size to an MLA10 fragment for which a crystal structure was previously determined fail to induce cell death and do not self-associate. Mutations in the truncated region also abolish self-association and cell-death signaling. Analysis of Sr33 and Sr50 CC domains fused to YFP and either nuclear localization or nuclear export signals in *N. benthamiana* showed that cell-death induction occurs in the cytosol. In stable transgenic wheat plants, full-length Sr33 proteins targeted to the cytosol provided rust resistance, whereas nuclear-targeted Sr33 was not functional. These data are consistent with CC-mediated induction of both cell-death signaling and stem rust resistance in the cytosolic compartment, whereas previous research had suggested that MLA10-mediated cell-death and disease resistance signaling occur independently, in the cytosol and nucleus, respectively.

wheat stem rust | cell death | plant immunity | resistance protein | signaling

Plants have evolved effective innate immune systems to defend themselves against fast-evolving pathogens (1, 2). Plant immunity relies in part on the recognition of specific pathogen molecules, termed “effectors,” that are involved in infection processes (3). Effectors are usually recognized by intracellular immune receptors called “resistance proteins”; this recognition leads to a strong immune response that often is associated with a localized programmed cell death called the “hypersensitive response” (HR).

Most known resistance proteins contain central nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains (4) and are known as “NLRs.” There are two major subclasses of plant NLRs: one containing N-terminal regions that resemble the intracellular domains of the *Drosophila* protein Toll and the mammalian interleukin-1 receptor protein (TIR domain), and one containing N-terminal coiled-coil (CC) domains. These N-terminal regions have been shown to be signaling domains in several plant NLRs (5–9).

Stem rust is a devastating disease caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) that threatens wheat production worldwide. Recently, two stem rust-resistance genes, *Sr33* and *Sr50*, that confer resistance to a broad spectrum of *Pgt* isolates were identified as orthologs of the barley powdery mildew resistance gene *MLA* (10, 11). More than 30 distinct *MLA* alleles have been isolated from different barley cultivars (12) and have been studied intensively. An N-terminal 160-amino acid fragment of

MLA10 containing the CC domain (CC_{1–160}) was sufficient to trigger cell death in transient expression assays (8). A larger CC–NB_{1–225} fragment was found to self-associate in yeast two-hybrid assays; a smaller CC_{5–120} fragment was reported to form a stable homodimer in solution, and its crystal structure revealed a rod-shaped dimer (8), suggesting that CC self-association may be important for MLA10 signaling. Cell-death signaling by the full-length MLA10 protein or its CC–NB_{1–225} domain required the cytosolic location of the protein, but in contrast, resistance to powdery mildew disease in a transient single-cell expression assay required the presence of the protein in the nucleus (13). This finding was proposed to indicate a bifurcation between cell-death signaling and resistance signaling immediately downstream of MLA10 activation.

A wheat ortholog of the *MLA* gene family, *TmMla1*, confers resistance to wheat powdery mildew (14). Likewise, *Sr33* and *Sr50* occur at the orthologous locus in diploid wheat (*Aegilops tauschii*) and rye (*Secale cereale*), respectively, and are the first members of the *MLA* family known to recognize a pathogen other than powdery mildew (10, 11). MLA10, Sr33, and Sr50 proteins share ~80% amino acid sequence identity. To understand better the similarities and differences in the way these resistance proteins operate, we undertook a functional analysis of Sr33 and Sr50 with a focus on their ability to self-interact and induce cell death or disease resistance in a cell compartment-dependent manner. This analysis revealed some significant similarities with *MLA* in terms of CC domain signaling and *in planta* self-association but also demonstrated important differences, with Sr33 requiring cytosolic localization to confer rust resistance.

Significance

Stem rust caused by the fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*) remains the major disease threat to wheat production. The Sr33 and Sr50 resistance proteins protect wheat against a broad spectrum of field isolates of *Pgt* and are closely related to the barley powdery mildew-resistance protein MLA10. Like MLA10, Sr33 and Sr50 possess signaling N-terminal domains that self-associate *in planta* and initiate cell-death signaling from the cytosol. However, Sr33 induces disease-resistance signaling from the cytosol but not from the nucleus of wheat cells, suggesting cytosolic activation of both cell death and stem rust resistance.

Author contributions: S.C., J.M., E.S.L., and P.N.D. designed research; S.C., J.M., C.C., D.W., S.P., R.M., and M.B. performed research; D.W. contributed new reagents/analytic tools; S.C., C.C., S.P., and P.N.D. analyzed data; and S.C. and P.N.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. Email: peter.dodds@csiro.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605483113/-DCSupplemental.

Results

The CC Domain of MLA10, Sr33, and Sr50 Is Sufficient for Cell-Death Induction

To investigate whether Sr33 and Sr50 function similarly to MLA in cell-death induction, we tested three N-terminal fragments of Sr33, Sr50, and MLA10, corresponding to the MLA10 1–120, 1–160, and 1–225 fragments (Fig. S1A). Using CC prediction software (15), we identified two coiled domains spanning roughly residues 20–50 and 115–144 in MLA10, Sr33, and Sr50 (Fig. S1B), suggesting that the 1–160 fragments should contain a full CC domain, whereas the 1–120 fragments may have a truncated CC domain. These N-terminal fragments were transiently expressed in *Nicotiana benthamiana* under the control of the 35S promoter and were fused to either a C-terminal HA or CFP tag. The full CC-containing constructs of Sr33 and Sr50 (amino acids 1–160 and 1–163, respectively) triggered a strong cell-death response that was visible 40 h after agro-infiltration when fused to either CFP or HA (Fig. 1A). Consistent with previous reports, MLA10_{1–160}:HA and MLA10_{1–160}:CFP also induced a strong cell-death response. A positive control protein, the rice autoactive CC–NLR RGA4, produced a strong cell-death response, but its inactive T₅₀₀M/Y₅₀₁H/G₅₀₂D mutant (rga4^{TYG/MHD}) did not (16, 17). Expression of the CC–NB fragments of Sr33 (residues 1–225), Sr50 (1–228), and MLA10 (1–225) also triggered a cell-death response (Fig. 1B), but the truncated CC domains of MLA10 (1–120), Sr33 (1–120), and Sr50 (1–123) fused to HA or CFP did not induce cell death (Fig. 1C and Fig. S2A). Western blot analysis indicated that all fusion proteins were properly expressed (Fig. 2 and Fig. S3A–C). Full-length Sr33 and Sr50 cDNA constructs also induced cell death when driven by the 35S promoter (Fig. 1C and Figs. S2B and S3D), whereas expression of the genomic constructs under their native cereal promoters led to very low or undetectable protein expression and no cell-death response in *N. benthamiana* (Figs. S2B and S3D).

The Sr33, Sr50, and MLA10 CC Domains Self-Associate in Planta

Although the truncated CC_{5–120} fragment of MLA10 was reported to homodimerize in solution, and its CC–NB_{1–225} domain was reported to self-interact in a yeast two-hybrid assay (8), self-interaction has not been demonstrated with the minimal active CC_{1–160} domain. In contrast to MLA10, self-interaction of the Sr33 CC–NB_{1–225} domain, the CC_{1–120} domain, and the CC_{1–160} domain was not detected by yeast two-hybrid assay (10). We tested the active CC and CC–NB fragments of MLA10 and Sr50 in a GAL4-based yeast two-hybrid system but did not observe evidence of self-interaction, although all fusion proteins were expressed (Fig. S4). Self-interaction was detected for the rice Resistance

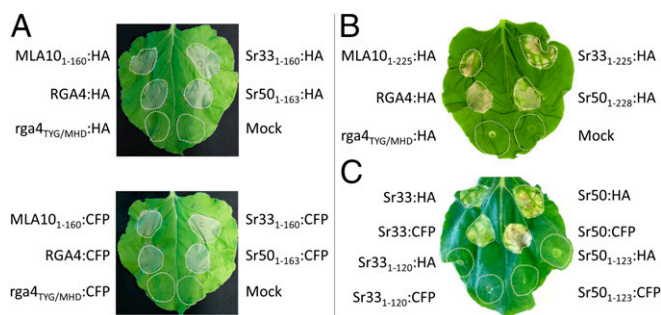


Fig. 1. The full CC domains of MLA10, Sr33, and Sr50 are sufficient for autoactivity *in planta*. Indicated fragments of the MLA10, Sr33, and Sr50 proteins fused to HA or CFP were transiently expressed in *N. benthamiana* leaves by *Agrobacterium tumefaciens* infiltration along with the autoactive RGA4:HA or RGA4:CFP positive controls and the inactive rga4^{TYG/MHD}:HA or rga4^{TYG/MHD}:CFP or mock inoculation as negative controls. Pictures were taken 3 d (A) or 5 d (B and C) after infiltration. Equivalent results were obtained in three independent experiments.

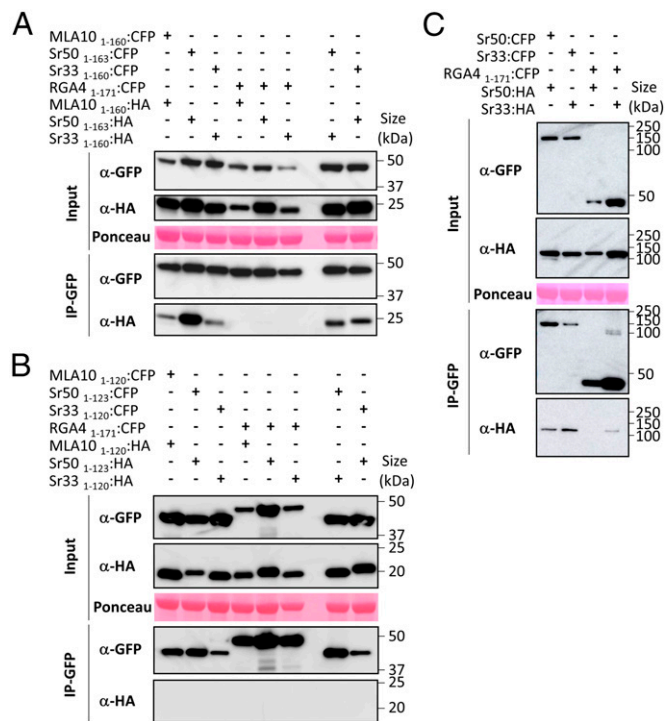


Fig. 2. The full CC domains of Sr33, Sr50, and MLA10 can self-associate *in planta*. (A) The full CC domains of MLA10, Sr33, and Sr50 fused to CFP or HA tags were transiently expressed in *N. benthamiana* leaves in the indicated combinations (+, agro-infiltrated construct; –, non-agro-infiltrated construct), and proteins were extracted after 24 h. Tagged proteins were detected in the extract (input) and after immunoprecipitation with anti-GFP beads (IP-GFP) by immunoblotting with anti-HA (α -HA) and anti-GFP (α -GFP) antibodies. The RGA4 CC fused to CFP was used as a control for specificity. Protein loading in the input is shown by Ponceau staining of the large RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) subunit. The experiment was carried out three times with identical results. (B) The truncated CC domains of MLA10, Sr33, and Sr50 fused to CFP or HA were transiently expressed in *N. benthamiana* leaves in the indicated combinations. Samples were processed as described in A. The experiment was carried out twice with identical results. (C) The procedure described in A was applied to the full-length Sr33 and Sr50 proteins.

Gene Analog 5 (RGA5) CC_{1–228} and L6-TIR domain, the positive controls in this experiment (Fig. S4) (7, 17).

To determine whether these CC domains could self-associate *in planta*, we performed coimmunoprecipitation experiments in *N. benthamiana* using the auto-active CC domains of Sr33, Sr50, and MLA10 fused to CFP or HA tags. Proper expression of all proteins was verified by immunoblotting using anti-GFP and anti-HA antibodies (Fig. 2A). CFP-fused proteins were enriched after immunoprecipitation with anti-GFP beads, and MLA10_{1–160}:HA, Sr33_{1–160}:HA, and Sr50_{1–163}:HA specifically coprecipitated with MLA10_{1–160}:CFP, Sr33_{1–160}:CFP, and Sr50_{1–163}:CFP, respectively, but not with RGA4_{1–171}:CFP (17), which was used as a negative control for binding specificity (Fig. 2A). These results show that the CC domains of MLA10, Sr33, and Sr50 form specific homo-complexes *in planta*. Interestingly, Sr33 and Sr50 CC domains also formed heterocomplexes when coexpressed *in planta* (Fig. 2A). This coexpression did not impair cell-death induction (Fig. S5A).

We also tested whether the truncated CC domains, which are inactive in cell-death signaling, would retain self-association or hetero-association in *N. benthamiana*. Although all fusion proteins were detected in leaf extracts by immunoblotting with anti-GFP or anti-HA antibodies, and CFP-tagged proteins were precipitated by anti-GFP beads, the HA-tagged proteins did not coprecipitate with any of the CFP-tagged proteins (Fig. 2B and Fig. S5B). Thus,

self-association *in planta* appears to be a property limited to the active signaling fragments of these proteins.

Full-Length Sr33 and Sr50 Proteins also Self-Associate *in Planta*. The full-length MLA1 protein was also shown to self-associate *in planta* (8), so we tested full-length Sr33 and Sr50 self-association by coimmunoprecipitation (Fig. 2C). Immunoblotting using anti-GFP and anti-HA antibodies showed expression of all proteins in the input, and immunoprecipitation with anti-GFP beads resulted in the pull-down of CFP-fused proteins and coprecipitation of the HA-tagged protein. Although Sr33:HA showed a low level of nonspecific binding to RGA4₁₋₁₇₁:CFP and to the anti-GFP beads (Fig. 2C and Fig. S5C), an enrichment of coimmunoprecipitated Sr33:HA protein was observed in the presence of Sr33:CFP (Fig. 2C and Fig. S5C). These results suggest that the Sr33 and Sr50 proteins also form homocomplexes *in planta*. Full-length Sr33 and Sr50 proteins also could form heterocomplexes in *N. benthamiana* (Fig. S5D), but this interaction did not inhibit cell-death induction (Fig. S5A).

Residues Within the 120–144 Region of Sr33 CC Domain Are Crucial for Self-Association and Autoactivity. The data presented above suggest that the region corresponding to MLA10 residues 120–160 is important for cell-death signaling activity and self-association. To test this suggestion further, we generated six different mutations within this region of Sr33 (Fig. 3A). In five mutants we exchanged conserved hydrophobic residues for negatively charged glutamate residues, and in one mutant we exchanged a conserved six amino acid hydrophilic stretch (RRDRNK) for alanine residues. These mutations were independently introduced in both the CC₁₋₁₆₀

domain and the full-length Sr33 protein, and transient expression experiments in *N. benthamiana* showed that all mutations impaired cell-death induction by both CC and full-length proteins (Fig. 3B), although all proteins were well expressed (Fig. 3C and Fig. S3E). In addition, coimmunoprecipitation assays revealed that all the mutations disrupted or strongly inhibited Sr33 CC self-association *in planta* (Fig. 3C). Taken together, these data indicate a crucial role of this region for both signaling and self-association.

Cytosolic Localization of MLA10, Sr33, and Sr50 CC Domains Is Required for Cell-Death Induction. The MLA10 full-length protein and its CC-NB₁₋₂₂₅ domain induce cell-death signaling in the cytoplasm of *N. benthamiana* cells (13). To test whether the smaller CC-containing autoactive domains also induce cell-death signaling in the cytosol, we transiently expressed MLA10₁₋₁₆₀:YFP, Sr33₁₋₁₆₀:YFP, and Sr50₁₋₁₆₃:YFP in *N. benthamiana* fused to a nuclear localization signal (NLS), a mutated NLS (nls), a nuclear export signal (NES), or a mutated NES (nes) (19). Upon expression of the YFP:NLS-fused CC domains, specific YFP fluorescence was detected exclusively in the nuclei of *N. benthamiana* cells (Fig. 4A and Fig. S6A). In contrast, the CC domains fused to YFP:NES were effectively, although not completely, excluded from nuclei, with fluorescence detected mostly in the cytosol. CC:YFP domains fused to mutated nls and nes variants fluoresced in both the cytosol and nuclei (Fig. 4A and Fig. S6A). Cell-death assays revealed that forced nuclear localization of all CC domains reduced their cell-death activity compared with other fusions with YFP:NES, YFP:nes, or YFP:nls (Fig. 4B). This cell-death reduction was less pronounced for the Sr50 CC domain than for the MLA10 and Sr33 CC domains (Fig. 4B). However, the strongest cell-death response triggered by the Sr50 CC domain was observed when it was fused to the NES, indicating that nuclear localization of Sr50 CC domain is not required for its signaling activity. Protein expression of all constructs was verified by immunoblotting (Fig. S3F). The NES-fused constructs consistently showed lower protein accumulation but nevertheless gave the strongest cell-death phenotype, suggesting that cell-death and protein-degradation processes had already been activated at the time of sampling. These results indicate that the MLA10, Sr33, and Sr50 CC domains activate cell-death signaling from the cytosol.

Sr33 Induces Stem Rust Resistance in the Cytosol of Wheat Cells. Previous reports showed that full-length MLA10 locates to both the cytoplasm and the nucleus of transiently transformed barley leaf epidermal cells (20) and that, in contrast to cell-death signaling, the nuclear pool is required for its powdery mildew resistance activity (13, 20). To examine the subcellular distribution of Sr33 and to identify the compartment in which it induces disease resistance, we stably transformed wheat with constructs carrying the genomic sequence of *Sr33* driven by its native promoter (*pSr33*) and fused with either YFP venus (YFPv), YFPv:NLS, YFPv:nls, YFPv:NES, or YFPv:nes, as well as a construct containing YFPv under the control of the *pSr33* promoter. Progeny of six independent T0 transgenic lines for each construct were assessed for resistance to *Pgt* race 98-1,2,3,5,6 (and Fig. 5A and Table S1). T1 plants derived from three independent T0 lines expressing *Sr33*:YFPv segregated for stem rust resistance, confirming the function of the YFPv-fused Sr33 protein. All transgenic lines carrying the *Sr33*:YFPv:NES, *Sr33*:YFPv:nes, or *Sr33*:YFPv:nls constructs gave rise to resistant T1 plants. However, all transformed lines containing the *Sr33*:YFPv:NLS construct were fully susceptible, similar to untransformed wheat or plants carrying the YFPv construct (Fig. 5A and Table S1). Immunoblot analysis revealed that all the YFP fusion constructs were expressed, with no YFPv cleavage observed (Fig. 5B).

Using confocal microscopy we observed that Sr33:YFPv accumulated mostly in the cytosol of leaf epidermal cells of the transgenic lines and was only weakly visible in a small proportion of nuclei (Fig. S6B). However, it was difficult to visualize Sr33:YFPv fluorescence in mesophyll cells, which are the main site of interaction with *Pgt* during infection (21). Therefore, we isolated

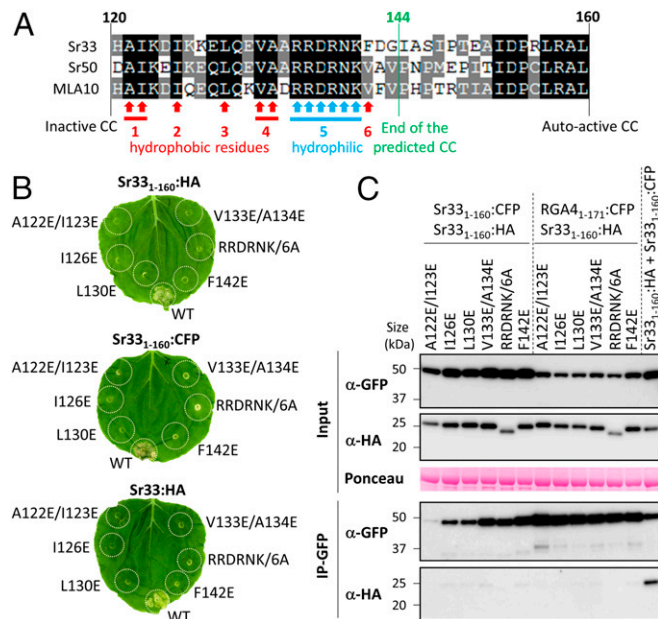


Fig. 3. Residues within the 120–144 CC region of Sr33 are crucial for self-association and autoactivity. (A) Alignment showing the 120–160 fragment of MLA10, Sr33, and Sr50. Six mutations (numbered 1–6) were generated in which conserved hydrophobic residues (red) were replaced by glutamate, or a hydrophilic stretch (blue) was replaced by alanines. (B) WT and mutated constructs of the Sr33 CC₁₋₁₆₀ domain or full-length protein fused to HA or CFP were transiently expressed in *N. benthamiana*, and cell death was visualized after 4 d. Equivalent results were obtained in three independent experiments. (C) The indicated mutants of the Sr33 CC₁₋₁₆₀ domain fused to CFP or HA tags were coexpressed in *N. benthamiana* and analyzed by coimmunoprecipitation as described in Fig. 2A. RGA4₁₋₁₇₁:CFP was used as a control for specificity, and WT Sr33₁₋₁₆₀:HA and Sr33₁₋₁₆₀:CFP were used as positive controls. Three independent experiments gave equivalent results.

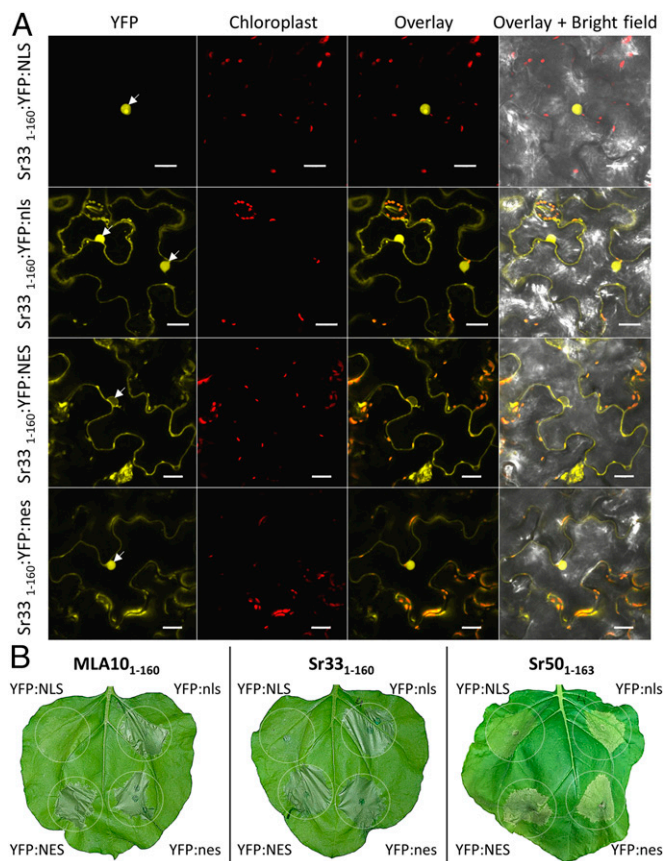


Fig. 4. The CC domains of Sr33, Sr50, and MLA10 induce cell-death signaling in the cytosol of *N. benthamiana* cells. (A) Fluorescence microscopy images (20 h postinfiltration) of the localization of the Sr33 CC₁₋₁₆₀ domain fused to YFP:NLS, YFP:nls, YFP:NES, or YFP:nls in transiently transformed *N. benthamiana* leaf epidermal cells. Fluorescence channels for YFP and chloroplasts are merged on the overlay picture, and all channels are merged on the overlay + bright-field picture. White arrows indicate nuclei. (Scale bars, 20 μ m.) (B) MLA10 CC₁₋₁₆₀, Sr33 CC₁₋₁₆₀, and Sr50 CC₁₋₁₆₃ domains fused to YFP:NLS, YFP:nls, YFP:NES, or YFP:nls were transiently expressed in *N. benthamiana* leaves by *A. tumefaciens* infiltration. Pictures were taken 40 h after infiltration. Equivalent results were obtained in three independent experiments.

mesophyll protoplasts from the T1 transgenic plants. We observed exclusive localization of YFPv fluorescence in the cytosol in these cells, indicating that Sr33:YFPv proteins are largely excluded from the nucleus (Fig. 6). By contrast, YFPv expressed under the control of the Sr33 promoter exhibited a nucleo-cytosolic localization pattern in both leaf epidermal cells and mesophyll protoplasts (Fig. 6 and Fig. S6B).

The Sr33:YFPv:NLS construct showed clear nuclear accumulation and undetectable cytosolic YFPv fluorescence in all epidermal or mesophyll cells, demonstrating that the NLS effectively targets Sr33 to the nucleus (Fig. 6 and Fig. 6B). Sr33:YFPv:nls proteins showed cytosolic and nuclear fluorescence in both epidermal and mesophyll cells, suggesting that the mutated nls retains some nuclear targeting activity (Fig. 6 and Fig. 6B). The subcellular fluorescence distribution of cells expressing Sr33:YFPv:NES and Sr33:YFPv:nls were indistinguishable from Sr33:YFPv, and these proteins were present exclusively in the cytosol (Fig. 6 and Fig. 6B). Taken together, these results show that Sr33 localizes to and induces stem rust resistance in the cytosol of wheat cells.

Discussion

In this study, we provide evidence that the CC domains of MLA10, Sr33, and Sr50 are active in cell-death signaling and self-associate *in planta*. Similarly, full-length Sr33 and Sr50 proteins trigger a

pathogen-independent cell-death response in *N. benthamiana* and self-associate *in planta* in the absence of recognized avirulence effector. These results are consistent with previous reports showing that MLA10 full-length protein displays autoactivity and self-associates in coimmunoprecipitation experiments (8, 13). However, because these proteins are autoactive in *N. benthamiana*, it is not clear whether this self-association represents a pre- or postactivation event, and it remains possible that the self-association of these NLR proteins is triggered by effector recognition. These results also are in line with the idea that plant NLR protein oligomerization plays an important role in their activation or repression processes. Indeed, together with MLA, previous studies on a number of immune receptors such as N, RPS5, Prf, RB, L6, RPS4/RRS1, or RGA4/RGA5 indicate that their N-terminal domains provide a self-association platform (7, 17, 22–26). In L6, MLA10, and RPS4, a clear link between CC/TIR self-interaction and cell-death signaling was observed (7, 8, 26), whereas for the NLR hetero-pairs RPS4/RRS1 and RGA4/RGA5, hetero-interactions are crucial to regulate the activity of the signaling-competent NLR partner (17, 26).

Although the CC domains of MLA10, Sr33, and Sr50 trigger cell death and self-associate *in planta*, their truncated CC domains do not self-associate in coimmunoprecipitation assays and are inactive in cell-death signaling. Furthermore, mutations within the 120–144 region of the predicted CC domain of Sr33 inhibited both cell-death induction and self-association. Therefore these truncated CC domains appear to lack important residues for cell-death induction and self-association. It was previously reported that a similar truncated domain of the MLA10 CC (5–120) forms a dimer in solution, and a dimeric crystal structure was solved (8). However, given that this domain lacks signaling activity, and does not self-associate *in planta*, it is not clear how these structural observations relate to the biological activity of the protein. We believe that further structural investigations of an active CC domain will be necessary to unravel the molecular bases for MLA10, Sr33, and Sr50 CC self-association.

We did not detect self-association of inactive truncated CC, active CC, or CC-NB domains of Sr33 (10), Sr50, or MLA10 using a Gal4-based yeast-two-hybrid assay. Self-interaction of the MLA10 CC-NB₁₋₂₂₅ domain was previously reported using a LexA-based yeast two-hybrid system (8), which may be more sensitive. However, it is possible that self-association of the active domains of MLA10, Sr33, and Sr50 may require additional uncharacterized plant proteins.

We also found that the Sr33 and Sr50 CC domains or full-length proteins can hetero-associate in coimmunoprecipitation assays. Because Sr33 and Sr50 are derived from diploid wheat and

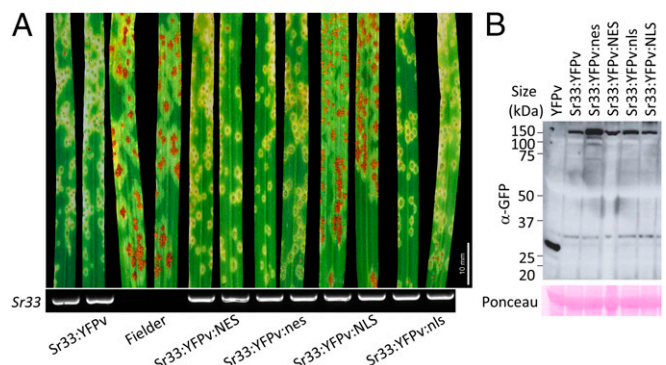


Fig. 5. Sr33 induces disease-resistance signaling from the cytosol of wheat cells. (A) Untransformed Fielder and two independent T1 transgenic lines per construct expressing Sr33 fused to YFPv, YFPv:NLS, YFPv:nls, YFPv:NES, or YFPv:nls plants were inoculated with *Pgt* strain 98-1,2,3,5,6 at the two-leaf stage. Pictures were taken 13 d after inoculation. The presence of the transgenes was determined by PCR with Sr33-specific primers. (B) Immunoblot of transgenic wheat seedlings expressing YFP-tagged proteins detected with anti-GFP.

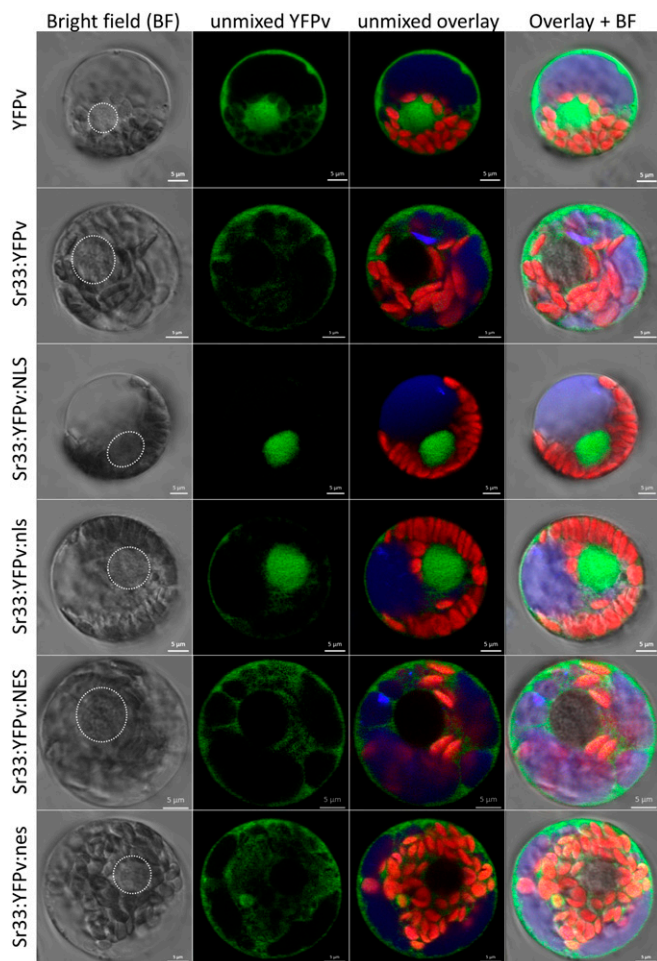


Fig. 6. Localization of Sr33 fusion proteins in wheat mesophyll protoplasts. (A) Linear unmixing confocal images showing the localization of Sr33 fused to the YFPv, YFPv:NLS, YFPv:nls, YFPv:NES, or YFPv:nes in transgenic wheat mesophyll protoplasts. Protoplasts were isolated from 10- to 14-d-old T1 transgenic plants and were observed within 8 h following isolation. YFPv-specific fluorescence appears in green; specific vacuolar and chloroplastic autofluorescence emissions are shown in blue and red, respectively. White circles mark nuclei locations on bright-field pictures. (Scale bars, 5 μ m.)

rye, respectively (10, 11), these genes do not naturally occur together in the same plant, but both have been introgressed into cultivated wheat to provide stem rust resistance. Their hetero-association *in planta* raises the possibility that they could interfere functionally, because such interference has been reported for the powdery mildew resistance gene *Pm8*, derived from rye, and its wheat ortholog *Pm3*, which also form heterocomplexes (27).

NLR proteins have been observed in a wide diversity of subcellular compartments. Many, such as N (28, 29), SNC1 (30), Pikh-1/Pikh-2 (31), Rx (32), Pb1 (33), MLA10 (20), and Rp1-D21, show a nucleo-cytoplasmic localization (34). Some, such as RGA4/RGA5, are localized mainly in the cytosol (17). In the presence of their corresponding effectors, R3a is relocalized from the cytosol to the endosome (35), whereas RRS1 is stabilized in the nucleus (36, 37). RPS4, which functions together with RRS1, distributes between endomembranes and nuclei, and its nuclear localization is required for AvrRPS4 recognition (38). Rpm1 (39), RPS2 (40), RPS5 (41), and Pit (42) are associated with the plasma membrane, whereas L6 and M are addressed to the Golgi membrane and tonoplast, respectively (43). This observation raises a fundamental question: What is the link between NLR localization and cell-death and/or disease-resistance signaling activity?

Here we found that the MLA10, Sr33, and Sr50 CC domains induce cell-death signaling in the cytosol of *N. benthamiana* cells. This finding is consistent with previous reports of cell-death induction by the full-length MLA10 protein or the MLA10 CC-NB in *N. benthamiana* (13). However, in stably transformed wheat, Sr33 confers stem rust resistance when it is located in the cytosol but not when it is confined to the nucleus. This effect is consistent with the cell-death signaling result but differs from the observations that nuclear-localized MLA10 is sufficient to confer resistance to *Blumeria graminis* in transient expression assays based on biolistic transformation of barley epidermal cells, while nuclear-excluded MLA10 cannot confer resistance (13, 20). Bai et al. (13) proposed a bifurcation of MLA10-triggered cell-death and disease-resistance signaling immediately after the receptor activation, so that MLA10 could directly trigger cell death in the cytosol and also could trigger a separate response leading to resistance in the nucleus. Another factor possibly contributing to these differing observations is that the corresponding effectors may have different localizations, with AvrMLA10 perhaps being localized to the nucleus and AvrSr33 to the cytosol and thus requiring nuclear or cytosolic receptors, respectively, for their recognition. It has been shown that exclusion of PopP2 from the nucleus impairs its recognition by RPS4 and RRS1, indicating that colocalization of the effector and NLR is required to trigger the HR in this system (44). Given that the HvWRKY1/2 and MYB6 transcription factors interact with the MLA10 CC domain (20, 45), it is possible that these nuclear proteins are guardees/decoys that are necessary for effector perception. Indeed, recent findings suggest that transcription factors such as WRKY and MYB are common integrated domains/decoys in plant NLR proteins, indicating that they could be common effector targets (46–48). Previous yeast two-hybrid assays did not detect any interaction of the Sr33 CC domain with HvWRKY1, HvWRKY2, or their wheat orthologs (10).

In terms of compartment-dependent activation, Sr33 is more similar to the potato CC-NLR Rx, a nucleo-cytoplasmic protein that recognizes the potato virus X (PVX) coat protein (CP) (32, 49). Rx interacts with the small cytosolic GTPase RanGAP2, which is required for Rx function (50, 51) and affects Rx nucleo-cytoplasmic distribution (49). Overexpression of RanGAP2 sequesters Rx in the cytosol, leading to enhanced Rx activity, whereas expression of NLS-fused RanGAP2 leads to Rx accumulation in the nucleus and inhibits Rx function (49). Likewise, fusion of an NLS to Rx significantly compromised its activity, whereas an NES fusion had a minimal effect (32). Furthermore, nuclear-targeted PVX CP does not induce the Rx-dependent HR, suggesting that both pathogen recognition and resistance signaling take place in the cytoplasm (32). However, unlike Sr33, Rx induces cell-death signaling through its NB domain, not through the CC domain (52). Therefore, although Rx and Sr33 display certain similarities, their signaling components and regulatory mechanisms may differ. Considering the wide diversity of NLR subcellular localizations and the existence of several identified signaling domains among NLRs, multiple disease-signaling pathways may exist.

Our study suggests that the AvrSr33 effector would be directly or indirectly recognized by the cytosolic pool of Sr33 and that the CC domain of the activated receptor would interact with cytosolic signaling partners to initiate both disease resistance and cell death. Subsequent downstream signaling pathway branches may be responsible for responses in different cellular compartments, such as transcriptional changes in the nucleus.

Methods

Details of the methods used are provided in *SI Methods*, including plant growth and infection assay conditions, constructs, yeast two-hybrid analysis, transient expression in *N. benthamiana*, protein extraction immunoblot and coimmunoprecipitation, wheat transformation, protoplast isolation, confocal microscopy, and accession numbers. Primers are listed in *Table S2*. Details of constructs are given in *Table S3*.

ACKNOWLEDGMENTS. We thank T. Richardson, S. Louis, D. Bhatt, X. Xia, and K. Newell for technical assistance; Drs. A. Ashton, M. Ayliffe, X. Zhang, and S. Williams for helpful comments and discussions; and Prof. P. Schulze-Lefert and Dr. S. Rivas for providing MLA10 cDNA and binary vectors, respectively. S.C. was supported by a Contrat Jeune Scientifique Postdoctoral

Fellowship from L'Institut National de la Recherche Agronomique. M.B. was a recipient of Discovery Early Career Research Award DE130101292 from the Australian Research Council. This work also was supported by funding from the Durable Rust Resistance in Wheat Project and Grains Research and Development Corporation Grant CSP00161.

- Dodds PN, Rathjen JP (2010) Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat Rev Genet* 11(8):539–548.
- Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444(7117):323–329.
- Kamoun S (2007) Groovy times: Filamentous pathogen effectors revealed. *Curr Opin Plant Biol* 10(4):358–365.
- Takken FL, Govers A (2012) How to build a pathogen detector: Structural basis of NB-LRR function. *Curr Opin Plant Biol* 15(4):375–384.
- Swiderski MR, Birker D, Jones JDG (2009) The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. *Mol Plant Microbe Interact* 22(2):157–165.
- Krasileva KV, Dahlbeck D, Staskawicz BJ (2010) Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22(7):2444–2458.
- Bernoux M, et al. (2011) Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. *Cell Host Microbe* 9(3):200–211.
- Maekawa T, et al. (2011) Coiled-coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. *Cell Host Microbe* 9(3):187–199.
- Wang G-F, et al. (2015) Molecular and functional analyses of a maize autoactive NB-LRR protein identify precise structural requirements for activity. *PLoS Pathog* 11(2): e1004674.
- Periyannan S, et al. (2013) The gene Sr33, an ortholog of barley Mla genes, encodes resistance to wheat stem rust race Ug99. *Science* 341(6147):786–788.
- Mago R, et al. (2015) The wheat Sr50 gene reveals rich diversity at a cereal disease resistance locus. *Nat Plants* 1(12):15186.
- Seeholzer S, et al. (2010) Diversity at the Mla powdery mildew resistance locus from cultivated barley reveals sites of positive selection. *Mol Plant Microbe Interact* 23(4):497–509.
- Bai S, et al. (2012) Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS Pathog* 8(6):e1002752.
- Jordan T, et al. (2011) The wheat Mla homologue TmMla1 exhibits an evolutionarily conserved function against powdery mildew in both wheat and barley. *Plant J* 65(4):610–621.
- Lupas A, Van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. *Science* 252(5009):1162–1164.
- Cesari S, et al. (2013) The rice resistance protein pair RGA4/RGA5 recognizes the *Magnaporthe oryzae* effectors AVR-Pia and AVR1-CO39 by direct binding. *Plant Cell* 25(4):1463–1481.
- Cesari S, et al. (2014) The NB-LRR proteins RGA4 and RGA5 interact functionally and physically to confer disease resistance. *EMBO J* 33(17):1941–1959.
- Lanford RE, Butel JS (1984) Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* 37(3):801–813.
- Wen W, Meinkoth JL, Tsien RY, Taylor SS (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell* 82(3):463–473.
- Shen Q-H, et al. (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315(5815):1098–1103.
- Garnica DP, Nemri A, Upadhyaya NM, Rathjen JP, Dodds PN (2014) The ins and outs of rust haustoria. *PLoS Pathog* 10(9):e1004329.
- Ade J, DeYoung BJ, Golstein C, Innes RW (2007) Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. *Proc Natl Acad Sci USA* 104(7):2531–2536.
- Gutierrez JR, et al. (2010) Prf immune complexes of tomato are oligomeric and contain multiple Pto-like kinases that diversify effector recognition. *Plant J* 61(3):507–518.
- Mestre P, Baulcombe DC (2006) Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *Plant Cell* 18(2):491–501.
- Chen Y, Liu Z, Halterman DA (2012) Molecular determinants of resistance activation and suppression by *Phytophthora infestans* effector IPI-O. *PLoS Pathog* 8(3):e1002595.
- Williams SJ, et al. (2014) Structural basis for assembly and function of a heterodimeric plant immune receptor. *Science* 344(6181):299–303.
- Hurni S, et al. (2014) The powdery mildew resistance gene *Pm8* derived from rye is suppressed by its wheat ortholog *Pm3*. *Plant J* 79(6):904–913.
- Burch-Smith TM, et al. (2007) A novel role for the TIR domain in association with pathogen-derived elicitors. *PLoS Biol* 5(3):e68.
- Caplan JL, Mamillapalli P, Burch-Smith TM, Czymmek K, Dinesh-Kumar SP (2008) Chloroplastic protein NR1P1 mediates innate immune receptor recognition of a viral effector. *Cell* 132(3):449–462.
- Cheng YT, et al. (2009) Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in *Arabidopsis*. *Plant Cell* 21(8):2503–2516.
- Zhai C, et al. (2014) Function and interaction of the coupled genes responsible for Pik-h encoded rice blast resistance. *PLoS One* 9(6):e98067.
- Slootweg E, et al. (2010) Nucleocytoplasmic distribution is required for activation of resistance by the potato NB-LRR receptor Rx1 and is balanced by its functional domains. *Plant Cell* 22(12):4195–4215.
- Inoue H, et al. (2013) Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through protein-protein interaction. *Proc Natl Acad Sci USA* 110(23):9577–9582.
- Wang G-F, Balint-Kurti PJ (2015) Cytoplasmic and nuclear localizations are important for the hypersensitive response conferred by maize autoactive Rp1-D21 protein. *Mol Plant Microbe Interact* 28(9):1023–1031.
- Engelhardt S, et al. (2012) Relocalization of late blight resistance protein R3a to endosomal compartments is associated with effector recognition and required for the immune response. *Plant Cell* 24(12):5142–5158.
- Deslandes L, et al. (2003) Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc Natl Acad Sci USA* 100(13):8024–8029.
- Tasset C, et al. (2010) Autoacetylation of the *Ralstonia solanacearum* effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in *Arabidopsis*. *PLoS Pathog* 6(11):e1001202.
- Wirthmueller L, Zhang Y, Jones JDG, Parker JE (2007) Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Curr Biol* 17(23):2023–2029.
- Gao Z, Chung E-H, Eitas TK, Dangl JL (2011) Plant intracellular innate immune receptor Resistance to *Pseudomonas syringae* pv. maculicola 1 (RPM1) is activated at, and functions on, the plasma membrane. *Proc Natl Acad Sci USA* 108(18):7619–7624.
- Axtell MJ, Staskawicz BJ (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* 112(3):369–377.
- Qi D, DeYoung BJ, Innes RW (2012) Structure-function analysis of the coiled-coil and leucine-rich repeat domains of the RPS5 disease resistance protein. *Plant Physiol* 158(4):1819–1832.
- Kawano Y, et al. (2014) Palmitoylation-dependent membrane localization of the rice resistance protein Pit is critical for the activation of the small GTPase OsRac1. *J Biol Chem* 289(27):19079–19088.
- Takemoto D, et al. (2012) N-terminal motifs in some plant disease resistance proteins function in membrane attachment and contribute to disease resistance. *Mol Plant Microbe Interact* 25(3):379–392.
- Sohn KH, et al. (2014) The nuclear immune receptor RPS4 is required for RRS15LH1-dependent constitutive defense activation in *Arabidopsis thaliana*. *PLoS Genet* 10(10): e1004655.
- Chang C, et al. (2013) Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. *Plant Cell* 25(3):1158–1173.
- Cesari S, Bernoux M, Moncuquet P, Kroj T, Dodds PN (2014) A novel conserved mechanism for plant NLR protein pairs: The “integrated decoy” hypothesis. *Front Plant Sci* 5(606):606.
- Kroj T, Chanclud E, Michel-Romiti C, Grand X, Morel J-B (2016) Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread. *New Phytol* 210(2):618–626.
- Sarris PF, Cevik V, Dagdas G, Jones JDG, Krasileva KV (2016) Comparative analysis of plant immune receptor architectures uncovers host proteins likely targeted by pathogens. *BMC Biol* 14:8.
- Tameling WIL, et al. (2010) RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *Plant Cell* 22(12):4176–4194.
- Tameling WI, Baulcombe DC (2007) Physical association of the NB-LRR resistance protein Rx with a Ran GTPase-activating protein is required for extreme resistance to Potato virus X. *Plant Cell* 19(5):1682–1694.
- Sacco MA, Mansoor S, Moffett P (2007) A RanGAP protein physically interacts with the NB-LRR immune receptor Rx, and is required for Rx-mediated viral resistance. *Plant J* 52(1):82–93.
- Rairdan GJ, et al. (2008) The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling. *Plant Cell* 20(3):739–751.
- Bernoux M, et al. (2008) RD19, an *Arabidopsis* cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the *Ralstonia solanacearum* PopP2 effector. *Plant Cell* 20(8):2252–2264.
- Heidrich K, et al. (2011) *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334(6061):1401–1404.
- Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 350:87–96.
- Kushnir VV (2000) Rapid and reliable protein extraction from yeast. *Yeast* 16(9):857–860.
- Richardson T, Thistleton J, Higgins TJ, Howitt C, Ayliffe M (2014) Efficient *Agrobacterium* transformation of elite wheat germplasm without selection. *Plant Cell Tissue Organ Cult* 119(3):647–659.
- Shan Q, et al. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 31(8):686–688.
- Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21):2947–2948.