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SIPK signaling controls multiple components of harpin-induced cell death in tobacco

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Summary

Harpin from *Pseudomonas syringae* pv. *phaseolicola* (HrpZ) elicits a rapid cell death response in tobacco plants. Multiple signaling components, including mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS) and salicylic acid (SA), have been reported to be involved in this cell death process, but the interaction between these molecules is poorly understood. Here we show through utilizing plants manipulated in SIPK expression levels that lack of SIPK results in increased sensitivity to harpin with concomitant accumulation of higher levels of ROS. Conversely, SIPK-overexpressing plants show reduced sensitivity to harpin relative to wild-type plants, and display reduced ROS accumulation. Harpin-induced cell death was found to be conditional on the ability of the plant to accumulate SA, whereas harpin induction of MAPK activation and ROS accumulation are not. However, harpin-induced ROS accumulation is required for activation of SIPK and wound-induced protein kinase. Transcriptional profiling revealed that suppression of SIPK signaling also affects early expression of a range of pathogen- and stress-responsive genes during harpin challenge.

Keywords: harpin, reactive oxygen species, SIPK, wound-induced protein kinase, salicylic acid, cell death.

Introduction

Phytopathogens have evolved multiple mechanisms for successfully colonizing and parasitizing their hosts, including virulence factors that directly or indirectly compromise the integrity and function of plant cells. One class of such factors consists of the harpins, a group of acidic, heat-stable, cell envelope-associated proteins encoded by members of the *hrp* gene clusters in bacterial phytopathogens such as *Erwinia amylovora* and *Pseudomonas syringae* (Bauer *et al.*, 1995; Wei *et al.*, 1992). Purified harpin applied to plant tissues causes rapid localized cell death, and induces systemic acquired resistance (SAR) in other parts of the challenged plant (Dong *et al.*, 1999; Lee *et al.*, 2001a). These responses are similar to the hypersensitive lesion response (HR) associated with classical gene-for-gene disease resistance (Desikan *et al.*, 1996), although there is no genetic evidence for a specific R-Avr interaction directly involving harpin. Nevertheless, *E. amylovora hrpN* mutants that fail to produce harpin are no longer pathogenic on pear, and fail to induce a hypersensitive response, indicating that the role of harpin is critical to the outcome of the disease (Wei *et al.*, 1992).

Detailed examination of the mode of action of harpin in plant cells indicates that it has an almost immediate effect on ion fluxes across the plasmalemma. In harpin-treated Arabidopsis cells, anion fluxes are suppressed within 1 min, while K^+ efflux is simultaneously stimulated (El-Maarouf *et al.*, 2001). The interaction of harpin with lipid bilayers *in vitro* results in rapid formation of cation-permeable pores (Lee *et al.*, 2001b). Whether this accurately models the initial *in vivo* response is unknown, but it is clear that harpin rapidly perturbs ion balances in challenged cells (Hoyos *et al.*, 1996; Xie and Chen, 2000). This perturbation is accompanied by other physiological responses, notably accumulation of reactive oxygen species (ROS; Desikan *et al.*, 1996; Ichinose *et al.*, 2001) and alteration of

mitochondrial functions such as induction of mitochondrial ROS accumulation, release of cytochrome *c* and inhibition of ATP synthesis (Krause and Durner, 2004; Xie and Chen, 2000). Harpin also rapidly induces activation of protein kinases (Desikan *et al.*, 1999) and expression of HR-related genes such as HIN1 (Gopalan *et al.*, 1996; Lee *et al.*, 2001a) and HSR201 (Czernic *et al.*, 1996), as well as more general stress-related genes such as PAL and GST (Andi *et al.*, 2001; Desikan *et al.*, 1998). If the harpin challenge is sufficiently severe, cell death ultimately ensues but it is unclear which of the initial responses induced by harpin are causally involved in this cell death outcome, or how they may be acting.

Numerous studies have reported the rapid activation of protein kinases in plant cells challenged with both crude and characterized elicitors (Romeis, 2001), and biochemical analysis has shown one of the major responding species to be a mitogen-activated protein kinase (MAPK), AtMPK6, in Arabidopsis (Desikan *et al.*, 2001) and the ortholog, SIPK, in tobacco (Zhang and Klessig, 2000). AtMPK6 activation in harpin-treated Arabidopsis cells is accompanied by activation of a second MAPK that was identified as AtMPK4 (Desikan *et al.*, 2001), although the second harpin-responsive MAPK in tobacco cells has been reported to be wound-induced protein kinase (WIPK; Zhang and Klessig, 2000), which is the ortholog of AtMPK3. Activation of MAPKs would appear to play a key role in controlling downstream impacts of harpin treatment, as application of either general protein kinase inhibitors or specific MAPKK inhibitors is able to block harpin-induced cell death in Arabidopsis (Desikan *et al.*, 1999).

By contrast, the role of ROS in mediating the cytotoxic effects of harpin is unclear. Harpin-induced accumulation of ROS such as superoxide anion or hydrogen peroxide might be sufficiently cytotoxic to cause cell death directly. Alternatively, altered levels of ROS might serve as second messengers to modulate a genetically programmed cell death (PCD) response, and/or to mobilize defense responses other than PCD. It is known that specific plant MAPKs (SIPK/AtMPK6) are rapidly and strongly activated by ROS (Samuel *et al.*, 2000; Yuasa *et al.*, 2001) in a process that requires both calcium transport and an active upstream MAPKK. However, harpin-induced activation of SIPK is unaffected by calcium transport inhibitors (Lee *et al.*, 2001a), which suggests that the harpin signal reaches the SIPK/MPK6 by a route somewhat different from that involved in oxidant-induced SIPK/MPK6 activation.

How activated SIPK/MPK6 affects the ultimate response of plant cells to harpin challenge remains largely undefined. These MAPKs appear to play a central role in harpin-induced cell death, and they also modulate cell death responses in tobacco (Samuel and Ellis, 2002) and Arabidopsis (G. Miles and B. Ellis, unpublished observations) plants challenged directly with oxidants. To gain further insight into the relationship between MAPK activation, ROS accumulation and cell survival in harpin-challenged cells, we have made use of transgenic tobacco lines in which SIPK has either been suppressed through RNAi technology, or ectopically overexpressed (Samuel and Ellis, 2002). The results provide new insights into the connection between harpin cytotoxicity, ROS and cell death, and help us to position both MAPK and salicylic acid (SA) signaling in that response matrix.

Results

Modification of SIPK affects harpin-induced cell death

In our previous study with SIPK-modified plants, we showed that both suppression (SIPK-RI plants) and overexpression (SIPK-OX plants) of SIPK leads to increased sensitivity of the plants to ozone, a potent oxidant, indicating a central role for SIPK during oxidant-stress tolerance (Samuel and Ellis, 2002). These transgenic plants were compared with the wild type (WT) plants for their response to infiltration with purified recombinant $harpin_{Psph.}$ Harpin (0.5 $\mu g\ \mu l^{-1}$), when infiltrated into leaves of WT plants, induced extensive cell death that led to collapse of the infiltrated zone within 24–36 h, followed by complete drying of the infiltrated zone within 72 h (Figure 1a). SIPK-RI plants consistently displayed more rapid cell death, with tissue collapsing as early as 8–12 h (Figure 1a,b). At lower harpin concentrations (0.3 $\mu g\ \mu l^{-1}$), WT plants showed no lesion formation, whereas lesions appeared within 48–72 h on SIPK-RI leaves (data not shown). In contrast, SIPK-overexpressing plants failed to develop lesions when infiltrated with harpin at a concentration of 0.5 $\mu g\ \mu l^{-1}$ (Figure 1a,b).

Commercial anti-pERK1/2 antibodies specifically recognize the activated forms of plant MAPKs (Samuel *et al.*, 2000). Western blot analysis of proteins extracted from the harpin-infiltrated zone at various times confirmed that the elicitor induced rapid and prolonged activation of two MAPKs in WT tissue (Figure 2a). This MAPK activation could be detected as early as 30 min following harpin elicitation (data not shown) and the kinases remained active for at least 4 h, after which the activation declined (Figure 2a). The identities of these activated MAPKs as SIPK and WIPK were confirmed through immunoprecipitation of SIPK and WIPK from protein extracts using SIPK- and WIPK-specific antibodies, followed by in-gel kinase assay (Figure 2c,d).

In the SIPK-suppressed (RI) genotype, harpin induced rapid and strong activation of WIPK, accompanied by relatively weak activation of residual SIPK (Figure 2a). The marked reduction in activated SIPK in the RI line was confirmed through immunoprecipitation of SIPK using SIPK-specific antibodies, followed by in-gel kinase assay (Figure 2c). A reciprocal pattern was observed in harpin-treated SIPK-OX tissue, where SIPK activation was sustained for 4 h following harpin infiltration and was accompanied by weak activation of WIPK (Figure 2b).

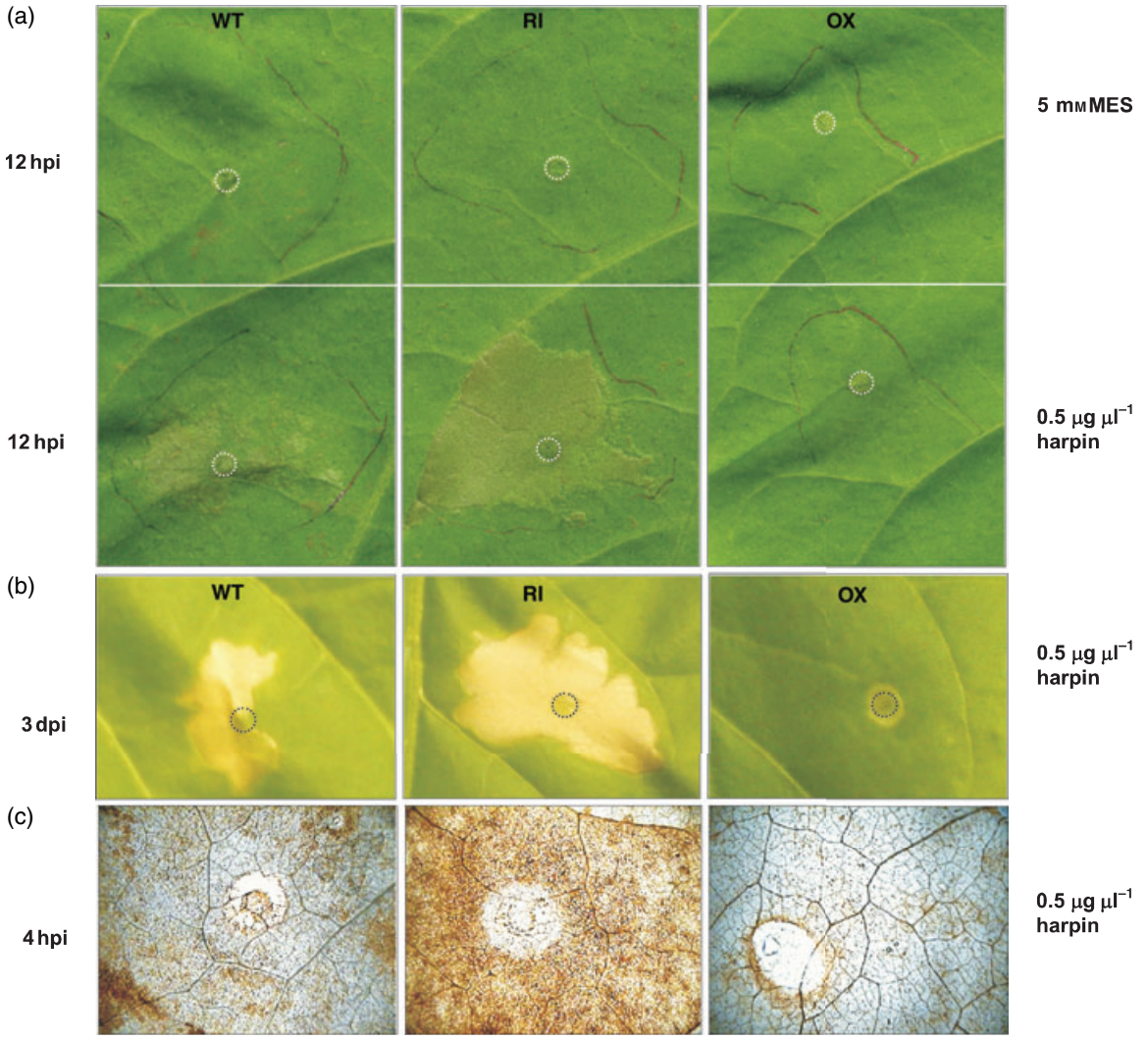

Figure 1. SIPK suppression leads to hypersensitivity to harpin.
(a, b) Pattern of lesion development in the SIPK genotypes at 12 h and 3 days after infiltration with harpin (0.5 μg μl^{-1}). The injection points are encircled by dotted lines.
(c) *In situ* detection of hydrogen peroxide by DAB staining in the leaves of the same genotypes as in (a), 4 h post-infiltration with harpin (0.5 μg μl^{-1}). Hydrogen peroxide accumulation is visualized as brown precipitates.

The heightened sensitivity to harpin of SIPK-RI tissue suggests that the suppression of SIPK (and/or the prolonged activation of only WIPK) was interfering with the ability of the challenged cells to control harpin-induced cell death.

SIPK activity is necessary for suppression of lesion formation in SIPK-OX plants

As plants over-expressing SIPK were resistant to harpin-induced cell death, we asked whether this outcome might reflect interference of increased levels of the ectopically expressed SIPK protein with normal SIPK signaling (dominant negative effect), or whether the increased resistance required the catalytically functional SIPK protein. To examine this, we employed stable transgenic tobacco lines that over-expressed a catalytically inactive form of SIPK (SIPK-KI) that can neither be phosphorylated by an upstream MAPKK, nor phosphorylate downstream targets. The sensitivity of these SIPK-KI plants to harpin infiltration was similar to that of WT plants (data not shown), indicating that the ability of ectopic SIPK to interact with its upstream and/or downstream effectors is essential to the cell death suppression observed in SIPK-OX plants.

Harpin induces hyper-accumulation of hydrogen peroxide in SIPK-suppressed tissues

Harpin treatment leads to hydrogen peroxide accumulation in tobacco and Arabidopsis cell cultures (Andi *et al.*, 2001; Desikan *et al.*, 1998), and hydrogen peroxide and related

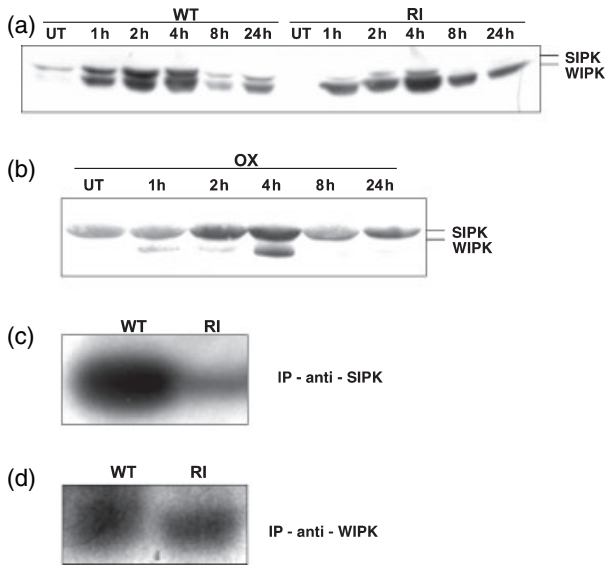

Figure 2. Harpin-induced MAPK activation profile in the SIPK-genotypes.
Protein samples (60 μg) extracted from SIPK genotypes either untreated (UT) or infiltrated with harpin (0.5 μg μl^{-1}) for different times were immunoblotted with anti-pERK antibody (a, b). The 2-h harpin (0.5 μg μl^{-1})-treated samples from wild type and SIPK-RI line were subjected to an in-gel kinase assay following immunoprecipitation (IP) with SIPK and WIPK-specific antibodies (c, d).

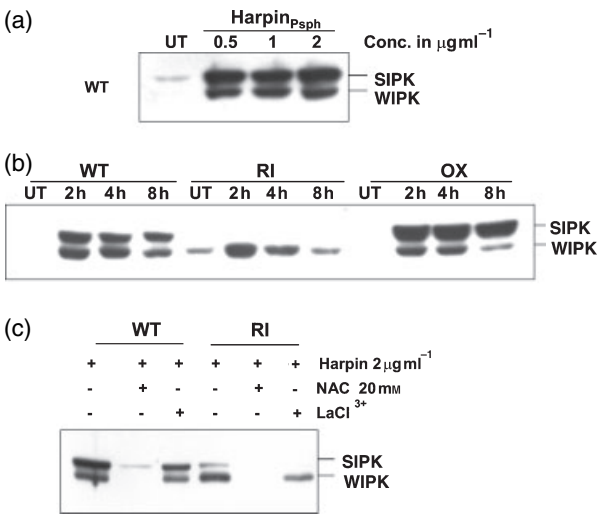

Figure 3. Harpin-induced MAPK activation is dependent partially on ROS accumulation and independent of extracellular calcium influx.
(a) Proteins (40 μg) extracted from WT suspension-cultured cells treated with different concentrations of harpin were immunoblotted using anti- pERK antibody.
(b) Temporal MAPK activation profile in the different SIPK genotypes was determined through immunoblotting of proteins extracted from harpin (2 μg ml^{-1})-treated cultures at various times with anti-pERK antibody.
(c) Extracted proteins (40 μg) from WT and RI suspension-cultured cells that had been pre-treated with either *N*-acetyl cysteine (10 mM) or $LaCl_3$ (5 mM) followed by elicitation with harpin (2 μg ml^{-1} for 30 min) were analyzed by immunoblotting using anti-pERK.

ROS are known activators of SIPK and AtMPK6 (Ahlfors *et al.*, 2004; Rentel *et al.*, 2004; Samuel *et al.*, 2000; Yuasa *et al.*, 2001). However, a causal link between harpin challenge, ROS accumulation and MAPK activation was questioned when harpin-induced activation of AtMPK6 in Arabidopsis cells was shown to be independent of the 'oxidative burst,' based on the failure of catalase and DPI treatments to block the activation process (Desikan *et al.*, 2001). To help clarify this issue, we examined the pattern of harpin-induced ROS accumulation in our SIPK-modified transgenic lines.

Leaves of the different tobacco genotypes were infiltrated with harpin (0.5 μg μl^{-1}) and tested histochemically 4 h later for hydrogen peroxide accumulation. Harpin treatment led to an increase in hydrogen peroxide levels in both the WT and SIPK-RI lines, but the SIPK-suppressed plants displayed visibly greater accumulation of this ROS (Figure 1c). In contrast, ROS accumulation in the SIPK-OX leaves appeared less pronounced than in WT plants (Figure 1c). Modulation of SIPK activity thus also has an effect on the levels of harpin-induced ROS, and the pattern suggests that one or more facets of ROS accumulation may normally be under negative control by SIPK.

Harpin also induces MAPK activation in tobacco cell suspension cultures

To further characterize the interplay between harpin challenge, ROS accumulation and MAPK activation in tobacco cells, we employed cell suspension cultures established earlier from the different SIPK genotypes (Samuel and Ellis, 2002). Treatment of WT cultures with different concentrations of harpin (0.5–2 μg ml^{-1}) for 30 min, followed by immunoblotting of extracted proteins with anti-pERK antibody, demonstrated that these treatments led to strong activation of both SIPK and WIPK, with a similar intensity of activation for both kinases at all elicitor concentrations used (Figure 3a). When harpin-induced MAPK activation was profiled over an extended time course (0–8 h), both kinases remained active in the WT cells over the entire period with decline in activation after 4 h (Figure 3b). In the SIPK-OX cultures, strong SIPK activation was observed that was sustained for the 8-h period, but WIPK activation declined after 4 h (Figure 3b). In the SIPK-suppressed line, only WIPK displayed activation.

As harpin elicits both MAPK activation and ROS accumulation in tobacco leaves and cultures, and ROS alone are capable of inducing MAPK activation, it seemed possible that these processes could be functionally related. Indeed, pre-treatment of suspension cultures of the different genotypes with *N*-acetyl cysteine (NAC), a free radical scavenger, completely blocked harpin-induced activation of WIPK in both the WT and SIPK-RI cultures, as well as effectively silencing the harpin-induced activation of SIPK in the WT cultures. However, harpin-induced MAPK activation in cells

pre-treated with the calcium channel blocker, La^{3+}, remained essentially unaltered, suggesting that harpin-induced MAPK activation involves an ROS-dependent, but calcium flux-independent, process (Figure 3c). This result is congruent with earlier reports that tobacco MAPK activation by harpin does not require calcium influx (Adam *et al.*, 1997; Lee *et al.*, 2001a).

Harpin-induced MAPK activation and ROS accumulation are SA-independent processes but cell death is SA-dependent

SA plays a central role in plant defense against pathogens, most prominently as an essential agent in establishment of SAR. As well as inducing cell death in Arabidopsis tissues, harpin induces SAR in Arabidopsis tissues. This SAR response is abolished in *nahG* plants, which are unable to accumulate SA (Dong *et al.*, 1999) The ability of harpin-treated Arabidopsis plants to mount a resistance response during a subsequent infection by a non-host bacterial pathogen is also compromised in the *nahG* genotype (Kariola *et al.*, 2003; van Wees *et al.*, 2003). These results suggest that part of the response pattern invoked by harpin in challenged plant cells requires the participation of SA, but a functional relationship between SA, MAPK activity and harpin-induced cell death has not been established.

When leaves of *nahG* tobacco plants were infiltrated with recombinant harpin (0.5 $\mu g\ \mu l^{-1}$), no lesions appeared over a 4-day period (Figure 4a). This insensitivity directly phenocopies the response of the SIPK-OX tobacco plants to harpin treatment (Figure 1b). However, the MAPK activation profile observed in tissues of harpin-treated *nahG* tobacco plants was similar to the profile found in WT plants (Figure 4c). SA accumulation is therefore not required for harpin-induced MAPK activation, similar to results obtained earlier for ROS-driven activation of SIPK in tobacco (Samuel *et al.*, 2000).

As cell death induced in WT tobacco leaf tissue by harpin infiltration is normally accompanied by increased ROS accumulation, and the harpin-insensitive SIPK-OX genotype also displayed reduced levels of ROS (Figure 1c), we anticipated that the *nahG* plants would behave in a similar fashion. Unexpectedly, however, this correlation was not observed. Four hours after harpin infiltration, histochemically detectable hydrogen peroxide accumulation in the *nahG* leaf tissue was similar to that seen in the WT genotype (Figure 4b), despite the marked difference in extent of cell death induced in the two tissues by harpin challenge.

The relationship between inability to accumulate SA and reduced sensitivity to harpin in the *nahG* genotype suggested that, by analogy, the absence of lesion formation in harpin-treated SIPK-OX plants might result from their

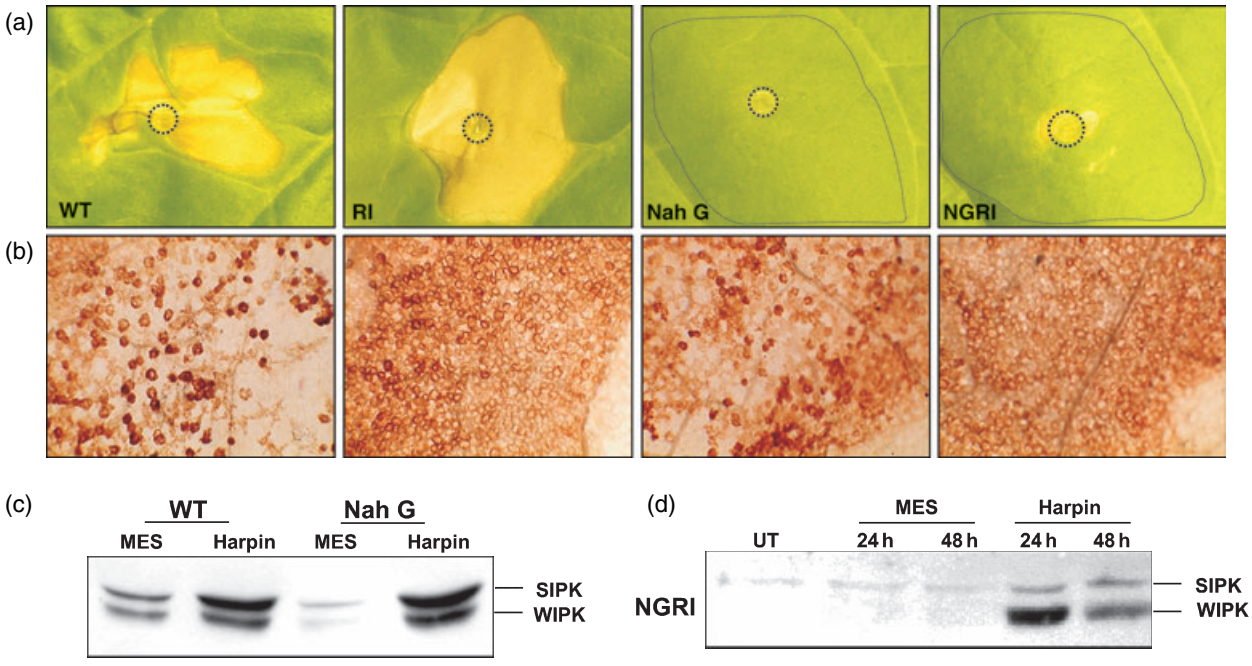

Figure 4. Harpin-induced cell death requires SA.
(a) WT, RI, Nah G and (Nah G × RI) NGRI lines were infiltrated with harpin (0.5 $\mu g\ \mu l^{-1}$) and infiltrated zones were photographed after 4 days. Injection points and infiltrated zones are shown in dotted black lines.
(b) DAB staining procedure was used to detect the extent of hydrogen peroxide accumulation at 4 h after harpin (0.5 $\mu g\ \mu l^{-1}$) infiltration.
(c) Protein samples extracted from WT and *nah G* plants treated with either MES or harpin for 30 min were immunoblotted with anti-pERK antibody.
(d) NGRI line was treated with either MES or harpin (0.5 $\mu g\ \mu l^{-1}$) for different times and the extracted proteins were immunoblotted with anti-pERK antibody.

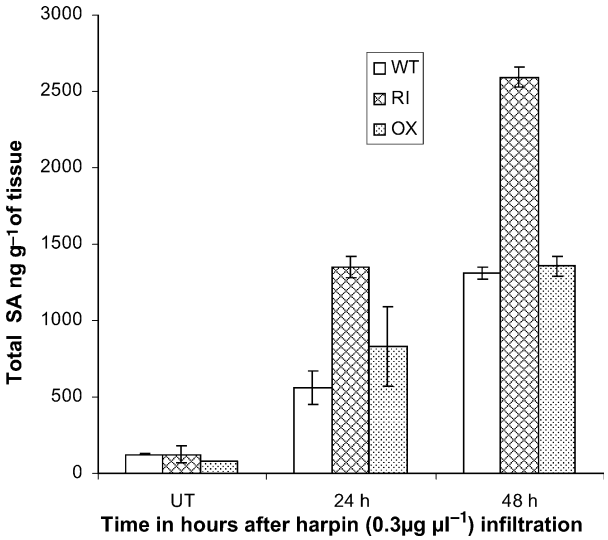

Figure 5. SIPK-RI line accumulates more SA following harpin treatment. WT, SIPK-RI, and SIPK-OX lines were treated with harpin (0.3 μg μl^{-1}) for various times and the infiltrated zones (four per genotype) were harvested and analyzed for SA accumulation. The experiment was repeated with consistent results and range bars are shown.

inability to accumulate SA. Conversely, increased sensitivity in the SIPK-RI plants might be associated with increased SA accumulation in the challenged tissue. This model would imply that increased SIPK activity serves as a negative regulatory checkpoint for SA accumulation.

We tested this hypothesis in two ways. First, we crossed *nahG* and SIPK-RI tobacco plants and selected four F_1 progeny that expressed both the *nahG* and SIPK-RI constructs (NGRI genotype). These plants displayed no visible phenotype under normal growth conditions. When leaves of the NGRI lines were infiltrated with harpin (0.5 μg μl^{-1}), none of the plants displayed lesions, i.e., they displayed a *nahG* phenotype (Figure 4a). We conclude that the increased harpin sensitivity displayed by SIPK-RI plants requires SA accumulation in order to be manifest. However, the MAPK activation profiles and ROS accumulation patterns induced in the NGRI plants by harpin were similar to those observed in the harpin-infiltrated RI genotype (Figure 4b,d), which is consistent with our earlier conclusion that neither MAPK activation nor ROS accumulation are reliant on SA signaling.

We next measured the total SA accumulation in untreated and harpin-treated tissues of the various genotypes (WT, SIPK-RI, *nahG*, SIPK-OX, NGRI) following infiltration with a harpin concentration (0.3 μg μl^{-1}) that leads to cell death over a period of 48–72 h in the SIPK-RI line. SA accumulation in WT and SIPK-OX plants followed a similar trajectory over a period of 48 h, whereas the levels attained in SIPK-RI plants were twofold higher (Figure 5). As expected, both the *nahG* and NGRI genotypes displayed low levels of SA accumulation (data not shown).

Loss of SIPK function alters harpin-induced gene expression profiles

Whether it acts at one or multiple sites within the cellular defense signaling network, SIPK activity clearly has a marked impact on the ultimate fate of cells challenged with harpin. This regulatory influence would be predicted to initially impact protein phosphorylation patterns within the affected cells leading, *inter alia*, to changes in gene transcription. To gain some preliminary insight into the early transcriptional events that are specifically dependent on upstream SIPK signaling following challenge with harpin concentration that elicited a significant cell death response, we compared gene expression profiles 4 h post-challenge in WT and SIPK-RI tobacco cell cultures. Although large-scale tobacco-specific DNA microarrays are not yet publicly available, The Institute for Genomic Research (TIGR) has produced 'Solanaceae' microarrays that carry approximately 10 000 replicated potato cDNAs, most of which would be expected to hybridize efficiently with homologous sequences from tobacco. RNA isolated from the WT and SIPK-RI cells in replicated experiments was provided to the TIGR microarray facility, which carried out the labeling and hybridization, and subsequently provided us with the resulting microarray image files.

When the expression profiles were filtered for high statistical reliability, a specific set of genes could be identified whose expression following harpin treatment was increased or decreased at least twofold in the SIPK-RI genotype relative to their behavior in WT cells (Table 1). The upregulated subgroup included a number of defense-related genes, such as those encoding enzymes involved in terpenoid phytoalexin biosynthesis (farnesyl diphosphate synthase; sesquiterpene synthase) (Brodelius *et al.*, 2003), as well as expression of WIPK and of the WRKY transcription factor, NtWIZZ (Table 1). The loss of SIPK expression also resulted in downregulation (relative to WT) of several harpin-induced genes encoding elicitor-inducible, stress-associated and oxidative stress management proteins (e.g., heat shock protein, monodehydroascorbate reductase, peroxidase precursor).

Discussion

Harpin-induces MAPK signaling in tobacco

Our results demonstrate that challenge with harpin rapidly activates MAPKs in tobacco cells, and that activated MAPKs play a central role in orchestrating the physiological responses of the cell. We confirmed that the two most prominent tobacco MAPK species activated within minutes of harpin application are SIPK and WIPK, and that, as expected for such an immediate post-challenge event, their activation is independent of SA accumulation, which is a much slower process.

Table 1 Harpin-induced changes in gene expression in SIPK suppressed line relative to WT at 4 h following treatment with 40 μg ml^{-1} harpin

StGI no.[a]	EST sequence similarity[b]	Tentative annotation (StGI)[c]	Fold-change in gene expression in SIPK suppressed line relative to WT[d]
TC62339	*Nicotiana tabacum*	WIZZ	3.66
TC60363	*Arabidopsis thaliana*	Cinnamyl-alcohol dehydrogenase CAD1	2.89
TC70686	*Arabidopsis thaliana*	Hypothetical protein F20P5.25	2.67
TC59043	*Lycopersicon hirsutum*	Sesquiterpene synthase 2	2.63
TC62891	*Arabidopsis thaliana*	Hypothetical protein	2.60
TC60010	*Nicotiana tabacum*	Avr9/Cf-9 rapidly elicited protein 264	2.55
TC60314	*Olea europaea*	Calcium-binding allergen Ole e 8	2.46
TC57829	*Lycopersicon esculentum*	Farnesyl-pyrophosphate synthetase FPS1	2.41
TC61932	*Arabidopsis thaliana*	Hypothetical protein F28A23.120	2.40
TC60200	*Arabidopsis thaliana*	SCARECROW gene regulator-like	2.40
TC60800	*Arabidopsis thaliana*	Inflorescence and root apice receptor-like kinase	2.38
TC70862	*Nicotiana tabacum*	Wound-induced protein kinase	2.37
TC61004	*Glycine max*	GMFP5	2.36
TC69439	*Glycine max*	Syringolide-induced protein B13-1-1	2.35
TC60733	*Arabidopsis thaliana*	Unknown protein	−2.31
TC70338	*Pharbitis nil*	Heat shock protein	−2.31
TC66804	*Galactowaldenase*	UDP-glucose 4-epimerase	−2.31
TC66648	*Gossypium hirsutum*	Ovule/fiber cell elongation protein Ghfe1	−2.31
TC68168	*Lycopersicon esculentum*	Heat shock protein	−2.32
TC63698	*Arabidopsis thaliana*	Putative chloroplast nucleoid DNA binding protein	−2.35
TC62220	*Lycopersicon esculentum*	Subtilisin-like proteinase	−2.36
TC67331	*Nicotiana tabacum*	Plasma membrane protein	−2.37
TC59206	*Arabidopsis thaliana*	Putative short-chain type dehydrogenase/reductase	−2.39
TC58395	*Lycopersicon esculentum*	Clone SENU1 – senescence-related	−2.40
TC66537	*Lycopersicon esculentum*	Monodehydroascorbate reductase (NADH) (cytosolic)	−2.42
TC71755	*Arabidopsis thaliana*	Receptor-like protein kinase	−2.42
TC67030	*Lycopersicon esculentum*	Lactoylglutathione lyase family protein/glyoxalase family protein	−2.61
TC69207	*Lycopersicon esculentum*	Cytochrome 1*b*	−2.63
TC66554	*Lycopersicon esculentum*	Peroxidase precursor, defense-related	−2.73
TC67271	*Arabidopsis thaliana*	Putative short-chain type dehydrogenase/reductase	−2.77
TC65924	*Lycopersicon esculentum*	Cysteine proteinase precursor	−2.82
TC58418	*Solanum tuberosum*	GAL83 protein	−2.98
TC69152	*Arabidopsis thaliana*	Hypothetical protein F18B13.24	−3.49
TC62407	*Arabidopsis thaliana*	Raffinose synthase family protein/seed, imbibition protein	−4.39
TC67573	*Nicotiana tabacum*	Elicitor-inducible protein	−4.78

[a]*Solanum tuberosum* Gene Index (StGI) tentative consensus (TC) numbers are associated with each potato EST on the 10 K potato cDNA array.
[b]Species names are based on ranking of best hits from BLAST searches performed by TIGR for each EST (non-singleton) in the StGI database TC Annotator (http://www.tigr.org/tigr-scripts/tgi/tc_ann.pl?db=potest).
[c]Annotations are based upon StGI database information for associated best hit in TC Annotator.
[d]Filtering of the gene list applied a fold-difference minimum of ±2.3 and a *t*-test *P*-value of 0.002.

The harpin-induced MAPK activation is abolished in the presence of an ROS-scavenging agent (NAC) (Figure 3c), indicating that an ROS signal forms an integral part of the activation process. However, unlike the previously observed activation of plant MAPKs by direct oxidants such as hydrogen peroxide or ozone, which is calcium influx-dependent (Samuel *et al.*, 2000), harpin-induced MAPK activation is essentially unaffected by lanthanum; predominantly a plasma membrane calcium ion channel blocker (Knight and Knight, 2000).

While extracellular calcium influx is apparently not required for harpin signaling to SIPK, calcium release from internal stores could still play a role in harpin-induced MAPK activation. Harpin from *Pseudomonas syringae* pv. *phaseolicola* induces a rapid increase in cytosolic calcium (Ca_{cyt}) levels in cultured parsley cells (Blume *et al.*, 2000). In aequorin-transformed tobacco cells, harpin treatment also led to a strong increase in Ca_{cyt}, which was blocked by application of an inhibitor of internal store release (Cessna and Low, 2001). This inhibition also blocked induction of an oxidative burst by harpin, which could place the harpin-induced oxidative burst downstream of calcium release. Because an ROS signal is required for harpin-induced MAPK activation, one model connecting harpin perception with MAPK signaling would involve harpin induction of a rapid increase in cytosolic calcium

levels derived from internal stores, which enables generation of the oxidative burst. This cascade of events would then converge on the MAPK pathway through one or more upstream kinases. Activation of SIPK both by harpin and by direct oxidants is interrupted by pre-treatment with the MAPKK inhibitor PD98059, while in Arabidopsis both a serine/threonine kinase (OXI1) (Rentel *et al.*, 2004) and the MAPKKK ANP1 (Kovtun *et al.*, 2000) appear to participate in ROS signal transmission to AtMPK6. Taken together, these findings are consistent with the presence in plant cells of one or more MAPK modules that link SIPK/AtMPK6 to upstream ROS-sensitive kinases.

SIPK and WIPK are not functionally redundant MAPKs

Although both SIPK and WIPK are often activated together by stress, and are the likely targets of the same upstream MAPKK(s) (NtMEK2/AtMKK4 and 5), these two terminal kinases appear to play somewhat different, but intertwined, biological roles (Kim and Zhang, 2004). Unlike SIPK/AtMPK6, WIPK/AtMPK3 transcription is very responsive to stress (Seo *et al.*, 1995; Liu *et al.*, 2003; Zhang *et al.*, 2000). Based on their analysis of this sensitivity within the NtMEK2/SIPK/WIPK sub-module in tobacco, Liu *et al.* (2003) proposed a hierarchical model in which activation of SIPK positively regulates transcription of WIPK, leading to an increase in WIPK protein levels. Phosphorylation of the resulting WIPK by NtMEK2 would activate this kinase, which would then serve as a positive effector of cell death.

However, this model is not entirely consistent with the observations that (i) long-term suppression of SIPK in SIPK-RI plants does not block the transcription (Table 1), synthesis or activation of WIPK, and (ii) SIPK-RI plants are more, rather than less, prone to stress-induced lesion formation (Samuel and Ellis, 2002; Figure 1). In addition, AtMPK3 transcription and activation respond normally to oxidant stress and elicitors in Arabidopsis mutants in which AtMPK6 is completely silenced (Liu and Zhang, 2004; J. Lee and B.E. Ellis, unpublished data).

Nevertheless, interpretation of the physiological effects of SIPK manipulation must bear in mind the apparently antagonistic functional interlock between SIPK and WIPK. Thus, loss of SIPK/AtMPK6 activity results in increased activation of WIPK in stressed cells and both phenomena are correlated with acceleration of lesion formation induced by either ozone (Samuel and Ellis, 2002), TMV infection (Zhang and Klessig, 1998), or harpin. This may explain why some of the genes that are most strongly upregulated in SIPK-suppressed tissues following harpin treatment (*HIN 1*(data not shown) and *NtWIZZ*) are also upregulated in transgenic tobacco expressing a constitutively active form of the upstream MAPKK, NtMEK2, which drives activation of both SIPK and WIPK (Kim and Zhang, 2004).

Harpin-induced ROS accumulation is negatively regulated by SIPK

Exposure to harpin normally induces accumulation of hydrogen peroxide in tobacco tissues, followed by death of the affected cells. Suppression of SIPK expression in SIPK-RI plants simultaneously enhanced both of these responses, while ectopic over-expression of SIPK had the reciprocal effect (Figure 1b). This pattern, together with the rapid activation of SIPK by ROS and the presence of several known antioxidant functions among the RI/WT differentially expressed genes (Table 1), is consistent with the hypothesis that one of the central functions of SIPK in the cell is to help monitor and regulate the cellular redox state. We had earlier observed that the gene product for another key antioxidant, cytosolic ascorbate peroxidase, is rapidly induced in SIPK-OX tobacco plants, and suppressed in the SIPK-RI lines (Samuel and Ellis, 2002). Arabidopsis plants lacking functional AtMKP1, the dual specificity phosphatase proposed to dephosphorylate and thus inactivate AtMPK6, also display constitutively increased levels of transcripts for various antioxidant enzymes (Ulm *et al.*, 2002).

However, this proposed function for SIPK does not immediately explain the observed correlation between SIPK expression and the plant's susceptibility to harpin-induced lesion formation. Although ROS accumulation and the 'oxidative burst' have frequently been associated with cell death phenomena such as the HR (Overmyer *et al.*, 2003), establishment of a convincing pro-PCD causal relationship for ROS has remained elusive (Dorey *et al.*, 1999). Our observation that suppression of SIPK in a *nahG* background is able to block harpin-induced cell death while leaving the increased accumulation of hydrogen peroxide apparently unaltered (Figure 4b) reinforces the point that elevated levels of ROS are not a reliable predictor of a cell death outcome.

Cell death induced by harpin is negatively controlled by SIPK and requires SA accumulation

As SIPK-RI tissue is less able than WT to survive harpin challenge, whereas SIPK-OX is more resistant, loss of this MAPK appears to undermine the cell's ability to successfully counter the abrupt homeostatic changes induced by the harpin elicitor. However, as seen in the NGRI genotype plants, ROS accumulation *per se* does not generate a commitment to the cell death pathway.

SA, on the other hand, clearly plays a key role in execution of the cell death process, cells in which SA accumulation is suppressed (i.e., *nahG* background) remain alive following challenge with concentrations of harpin that consistently kill WT cells, and this cell death suppression is able to override the increased harpin sensitivity otherwise conditioned by SIPK silencing (i.e., NGRI background). This places the

negative regulatory effect of MAPKs on cell death upstream of SA. It is possible that the two phenomena may be cross-regulated, as harpin-treated SIPK-RI plants display accentuated SA accumulation. However, SIPK-OX tobacco plants are as tolerant of harpin as the *nahG* genotype, even though they are unaltered in their ability to synthesize and accumulate SA following elicitation. Suppression of the harpin-induced cell death pathway in SIPK-OX plants must therefore have origins other than removal of SA from the signaling pathway.

In summary, our results show that tobacco cells challenged with harpin rapidly mobilize MAPK signaling, a process that is probably mediated through the early ROS burst elicited by this microbial protein. The activated MAPKs modulate the challenged cells' subsequent responses to harpin, including SA accumulation and induction of cell death. Both of the latter processes appear to be negatively regulated by SIPK in elicitor-treated tobacco. There is increasing evidence that SIPK and its orthologs are involved in a wide range of other biological processes as well as elicitor and oxidant response. These include environmental stress sensing (Droillard *et al.*, 2004; Teige *et al.*, 2004), ethylene biosynthesis (Liu and Zhang, 2004), and cellular differentiation (Samaj *et al.*, 2003). This scope, as well as the diversity of regulatory proteins represented in the RI/WT differential gene list (Table 1), emphasizes the highly integrated nature of the regulatory networks supported by MAPK signal transduction in plants. Elucidation of these complex and often subtle relationships will require application of the full power of both genetic and biochemical approaches.

Experimental procedures

Plant material and treatment

Tobacco (*Nicotiana tabacum* cv. *Xanthi-nc*) plants of all genotypes were grown for 6 weeks in soil under controlled environmental conditions (25/20°C 16 h light/8 h dark) as described previously (Orvar *et al.*, 1997). Six-week-old plants were used for the treatments. The third and fourth fully developed leaves were infiltrated with different concentrations of harpin using a hypodermic syringe without needle. The infiltrated zones were either observed for lesion development or harvested using a scalpel and stored at −80°C for further analysis.

Cell culture treatments

Tobacco (Xanthi-nc, SIPK-OX, and SIPK-RI) suspension cultures were established and maintained as described in Samuel *et al.* (2000). To test potential inhibitors, suspension-cultured cells were pre-treated with $LaCl_3$ (5 mM) for 10 min and NAC (10 mM) (Sigma-Aldrich Corp., St Louis, MO, USA) for 45 min followed by treatment with harpin (2 μg ml^{-1}). The cells were harvested by vacuum filtration, frozen in liquid nitrogen, and stored at −80°C to await analysis.

Generation of SIPK-KI and NahG/RI (NGRI) lines

Stable transgenic SIPK-KI lines that ectopically expressed a mutant version of SIPK that can neither be phosphorylated (T218A/Y220F) nor can phosphorylate (K89R) downstream substrates were generated through *Agrobacterium*-mediated transformation. Site-directed mutagenesis, transformation, and screening procedures were followed as described previously (Samuel and Ellis, 2002).

NGRI lines were created through fertilizing SIPK-RI pistils with *nah G* pollen followed by screening the kanamycin-resistant F_1 lines for the presence of both insertions through PCR, using 35S forward primer in combination with either SIPK-specific reverse primer (Samuel and Ellis, 2002) or *nah G*-specific reverse primer (5′ GTC GCG CAA CTC GTA TAA CTC 3′) (Gaffney *et al.*, 1993).

Recombinant harpin production

Recombinant harpin from BL-21 cells harboring the pT7-7 plasmid containing the DNA fragment encoding $harpin_{Psph}$ was purified essentially according to Lee *et al.* (2001a) except 40% saturation of ammonium sulfate was used for the precipitation, and the following desalting and concentration was achieved through dialysis (14–18 kDa cutoff).

Protein extraction, Western blotting, and in-gel kinase assays

Total protein extracts (40–80 μg) were prepared and used for Western blotting and in-gel kinase assays as described earlier (Samuel and Ellis, 2002; Samuel *et al.*, 2000).

In situ *staining for hydrogen peroxide*

Hydrogen peroxide was visualized *in situ* by 3,3′-diaminobenzidine staining performed essentially according to Torres *et al.* (2002) and Samuel and Ellis (2002).

Solanaceae-expressing profiling

Four-day-old cell suspension cultures of WT and SIPK-RI line were used for the experiment. For each genotype, six 25 ml cultures were randomly assigned as three controls (no inoculation) or three treatments (40 μg ml^{-1} purified hrpZ). After 4 h, RNA_{total} was extracted from 1.5 g of cell culture from each flask by the Trizol™ method followed by assessment of purity (GeneQuant™ 260/280; Amersham, Piscataway, NJ, USA) and degradation (Agilent bioanalyzer™; Agilent Technologies, Mississauga, ON, Canada). Extracted RNAs were submitted to The Institute for Genomic Research (TIGR) Solanaceae Gene Expression Profiling Service (http://www.tigr.org/tdb/potato/profiling_service2.shtml).

Background correction, log transformation, and normalizations were performed for all slides in the R environment (version 1.8.1) using standard tools (LOWESS-locally weighted regression) and a supplemental Bioconductor package for cDNA microarray analysis in R (modreg library).

Student's *t*-test was used in per-gene comparisons between the treatment groups (each having three hybridizations of mutant-to-wild type) for the determination of genes with significant differentials. Technical replicates for each of the three treatment group hybridizations were used to determine $\log_2$ mean expression ratios

for each treatment group and thus the fold change between of the treatment groups. Filtering of the gene list applied a fold-difference minimum of ±2.3 and a *t*-test *P*-value of 0.002.

SA measurements

Harpin-infiltrated zones (four per genotype/treatment/time point) were pooled and 0.5–1 g of tissue was used for measuring free and total SA as described previously (Li *et al*., 1999).

Upon request, all novel materials described in this article will be made available in a timely manner for non-commercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for non-commercial research purposes.

Unless otherwise indicated, all experiments were repeated with consistent results.

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