

chromosome, which is typically random in female mammals, is skewed in cloned female mice (22, 23), causes recurrent spontaneous abortions in some pregnant women (24), and could result in misexpressions in NT-hESCs derived from women. Finally, the genomic stability of NT-hESCs, as well as their differentiation fidelity, including aging and telomerase/telomere behavior, also require rigorous investigations.

The somatic cell's adaptation to in vitro conditions may predispose human NT embryos to cell culture proliferation, with negligible potentials for implantation and none for normal development. Neither NT embryonic development nor NT-hESC establishment rates provide any encouragement for dangerous human reproductive cloning attempts. Cloned animals have adverse pregnancy outcomes, so regardless of cruel hoaxes (25), scientific evidence should further discourage reckless notions regarding human reproductive cloning. Human SCNT was optimized from porcine SCNT procedures in which ~150 NT embryos were transferred for pregnancy establishment (26–28). Furthermore, in rhesus monkeys, 135 cloned embryos transferred into 25 surrogates using some of these improved SCNT techniques (29) did not result in any pregnancies, although rhesus NT blastocysts developed and NT-ICMs were isolated.

Our work described here shows that stem cell lines can be generated using somatic cells from patients with disease and injury. It may also be possible to generate NT-hESC lines from patients with diseases and disorders of unknown causes. For example, NT-hESCs derived from early-onset Alzheimer's disease or autism patients might prove invaluable for mechanistic studies in vitro after differentiation into neuroprogenitors (30, 31). In addition, biological insight gained through studying hESCs might find application to ART and assist in understanding genomic imprinting. The derivations of patient-specific NT-hESCs grown without animal cell co-culture may advance cell transplantation therapies as well as aid in the discovery of human developmental processes and the causes of many complex diseases.

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8. Before beginning any experiments, we obtained approval for this study from the IRB for Human Subjects Research and Ethics Committee at Hanyang University Hospital, Seoul, Korea, which was required by existing regulations that were in place up to 31 December 2004. IRB approvals are included in (9). Oocyte and/or somatic cell donors were counseled by two IRB mem-

- bers to ensure that they were fully aware of the scope of the investigation, and each donor signed informed consent forms. Both of the parents of children under 18 years old donating somatic cells were similarly counseled, and each signed informed consent forms on behalf of their child. On 1 January 2005, the Republic of Korea's new regulation entitled Bioethics and Biosafety Act—Act No. 7150, requiring governmental licensing of SCNT using human oocytes and subsequent derivation of NT-hESCs (Therapeutic Cloning), became effective. On 12 January 2005, we received governmental approval in accordance with this new stem cell law. This law also required IRB approval from the College of Veterinary Medicine, Seoul National University, which was granted on 25 January 2005. When our previous report on NT-hESCs appeared online on 12 February 2004 (6), we imposed a voluntary moratorium on new NT-hESC derivations. In September 2004, we announced that we were again performing SCNT and deriving NT-hESCs, under the auspices and oversight of the Hanyang University IRB for Human Subjects Research and Ethics Committee. All IRB documents are included in (9).
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Supporting Online Material

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 Materials and Methods  
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 Tables S1 to S4  
 References

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## Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for Cf-2–Dependent Disease Resistance

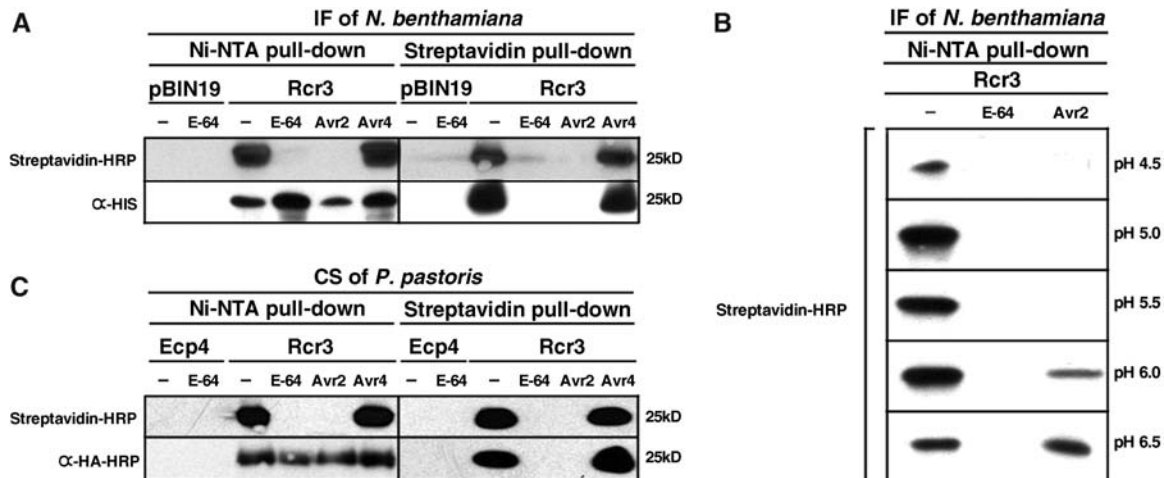
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How plants recognize pathogens and activate defense is still mysterious. Direct interaction between pathogen avirulence (Avr) proteins and plant disease resistance proteins is the exception rather than the rule. During infection, *Cladosporium fulvum* secretes Avr2 protein into the apoplast of tomato leaves and, in the presence of the extracellular leucine-rich repeat receptor-like Cf-2 protein, triggers a hypersensitive response (HR) that also requires the extracellular tomato cysteine protease Rcr3. We show here that Avr2 binds and inhibits Rcr3 and propose that the Rcr3-Avr2 complex enables the Cf-2 protein to activate an HR.

Plant disease resistance (*R*) genes mediate race-specific recognition of pathogens via perception of avirulence (*Avr*) gene products (*I*).

Tomato (*Lycopersicon esculentum*) *Cf* genes confer resistance to leaf mold caused by *Cladosporium fulvum* and encode trans-

**Fig. 1.** The Rcr3 cysteine protease of tomato is inhibited by Avr2 of *C. fulvum*. (A) Inhibition of Rcr3 produced in *Nicotiana benthamiana* by Avr2. IF was isolated from *N. benthamiana* expressing either the empty vector (pBin19) or Rcr3-His-HA (Rcr3). Protease activity profiling with 220 nM DCG-04 was performed in the absence of inhibitor (-) or in the presence of E-64, His-FLAG-Avr2 (Avr2), or His-FLAG-Avr4 (Avr4). Rcr3-His-HA was captured (pulled down) by Ni-NTA beads (left) or by streptavidin beads (right), electrophoresed on an SDS gel, and detected with streptavidin-HRP or His-specific antibodies ( $\alpha$ -His) (28). Detection with streptavidin-HRP reveals that Rcr3 is not biotinylated in the presence of E-64 or Avr2, whereas biotinylation of Rcr3 occurs without inhibitor or with Avr4, indicating that, like E-64, Avr2 inhibits Rcr3 cysteine protease activity.  $\alpha$ -His always detects Ni-NTA-captured Rcr3-His-HA irrespective of whether Rcr3 is inhibited or not (left), whereas  $\alpha$ -His only detects biotinylated Rcr3-His-HA when Rcr3 is not inhibited by E-64 or Avr2 (right). No biotinylated cysteine proteases were detected in the empty vector control (pBin19). (B) Inhibition of Rcr3 by Avr2 is pH-dependent. IF from *N. benthamiana* containing Rcr3-His-HA was profiled with 220 nM DCG-04 in the absence of inhibitor (-), and in the presence of E-64 (1120 nM) or Avr2 (140 nM), over a pH range from 4.5 to 6.5. Rcr3-His-HA was captured by Ni-NTA beads and detected with streptavidin-HRP to demonstrate biotinylation (28). Rcr3 is biotinylated in



the absence of inhibitor (-), with highest amounts of biotinylation at pH values between 5.0 and 6.0. Inhibition of biotinylation of Rcr3 by E-64 is complete at all pH values, whereas inhibition by Avr2 decreases at pH values above 6.0. (C) Inhibition of Rcr3 produced in *Pichia pastoris* by Avr2. Culture supernatant (CS) was isolated from *P. pastoris* cultures expressing either His-FLAG-Ecp4 (Ecp4) or Rcr3-His-HA (Rcr3), and protease activity was profiled with 220 nM DCG-04 in the absence of inhibitor (-) or in the presence of E-64, His-FLAG-Avr2 (Avr2) or His-FLAG-Avr4 (Avr4). Subsequently, Rcr3 was captured by Ni-NTA beads (left) or by streptavidin beads (right), electrophoresed on an SDS gel, and detected with streptavidin-HRP or HA-specific antibodies ( $\alpha$ -HA-HRP) (28). Detection with streptavidin-HRP reveals that in the presence of E-64 or Avr2, Rcr3 is not biotinylated, whereas biotinylation of Rcr3 occurs in the absence of inhibitor or in the presence of Avr4, indicating that, similar to E-64, Avr2 inhibits Rcr3 cysteine protease produced in *P. pastoris* in a similar way as Rcr3 produced in *N. benthamiana*.

membrane receptor-like proteins (RLPs) with extracellular leucine-rich repeats (LRRs) that mediate recognition of fungal Avr2s secreted during infection (2). *Cf*-dependent perception of Avr2s activates plant defense, including the HR, which results in host cell death at the site of penetration and limits pathogen ingress (3, 4). How *Cf* proteins enable tomato to perceive Avr2s is unknown. So far, no direct interaction between *Cf* proteins and Avr2 proteins has been detected (5). A direct interaction has only been demonstrated for two Avr2s and LRR-containing proteins (6, 7). The lack of a direct interaction led to the formulation of the guard hypothesis (8, 9), proposing that Avr2s are virulence factors that interact with host targets to facilitate pathogen growth in the host and that R proteins monitor the status of these host targets (10).

*Cf-2*, which originates from the wild tomato variety *L. pimpinellifolium*, confers resistance to *C. fulvum* in tomato (11) on the basis of perception of Avr2, a cysteine-rich protein secreted by the fungus (12). *Cf-2* function also requires Rcr3 (13), a secreted tomato cysteine

protease (14) that is not required by other *Cf* genes, including the highly homologous *Cf-5* gene (13, 14). The *L. esculentum* allele encodes the Rcr3<sup>esc</sup> protein that weakly activates *Cf-2*-dependent HR in tomato leaves in the absence of Avr2. The *L. pimpinellifolium* allele encodes Rcr3<sup>pim</sup>, required for *Cf-2* to confer an Avr2 response (14).

We hypothesized that Rcr3 is a target of Avr2. To test this hypothesis, we produced Rcr3 as a C-terminal 6xHistidine (His)- and hemagglutinin (HA)-tagged protein fusion (Rcr3-His-HA) both in *Nicotiana benthamiana* and in *Pichia pastoris*. Mature Rcr3 was recovered from intercellular fluid (IF) of *N. benthamiana* leaves by using the tags on the fusion protein (14). To monitor Rcr3 activity, we applied protease activity profiling at pH = 5 by using DCG-04, a biotinylated derivative of the irreversible cysteine protease inhibitor E-64 that has been used to profile cysteine protease activities from mammals (15), insects (16), and plants (17). DCG-04 treatment leads to irreversible labeling of cysteine proteases with biotin. Labeling of Rcr3 with 220 nM DCG-04 was assayed in the presence or absence of different concentrations of E-64 as a competitive inhibitor. After reaction with DCG-04, Rcr3-His-HA was precipitated with the use of Ni-nitrilotriacetic acid (NTA) (binding to His tag) or streptavidin (binding to biotin) beads. In the absence of E-64, DCG-04 biotinylates Rcr3, confirming that Rcr3 is a cysteine protease,

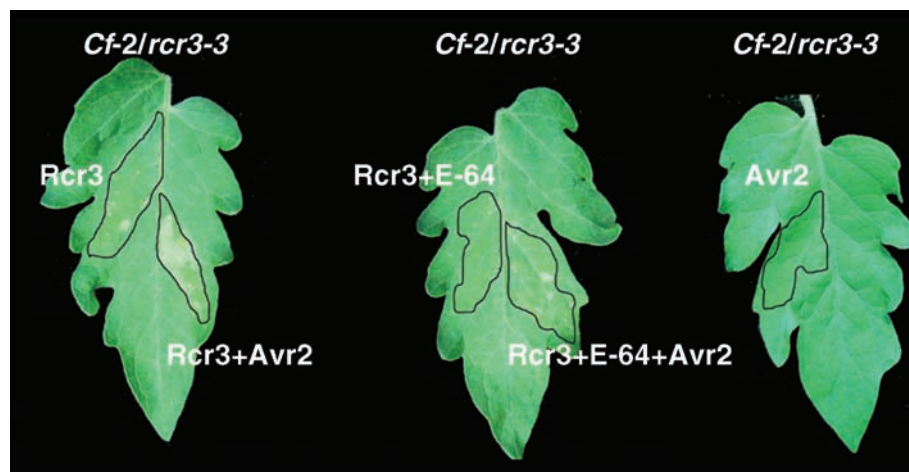
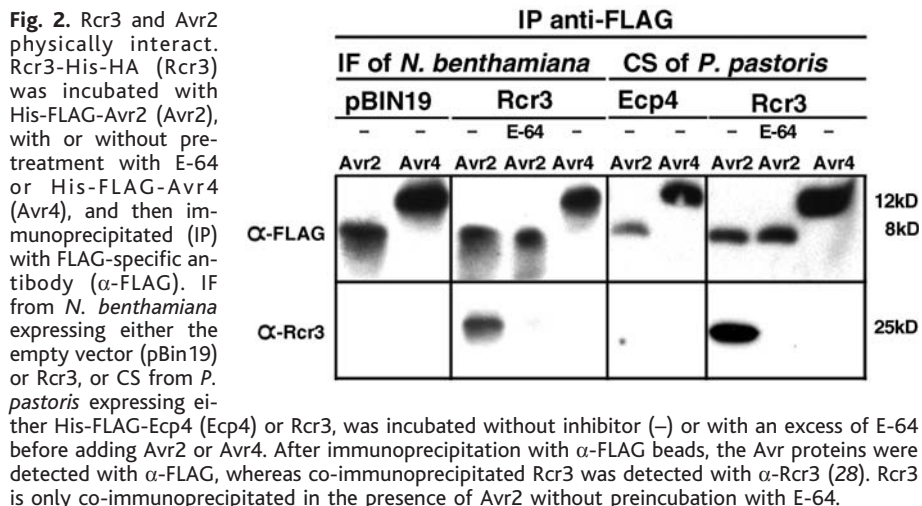
whereas in the presence of 1120 nM E-64, Rcr3 is not biotinylated (Fig. 1A).

We tested whether Avr2 could inhibit biotinylation of Rcr3 by DCG-04. As a negative control, *C. fulvum* Avr4, which triggers *Cf-4*-dependent HR (18), was included. Both Avr2s were expressed in *P. pastoris* as N-terminal His-FLAG-fusions and purified on a Ni-NTA column. In the presence of 140 nM Avr2, Rcr3 is not biotinylated (Fig. 1A), indicating that Avr2 inhibits Rcr3 activity (fig. S1). In the presence of Avr4, Rcr3 is biotinylated, showing that inhibition of Rcr3 by Avr2 is specific (Fig. 1A and fig. S1).

IF obtained from tomato has a pH of about 5 (19). To investigate the pH dependence of Rcr3 activity and its inhibition by Avr2, we incubated *N. benthamiana* IF containing Rcr3 with DCG-04 in the absence or presence of an excess of E-64 or Avr2 over a pH range from 4.5 to 6.5. Rcr3 activity is highest at pH of 5 to 6 and strongly decreases outside this range (Fig. 1B). Inhibition by E-64 is effective over the whole pH range, whereas inhibition by Avr2 is only effective below pH = 6 (Fig. 1B), indicating that the pH optimum for Rcr3 activity and its inhibition by Avr2 coincides with the pH of the apoplast of tomato (pH = 5). Rcr3 produced as a C-terminal His-HA fusion in *P. pastoris* is also inhibited by E-64 and Avr2 (Fig. 1C), indicating that Avr2 alone is sufficient to inhibit Rcr3 and that no additional plant factors are required. No biotinylation by

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**Fig. 3.** Cf-2-mediated HR requires physical interaction between Rcr3 and Avr2. Fully expanded leaves of 5-week-old *Cf-2/rcr3-3* tomato were infiltrated with Rcr3-His-HA (Rcr3) produced in *N. benthamiana* (either alone or in combination with His-FLAG-Avr2 (Avr2), E-64, or E-64 and Avr2) or infiltrated with Avr2 alone (28). Leaves were photographed 3 days postinfiltration. The infiltrated sectors are outlined and the infiltrated compounds indicated. The HR is only triggered when Rcr3 and Avr2 can interact, whereas the HR is blocked when interaction of Rcr3 with Avr2 is prevented by preincubation of Rcr3 with E-64. Rcr3 produced in *P. pastoris* treated with the same compounds gave similar results (21).

DCG-04 of other cysteine proteases was observed in control IF from *N. benthamiana* nontransgenic for Rcr3 (Fig. 1A) or in control culture supernatant (CS) from *P. pastoris* nontransgenic for Rcr3 but expressing Ecp4, another protein secreted by *C. fulvum* (20) (Fig. 1C). This indicates that, at the DCG-04 concentration used (220 nM), no biotinylation of endogenous extracellular cysteine proteases could be detected.

Because Avr2 inhibits Rcr3, we expected a physical interaction between the two proteins. This was investigated by co-immunoprecipitation studies. His-FLAG-Avr2 or His-FLAG-Avr4 was added to *N. benthamiana* IF or culture supernatant (CS) of *P. pastoris* containing Rcr3-His-HA and immunoprecipitated with a FLAG-specific antibody ( $\alpha$ -FLAG). As a control, Rcr3-His-HA was also preincubated with E-64 to block the active site

before adding His-FLAG-Avr2. After immunoprecipitation, the samples were run on SDS gels and blotted, and Avr proteins and Rcr3 were detected with use of  $\alpha$ -FLAG and an Rcr3-specific antibody ( $\alpha$ -Rcr3), respectively (Fig. 2). Rcr3 co-immunoprecipitates with Avr2 but not with Avr4 (Fig. 2), indicating a specific interaction between Avr2 and Rcr3. Blocking the active site of Rcr3 by E-64 eliminates this interaction (Fig. 2). In the presence of Avr2,  $\alpha$ -FLAG co-immunoprecipitates Rcr3 irrespective of the source of Rcr3, again indicating that the interaction between Avr2 and Rcr3 is independent of additional plant factors (Fig. 2). No signals were detected on blots probed with  $\alpha$ -Rcr3 after immunoprecipitation of proteins from control *N. benthamiana* IF (marked pBin19 in Fig. 2) or control CS of *P. pastoris* nontransgenic for Rcr3 (marked Ecp4 in Fig. 2), indicating that  $\alpha$ -Rcr3 is specific.

We tested whether native Rcr3 in tomato IF can be detected and inhibited by E-64 and Avr2. IF (6 ml) from different Cf tomato plants producing Rcr3 were labeled with 2.2  $\mu$ M DCG-04 in the presence or absence of E-64 (28.6  $\mu$ M) or Avr2 (6.9  $\mu$ M). Biotinylated proteins were captured on streptavidin beads, run on an SDS gel, and probed with  $\alpha$ -Rcr3 or streptavidin-horseradish peroxidase (HRP) (fig. S2). Native Rcr3 is detected by  $\alpha$ -Rcr3 in Cf0 and Cf2 tomato lines, and its biotinylation by DCG-04 is inhibited by Avr2, whereas Rcr3 is absent in *Cf-2/rcr3-3* plants (13, 14) (fig. S2; upper panel). In addition to Rcr3, several other apoplastic cysteine proteases are biotinylated that can be inhibited by Avr2 (fig. S2; lower panel).

To determine whether inhibition of Rcr3 by Avr2 is sufficient to trigger Cf-2-dependent HR, we infiltrated Rcr3 produced in *N. benthamiana*, either alone or in combination with Avr2, E-64, or E-64 and Avr2, or we infiltrated Avr2 alone, into *Cf-2/rcr3-3* tomato leaves (Fig. 3). Infiltration of Avr2 or Rcr3 alone or Rcr3 incubated with E-64 does not trigger an HR, whereas infiltration of Rcr3 incubated with Avr2 does. However, Rcr3 preincubated with an excess of E-64 to saturate the active site, and subsequently incubated with Avr2, does not trigger Cf-2-mediated HR, indicating that Cf-2 specifically recognizes the Rcr3-Avr2 complex. Similar results were obtained with *P. pastoris*-produced Rcr3 pretreated with the same compounds (21).

Inhibition of Rcr3 activity by Avr2 could be caused by either Avr2 acting solely as an inhibitor of Rcr3 or Avr2 being both a substrate and an inhibitor. However, we observed no degradation of Avr2 upon incubation with Rcr3 (Fig. 2), suggesting that Avr2 is not a substrate for Rcr3. Furthermore, if processing of Avr2 by Rcr3 were required for Cf-2-mediated HR, then Avr2 present in IF from Cf0 tomato plants (containing Rcr3) infected by Avr2-producing *C. fulvum* strains would induce an HR in *Cf-2/rcr3-3* tomato. This was not observed (21), indicating that Avr2 is an inhibitor, not a substrate, of Rcr3. However, inhibition of Rcr3 activity is not sufficient to initiate Cf-2-mediated HR (Fig. 3). Therefore, we propose that inhibition of Rcr3 by Avr2 induces a conformational change in Rcr3 that triggers the Cf-2 protein to activate HR. This model is consistent with the observation that the Rcr3<sup>esc</sup> protein alone provokes a weak Cf-2-dependent, Avr2-independent HR (14). We suggest that Rcr3<sup>esc</sup>, which differs from Rcr3<sup>pim</sup> in one amino acid deletion and six amino acid changes, constitutively mimics the conformational change imposed on Rcr3<sup>pim</sup> (present in Cf-2 plants) by Avr2 binding and weakly activates Cf-2-dependent HR in the absence of Avr2.

The role of Rcr3 cysteine protease activity for tomato and the importance of its inhibition by Avr2 for *C. fulvum* during infection are unknown, but secreted plant cysteine pro-

teases possibly have antimicrobial activity. *Rcr3* transcription is induced faster and transcripts accumulate to higher concentrations in incompatible compared with compatible interactions between tomato and *C. fulvum* (14), as do transcripts for pathogenesis-related proteins after infection by this fungus (22). *Rcr3* is also induced in the absence of Cf-2, consistent with a role for *Rcr3* in basal host defense (14). Furthermore, in addition to *Rcr3*, several other apoplastic cysteine proteases are inhibited by *Avr2*, suggesting that *Avr2* is a general virulence factor facilitating growth of *C. fulvum* in the apoplast. Recently, it has been shown that a protease inhibitor from *Phytophthora infestans* interacts with and inhibits the plant serine protease P69B, which is induced during infection of tomato by this pathogen (23). Thus, inhibition of plant proteases may represent a general counter-defense used by invading pathogens.

The role of *Rcr3* in the perception of *Avr2* by Cf-2 is consistent with the guard hypothesis. The *Rcr3-Avr2* complex, but not other *Avr2*-cysteine protease complexes, activates Cf-2. So far, all bacterial pathogens colonizing the apoplast of plants deliver their effector proteins into the plant cell by the type III secretion system where they interact with cytoplasmic virulence targets (10, 24–27). In the case of RPM1- and RPS2-mediated resistance in *Arabidopsis*, the action of the *Avr* proteins *AvrB*, *AvrRpm1*, and *AvrRpt2* on the guard protein RIN4 is thought to trigger the activation of RPM1 (resistance to *Pseudomonas syringae* p. *maculicola* expressing *AvrRpm1*) or RPS2 (resistance to *P. syringae* pv. *tomato* expressing *AvrRpt2*) proteins (24–27). Similarly, in RPS5-mediated resistance in *Arabidopsis* the cysteine protease activity of the *Avr* protein, *AvrPphB*, on the guard protein PBS1 is required to trigger the HR (28). Such indirect interactions between pathogen *Avrs* and plant R proteins may be more difficult for the pathogen to circumvent without a virulence penalty than direct interactions (8, 9). In addition to *Rcr3*, other tomato cysteine proteases are inhibited by *Avr2* that are not guarded by known Cf proteins. Characterization and functional analysis of these proteases will be the subject of future studies.

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29. Materials and methods are available as supporting material on *Science* Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/1111404/DC1  
 Materials and Methods  
 Tables S1 and S2  
 Figs. S1 and S2  
 References

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# Nodulation Signaling in Legumes Requires NSP2, a Member of the GRAS Family of Transcriptional Regulators

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Rhizobial bacteria enter a symbiotic interaction with legumes, activating diverse responses in roots through the lipochito oligosaccharide signaling molecule Nod factor. Here, we show that *NSP2* from *Medicago truncatula* encodes a GRAS protein essential for Nod-factor signaling. *NSP2* functions downstream of Nod-factor-induced calcium spiking and a calcium/calmodulin-dependent protein kinase. We show that *NSP2*-GFP expressed from a constitutive promoter is localized to the endoplasmic reticulum/nuclear envelope and relocalizes to the nucleus after Nod-factor elicitation. This work provides evidence that a GRAS protein transduces calcium signals in plants and provides a possible regulator of Nod-factor-inducible gene expression.

The legume/rhizobial symbiosis plays a crucial role in the introduction of fixed nitrogen into both agricultural and natural systems. Legumes form specialized organs, usually on the roots, and these “nodules” provide the low-oxygen environment required for the activity of bacterial nitrogenase. Within nod-

ules, the bacteria reside in membrane-bound compartments within plant cells and differentiate into bacteroids, a specialized symbiotic form of the bacteria. Nod factor is central to the establishment of this symbiotic interaction. This lipochito oligosaccharide signal is produced by the bacteria in response to plant phenolics (1). Nod factor alone is sufficient to activate the majority of the early responses in the plant normally seen during the interaction with the bacteria, including the activation of cytosolic calcium spiking associated with the nucleus of epidermal root cells (2) and a wide range of plant genes (3). The recent identification of a gene (*DMI3*) encoding a calcium/calmodulin-dependent protein kinase (CCaMK) that functions in Nod-factor signaling (4, 5) downstream of calcium

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