Regulatory Roles of NPR1 in Plant Defense: Regulation and Function

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Overcoming infection is a struggle that all eukaryotic organisms have to face in order to survive and evolve among ubiquitous microorganisms. Extensive research on plant defenses has revealed that defense signal transduction pathways form an interconnected network in which the signaling molecules salicylic acid (SA) and jasmonic acid (JA) play key roles. Previously, SA and JA signaling pathways have been shown to cross-communicate (Felton and Korth 2000; Pieterse et al. 2001; Kunkel and Brooks 2002). Cross-talk between defense signaling pathways is thought to provide the plant with a mechanism to activate defenses that are specifically active against the invader encountered.

The Arabidopsis NPR1 protein is a master regulator of both SA- and JA-dependent defense signaling pathways (Dong 2004; Pieterse and Van Loon 2004). Mutant *npr1* plants were identified in a genetic screen for loss of SA-induced expression of *pathogenesis-related* (*PR*) genes and systemic acquired resistance (SAR) (Cao et al. 1994; Delaney et al. 1995). Cloning of *NPR1* revealed that it encoded a protein with an ankyrin repeat and BTB/POZ domain (Cao et al. 1997), which have been implicated in protein-protein interactions. SA induces nuclear localization of NPR1 through a C-terminal NLS sequence, a process shown to be necessary and required for the induction of *PR* genes and associated SAR (Kinkema et al. 2000). Moreover, upon SAR induction TGA transcription factors are thought to be recruited by NPR1 to selective sequences in the *PR* gene promoters to

activate transcription (Fan and Dong 2002; Johnson et al. 2003). Thus, NPR1 functions as an essential activator of genes that collectively establish SAR.

In contrast to its positive regulatory role in SAR, we have previously shown that NPR1 also functions as a potent negative regulator of JA-responsive defense genes. SA-activated NPR1 inhibits the pathogen-induced expression of JA-responsive genes, including genes that encode JA biosynthesis enzymes, to prevent JA synthesis (Spoel et al. 2003).

Regulatory Mechanisms That Control NPR1 Activity

The presence or absence of functional NPR1 protein has a striking effect on disease resistance, indicating that NPR1 is an essential defense modulator. But how does NPR1 distinguish between its positive and negative regulatory roles in plant defense? To answer this question we are studying the mechanisms that regulate NPR1 protein and its function.

SIGNAL-INDUCED REDOX REGULATION

Previously, we have shown that inducers of SAR alter the redox status of the plant cell. Together with the fact that NPR1 contains 10 conserved cysteines among NPR1-like proteins in different plant species, this led us to hypothesize that redox changes may influence NPR1 conformation. Indeed, induction of SAR led to a rapid biphasic change in cellular redox potential, resulting in the reduction of NPR1 from an oligomeric to a monomeric form (Mou et al. 2003). NPR1 monomer is translocated to the nucleus and induces *PR* gene expression (Kinkema et al. 2000; Mou et al. 2003). Mutation of two cysteines resulted in accumulation of NPR1 monomer and constitutive activation of *PR* genes in resting cells (Mou et al. 2003). Thus, redox-mediated control of NPR1 conformation is a key regulatory step in the activation of SAR.

CELLULAR LOCALIZATION AS A REGULATORY MECHANISM

Mutation of the C-terminal NLS sequence in NPR1 results in failure to localize to the nucleus and failure to express *PR* genes (Kinkema et al. 2000), indicating that nuclear localization is important for its positive regulatory function. Using plants expressing the NPR1-GFP fusion protein, we recently discovered that the SA-induced nuclear localization of NPR1 was greatly reduced in the presence of JA. This suggests that cellular localization of NPR1 may be altered to support its suppressive function on JA signaling. Indeed, experiments with a transgenic line expressing a fusion

of NPR1 to the rat glucocorticoid receptor (GR), allowing control of the nucleocytoplasmic localization of the NPR1-GR fusion protein, showed that the negative and positive functions of NPR1 are separable. Retention of NPR1-GR in the cytoplasm completely abolished its ability to induce *PR* gene expression, whereas suppression of JA signaling was unaffected (Spoel et al. 2003). Moreover, cytoplasmic retention of NPR1-GFP by mutation of the NLS yielded similar results. Together, these data strongly suggest that NPR1 exerts its function as a suppressor of JA signaling, at least partly, in the cytoplasm. Thus, cellular trafficking of NPR1 between the cytoplasm and the nucleus is a crucial regulatory step that is tightly associated with and perhaps determines function.

SIGNAL-INDUCED PROTECTION FROM PROTEASOME-MEDIATED DEGRADATION

NPR1 shows striking structural similarity to the animal protein IκBα (Ryals et al. 1997). IκB functions as a cytoplasmic inhibitor of the transcriptional activator NF-κB. Cellular stress promotes the signal-induced phosphorylation of IκB. Phosphorylated IκB is targeted for degradation by the proteasome, which results in the release of NF-κB from IκB. Subsequently, NF-κB localizes to the nucleus where it activates pro-inflammatory genes (Baldwin 1996). Thus, control of proteasomal degradation of IκB is a key regulatory step in the activation of stress responses in animals. Because of its structural similarity to animal IκB, we investigated if NPR1 is also regulated by proteasome-mediated degradation. Indeed, we observed that NPR1 was constitutively degraded in an *in vitro* cell-free degradation assay. In addition, treatment of plants with the protein synthesis inhibitor cycloheximide dramatically lowered cellular NPR1 levels. Conversely, we treated plants with proteasome inhibitors and observed that NPR1 levels increased. These results indicate that constitutive degradation of NPR1 occurs *in vivo* and is mediated by the proteasome. Interestingly, when we prepared protein from proteasome-treated plants under non-reducing conditions, we observed that NPR1 monomer levels increased rather than NPR1 oligomer levels. This suggests that monomeric NPR1 is the preferred conformational form for degradation.

Monomeric but not oligomeric NPR1 can translocate to the nucleus (Mou et al. 2003). Therefore, we hypothesized that degradation of NPR1 occurs in the nucleus. Transgenic *npr1-nls-GFP* plants (in *npr1*), expressing a cytoplasmic NLS-mutant of NPR1-GFP, lacked degradation of npr1-nls-GFP. Moreover, cytoplasmic retention of NPR1-GR in *NPR1-GR* plants (in *npr1*) did not result in degradation of NPR1-GR. In contrast, targeting NPR1-GR to the nucleus by dexamethasome treatment resulted in complete degradation of NPR1. These data point out that only nuclear localized monomeric NPR1 is degraded by the proteasome.

Because SA is a potent inducer of SAR and activates NPR1, we examined the effect of SA on constitutive proteasome-mediated degradation of NPR1. To that end we expressed *NPR1-GFP* in genetic backgrounds that are deficient in SA accumulation. Deficiency in SA accumulation dramatically decreased cellular NPR1-GFP levels. Remarkably, SA and SA analogues completely rescued NPR1-GFP to wild-type levels. In addition, SA treatment rendered NPR1-GFP resistant to degradation in the presence of cycloheximide. These findings clearly indicate that SA can stabilize NPR1 and prevents its nuclear degradation to promote SAR.

Functional Significance of Proteasome-Mediated Degradation of NPR1

NPR1 is a key positive regulator of SAR. So why is NPR1 subject to proteasome-mediated degradation? Our results described above show that the NPR1 oligomer-to- monomer switch is leaky, resulting in monomer being degraded by the proteasome in the absence of an SAR inducing signal. Together with the fact that SAR inducers protect NPR1 from degradation, this may indicate that protection from degradation is a rapid way of turning on SAR. Stabilization of NPR1 monomer may occur before SA-induced changes in cellular redox stimulate NPR1 monomer formation. Thus, control of degradation may be a means to rapidly *switch on* SAR.

Activation of defense is associated with high fitness costs (Heidel et al. 2004). Therefore, degradation of NPR1 may also function to switch off SAR once pathogen attack subsides. To test this hypothesis we pretreated *NPR1-GFP* plants (*in npr1*) with SA and subsequently treated with either water or proteasome inhibitor. As expected, treatment with SA resulted in formation of NPR1-GFP monomer and expression of SA-responsive *PR* gene expression. However, when plants were treated with water after the SA-pretreatment, NPR1-GFP monomer levels rapidly decreased and *PR* gene expression shut down. In contrast, treatment with proteasome inhibitors following SA-pretreatment, resulted in the maintenance of both NPR1-GFP monomer levels and high *PR* gene expression. These data indicate that degradation of NPR1 rapidly *switches off* SAR once pathogen infection and the SA signal subside.

As described above, SA-activated NPR1 is a strong negative regulator of JA signaling. At present it is unclear how NPR1 suppresses the JA signal. It is plausible that proteasome-targeted NPR1 directs a positive regulator of JA signaling for degradation, thereby blocking JA signaling. However, above we described that SA stabilizes NPR1 by protecting it from proteasome-mediated degradation. To investigate this apparent discrepancy, NPR1 stability was investigated in SA/JA cross-talk conditions. To our surprise we observed that SA lost its ability to protect NPR1 from

proteolysis in the presence of JA. These findings support a model in which the JA signal is repressed by NPR1-mediated targeting of a positive regulator of JA signaling for degradation in the presence of SA.

We showed that NPR1 degradation occurs in the nucleus, but nuclear localization of NPR1 is not required for suppression of JA-responsive gene expression. So how could NPR1 suppress JA signaling by targeting a positive regulator for proteolysis? One explanation is that NPR1 sequesters a positive regulator of JA signaling as a short-lived cytosolic complex, which is subsequently rapidly translocated to the nucleus for degradation.

Conclusions

In summary, our data shows that cellular redox, localization, and control of proteasome-mediated degradation are all key regulatory steps in the regulation of NPR1 function in plant defense. Currently, we are investigating the molecular mechanisms by which SA protects NPR1 from degradation. The many regulatory steps that control NPR1 function emphasize the presence of several layers of complexity in signaling to rapidly and efficiently control plant defense responses.

Literature Cited

Baldwin, A.S., Jr. 1996. The NF-κB and IκB proteins: New discoveries and insights. Annu. Rev. Immunol. 14:649-683.

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.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88:57-63.

Delaney, T.P., Friedrich, L., and Ryals, J.A. 1995. Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. U.S.A. 92:6602-6606.

Dong, X. 2004. NPR1, all things considered. Curr. Opin. Plant Biol. 7:547-552.

Fan, W., and Dong, X. 2002. In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell 14:1377-1389.

Felton, G.W., and Korth, K.L. 2000. Trade-offs between pathogen and herbivore resistance. Curr. Opin. Plant Biol. 3:309-314.

Heidel, A.J., Clarke, J.D., Antonovics, J., and Dong, X. 2004. Fitness costs of mutations affecting the systemic acquired resistance pathway in Arabidopsis thaliana. Genetics 168:2197-2206.

Johnson, C., Boden, E., and Arias, J. 2003. Salicylic acid and NPR1 induce the recruitment of *trans*-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell 15:1846-1858.

Kinkema, M., Fan, W., and Dong, X. 2000. Nuclear localization of NPR1 is required for activation of *PR* gene expression. Plant Cell 12:2339-2350.

Kunkel, B.N., and Brooks, D.M. 2002. Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. 5:325-331.

Mou, Z., Fan, W., and Dong, X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113:935-944.

Pieterse, C.M.J., Ton, J., and Van Loon, L.C. 2001. Cross-talk between plant defence signalling pathways: Boost or burden? AgBiotechNet 3: ABN 068, www.agbiotechnet.com.

Pieterse, C.M.J., and Van Loon, L.C. 2004. NPR1: the spider in the web of induced resistance signaling pathways. Curr. Opin. Plant Biol. 7:456-464.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., and Vos et, a. 1997. The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell 9:425-439.

Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M.J. 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell 15:760-770.