Commentary

Plant mitogen-activated protein kinase cascades: Negative regulatory roles turn out positive

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Mitogen-activated protein (MAP) kinase cascades are a standard player in the signal transduction literature for diverse organisms (1–3). Previous work has shown that specific MAP kinases become activated during plant responses to pathogens, suggesting a role for MAP kinase cascades in disease resistance. However, no function-blocking experiments had demonstrated a causal role until the work of Frye *et al.* (4) on the *Arabidopsis EDR1* gene that was presented in a recent issue of PNAS. In a related development, Petersen *et al.* (5) have just published work showing that disruption of the *Arabidopsis MPK4* MAP kinase gene also alters plant defense activation. It is surprising that, for all plant species, these are only the second and third characterized mutants known to carry a disrupted kinase from a MAP kinase cascade. Equally striking, both papers revisit a theme established by the first known plant MAP kinase cascade mutant and reinforced in a separate study (6, 7): the MAP kinase cascade as a negative regulator of the relevant biological response. However, the probability of crosstalk among MAP kinase cascades, discussed below, suggests that this negative regulatory activity of MAP kinase cascades could be misleading.

MAP kinase cascades, which transduce a wide variety of extracellular signals, are known as much for their laborious acronyms as for their important biological functions. MAP kinase kinase kinase (MAPKKK) proteins, including *Raf*-like kinases and MEKK proteins, phosphorylate MAP kinase kinases (MAPKKs, or MEKs) that phosphorylate MAP kinase (MAPK) proteins. Upon activation, MAPKs are often transported to the nucleus where they phosphorylate specific transcription factors. No single plant MAP kinase cascade has been characterized to the extent that a functional relationship among three sequential kinase participants has been confirmed (3). However, numerous plant gene products with the conserved residues of these kinases have been identified, activation of specific MAPK proteins has been observed in plants responding to hormones or environmental stimuli such as cold, touch, infection or wounding, and some plant MAPKs, MAPKKs, or MAPKKKs have been shown to complement yeast mutants at the appropriate level of the yeast kinase cascades (3). Until now, most plant MAPK proteins have been implicated in a particular biological response solely because of their activation during that response.

Previously, the only known mutations affecting a protein kinase from a plant MAP kinase cascade disrupted *Arabidopsis CTR1*, which encodes a *Raf*-like MAPKKK protein (6). *Arabidopsis ctr1* mutants exhibit constitutive activation of ethylene hormone–response pathways. Exposure to ethylene is hypothesized to inactivate a *CTR1* MAP kinase cascade, triggering plant ethylene responses (8). The importance of ethylene in plant biology has prompted extensive, near-saturating, and very successful mutational analysis in *Arabidopsis*, and it is intriguing that no MAPKK or MAPK participating in the ethylene–response pathway has yet been reported (8). Although the possible lethality of such mutations cannot be dismissed, a more likely explanation is that functional redundancy among multiple CTR1 targets has precluded mutant identification. A third possibility, that no such MAPKK or MAPK partners exist, is discussed below.

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The *EDR1* MAPKKK gene described by Frye *et al.* (4) was first identified in a mutational screen for *Arabidopsis* plants expressing elevated resistance to a *Pseudomonas syringae* bacterial pathogen (9). Many other such mutants had previously been described, but those mutants display constitutively elevated levels of salicylic acid (SA, a key defense signal transduction mediator) and constitutive pathogenesis-related gene expression (10). These phenotypes are often associated with dwarfing of the plant and/or aberrant cell death (lesion formation on uninfected leaves), but the *edr1* mutant exhibits none of the above traits. Resistance in *edr1* plants is even more effective against the fungal powdery mildew pathogen *Erysiphe cichoracearum* than against *P. syringae*, but is ineffective against virulent *Peronospora parasitica* that cause downy mildew disease. Resistance is manifested late in pathogen development; *Erysiphe* germinates and forms a hyphal mat equally well on both wild-type and *edr1* lines, but *edr1* plants arrest most conidiophore development before release of disease-propagating spores. The *edr1* resistance phenotype is recessive to wild type (9).

In the paper Frye *et al.* (4) used the increasingly accessible positional cloning/candidate gene approach to isolate the *EDR1* gene. *EDR1* was found to encode a putative MAPKKK that is similar to the product of the *CTR1* gene discussed above. Frye *et al.* also showed that the defense phenotype depends on SA and the salicylate-associated signal transduction protein NPR1/NIM1. This latter result is significant because, in combination with earlier work on the *edr1* mutant, it shows that disruption of the *EDR1* MAPKKK does not cause constitutive activation of SA-mediated defense pathways, but rather, causes heightened responsiveness to pathogen induction of those pathways. Like the *ctr1* mutants, *edr1* mutants reveal a plant *Raf*-like MAPKKK as a negative regulator of a critical response pathway.

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But in this case, the response pathway apparently requires additional factors before SA-mediated signaling and downstream defense responses are activated.

A number of biochemical studies have implicated two plant MAPK protein families, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), in pathogen-induced defense signal transduction (11–14). SIPK and/or WIPK proteins have been characterized in tobacco, tomato, parsley, and *Arabidopsis*. Activation of SIPK can occur not only in response to wounding or SA, but also in response to pathogen-derived peptide elicitors or specific *R*/*avr*-mediated plant–pathogen interactions (11). An obvious hypothesis is that these proteins are positive signal transduction mediators that activate defense gene expression.

The above sets up a possibly confusing contrast. Positive activation of specific MAP kinases is observed biochemically after elicitation of a defense response. Yet a negative role of MAP kinase cascades is indicated when such responses are elicited by kinase-disrupting mutations (4, 5) or are blocked by overexpression of a functionally active MAPKKK (7). The mechanistic relationship of the *EDR1* MAPKKK to SIPK and WIPK remains an important unanswered question. In the meantime, the recent work of Petersen *et al.* (5) on an *Arabidopsis MPK4* MAPK mutant may help explain this positive-regulation/negative-regulation contrast.

The *mpk4* MAPK mutant plants described by Petersen *et al.* exhibit the phenotypes common among many *Arabidopsis* elevated resistance mutants: dwarf phenotype, constitutively elevated SA levels, constitutive pathogenesis-related gene expression, and elevated resistance to virulent *P. syringae* and *P. parasitica* (5, 10). These *mpk4* mutant plant phenotypes are shown to depend on SA. Petersen *et al.* also report that in wild-type plants activated MPK4 protein is constitutively present. Mirroring the *ctr1* and *edr1* stories, it is loss of the *MPK4* MAP kinase cascade protein that turns on the plant response. But curiously, the *Arabidopsis* orthologs that encode likely SA-responsive SIPK and/or WIPK proteins are not *MPK4*, but rather *MPK6* (SIPK) and *MPK3* (WIPK) (see, e.g., ref. 12).

Particularly relevant to an explanation of the positive-regulation/negative-regulation contrast, plants homozygous for the recessive *mpk4* mutation were activated for SA responses but blocked for jasmonate-responsive gene expression. Finally!—Mutational evidence implicating a plant MAP kinase cascade in positive regulation of a plant response! Jasmonic acid mediates responses to wounding, insect chewing, and other stimuli and induces a set of responses distinct from those induced by SA. Mutant *mpk4* plants responded normally to a range of other stimuli suspected to be mediated by MAP kinases, indicating that mutation of *MPK4* is not excessively pleiotropic. The most straightforward interpretation of this jasmonate pathway disruption is that MPK4 protein mediates jasmonate-responsive signal transduction.

Increasingly, cellular signaling is being viewed as a web or a net, with proteins at each node capable of receiving inputs from multiple partners and pathways.

An alternative interpretation, that *mpk4* mutation causes broad physiological disruption that indirectly blocks jasmonate signal transduction, is argued against (5). Microarray analysis of >8,000 genes revealed a 5-fold or greater increase in constitutive mRNA abundance for only 16 genes, many related to SA-induced defenses, and not for genes related to other cellular processes. But do elevated SA levels or SA responses cause indirect blockage of jasmonate signaling in the *mpk4* mutant? Such blockage can occur (15), but apparently the effect of the *mpk4* mutation on jasmonate signaling is more direct. Jasmonate signaling was still disrupted in *mpk4* mutants even if SA was transgenically degraded and the SA responses were accordingly suppressed (5).

Another hypothesis explaining the apparent negative regulation of SA responses by MPK4 can be proposed: What if wild-type MPK4 protein mediates jasmonate signal transduction, and physically blocks the MAPK that mediates SA responses (*Arabidopsis MPK6* SIPK?) from productive association with other MAP kinase cascade proteins? Mutation of *MPK4* would knock out jasmonate responses and could free that MAP kinase cascade complex for abnormal, constitutive-signaling interaction with SIPK.

There is precedent for this latter hypothesis. In yeast and other systems, crosstalk between different MAP kinase cascades has been observed (1, 2). Multiprotein complexes apparently hold wild-type MAP kinase cascade proteins in physical proximity, ensuring specificity of signaling. For the yeast Fus3p MAPK, single amino acid mutations that disrupt kinase activity do not allow inappropriate use of the complex for signaling via the "wrong" kinase, whereas null mutations do allow inappropriate signaling, apparently by leaving a physical opening in the Fus3p-specific MAP kinase cascade protein complex that can be filled by the closely related MAPK Kss1p (2). In other words, the Fus3p protein, in addition to a positive signaling role, has an inhibitory role that maintains signaling specificity by preventing activation of closely related but functionally distinct kinases on stimulation by Fus3p-activating signals.

Petersen *et al.* (5) found that kinase activity, rather than simple presence of the MPK4 protein, is required to negatively regulate expression of SA-mediated responses. But, in this system, kinase activity may be required for persistent association of MPK4 protein with other MAP kinase cascade proteins or accessory factors. As outlined above, this association could negatively regulate SA-mediated signal transduction by competing for factors that might otherwise be used by MAPK proteins such as SIPK.

If this scenario is valid, the mutational results with *edr1* and *mpk4* could be misleading. The mutations may not reveal the MAP kinase cascade proteins that participate in the activated plant response in a wild-type plant. Formally, negative regulatory roles of specific MAP kinase cascade proteins are indicated by these mutations. But the results may be consistent with predominantly positive regulation in wild-type plants via other MAP kinase cascades.

Could this hypothesis also explain why mutation of *ctr1* activates ethylene responses, or why overexpression of functional *NPK1* blocks auxin responses (6, 7)? Does the *ctr1* mutation tell us, as widely hypothesized, that ethylene-response signal transduction flows through CTR1 protein in a normal wild-type plant? Or is the effect of mutant *ctr1* on ethylene signaling a coincidental side effect because of atypical availability of MAP kinase cascade proteins, with CTR1 normally participating in some other presently unidentified plant process? The latter would imply that the true ethylene response-mediating MAP kinase cascade proteins have not been identified.

The above scenario can be extended to propose that normal ethylene signal transduction may not involve a MAP kinase cascade. Mutation of CTR1 may cause aberrant activation of ethylene signal transduction downstream of the ethylene receptor but upstream of many other response components. In the absence of other experimental data linking MAP kinase cascades to the many known ethylene signal transduction gene products, this would seem to be a viable hypothesis. The demonstration of direct interaction between CTR1 protein and plant ethylene receptor proteins indicates that the hypothesized direct, negative regulatory role of CTR1 in ethylene signaling may in fact be valid (16). But the above possibilities

provide a cautionary example regarding the interpretation of MAP kinase cascade findings in any biological system.

Turning to a separate matter, earlier work showing the convergence of signaling at tobacco SIPK in response to pathogen-derived elicitor, *R/avr* interaction, wounding, or SA stimuli raised two additional questions (14): (*i*) Do these distinct signaling pathways merge at the MAP kinase, or upstream? (*ii*) How can stimuli that are mediated by the same MAP kinase cause different responses? These questions remain unanswered, but many have speculated that the same MAP kinase could mediate disparate responses if it interacts with other proteins or molecules that are differentially present under different stimuli (1, 2, 11, 14). Indeed, if MPK4 is constitutively activated in wild-type nonstimulated plants (as reported), jasmonate pathways should be constitutively on unless activation requires other factors beyond MPK4. The above questions remind us that pathways unrelated to MAP kinase cascades are very likely to contribute centrally to pathogen- or wound-induced signal transduction (e.g., ref. 17).

Work on *EDR1* and *MPK4*, as well as SIPK and WIPK, raises interest in identifying the relevant interacting proteins. A MAPKK that interacts with tobacco SIPK has been identified (18). MPK4 protein is activated *in vitro* by the MAPKK AtMEK1 (19). In *Arabidopsis* protoplast experiments that establish a strong model for future work, Kovtun *et al.* (20) recently associated expression of three closely related MAPKKKs (ANP1, ANP2, or ANP3) with activation of AtMPK3 and AtMPK6 (WIPK and SIPK). It is suggested that ANP-associated MAP kinase cascades mediate both H_2O_2-stimulated activation of AtMPK3/6 and negative regulation of auxin-induced gene expression. Direct cause-and-effect relationships are not demonstrated, and the use of constitutively active MAPKKK proteins and/or overexpression constructs raises previously expressed concerns about oversimplified interpretation of the experimental results. But are any better experimental tools available to address the question? Perhaps such dual specificity is how things work. However, the alternative hypothesis—altered balance among different MAP kinase cascades—must be entertained. For plant responses such as jasmonate/salicylate defense signaling (as well as ethylene signaling), competition among MAP kinase cascades may be a central mechanism determining the degree to which different downstream responses are activated (see also refs. 21–23).

Even if the MAP kinase cascade controlled by wild-type *EDR1* is not normally involved in heightened responsiveness to pathogen, the work of Frye *et al.* (4, 9) may provide a number of avenues for further dissection of defense signal transduction. What defense-pathway protein(s) does the *edr1* mutation act on? Interaction cloning, protoplast experiments, and genetic suppressor screens are likely to be pursued in that regard.

More practically, work with *EDR1* suggests novel methods for disease control, in particular because mutant *edr1* plants appear phenotypically normal in the absence of pathogen. Putative orthologs of *EDR1* were identified in rice, barley, and other plants. Constitutive expression of a dominant negative or gene-silencing form of the *EDR1* gene could enhance disease resistance. Alternatively, conditional expression could heighten defense activation only in infected plants. Analogous studies are likely to arise from the work on *MPK4*.

Increasingly, cellular signaling is being viewed as a web or a net, with proteins at each node capable of receiving inputs from multiple partners and pathways (1, 2). Overstimulation or understimulation at a given node, or loss of a single node, can cause abnormal activity in adjacent regions of the net. These abnormal activities may functionally substitute for a defective node (genetic redundancy), but also can foster inappropriate phenotypes that mislead scientists seeking to understand the normal function of that node or protein.

The findings of Frye *et al.* (4) and Petersen *et al.* (5) suggest new interpretations of the negative role of MAP kinase cascades in the web of plant signal transduction. Their work provides new tools for signal transduction research and also provides new openings for the possible improvement of plant disease resistance.

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