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ORIGINAL ARTICLE

Role of α-tocopherol in cellular signaling: α-tocopherol inhibits stress-induced mitogen-activated protein kinase activation

Tae Kyung Hyun · Kundan Kumar · Kudupudi Prabhakara Rao · Alok Krishna Sinha · Thomas Roitsch

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Abstract Tocopherols belong to the plant-derived poly phenolic compounds known for antioxidant functions in plants and animals. Activation of mitogen-activated protein kinases (MAPK) is a common reaction of plant cells in defense-related signal transduction pathways. We report a novel non-antioxidant function of α-tocopherol in higher plants linking the physiological role of tocopherol with stress signalling pathways. Pre-incubation of a low concentration of 50 μM α-tocopherol negatively interferes with MAPK activation in elicitor-treated tobacco BY2 suspension culture cells and wounded tobacco leaves, whereas pre-incubated BY2 cells with α-tocopherol phosphate did not show the inhibitory effect on stimuli-induced MAPK activation. The decreased MAPK activity was neither due to a direct inhibitory effect of α-tocopherol nor due to the induction of an inhibitory or inactivating activity directly affecting MAPK activity. The data support that the target of α-tocopherol negatively regulates an upstream component of the signaling pathways that leads to stress dependent MAPK activation.

Keywords α-Tocopherol · MAPKs · Non-antioxidant · Stress · E-Fol

Abbreviations

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
E-Fol	An elicitor preparation of *Fusarium oxysporum lycopersici*

Introduction

Reactive oxygen species (ROS) derived from molecular oxygen can accumulate in plants under a variety of biotic and abiotic stress conditions, and cause oxidative damage to the cellular compounds including proteins, chlorophyll, and lipids. To survive in these conditions, plants have developed two general protective mechanisms, enzymatic and non-enzymatic detoxification (Alscher et al. 2002).

Tocopherols, better known as vitamin E, are lipophilic anitioxidants synthesized by plants, algae, fungi, and some cyanobacteria. There are four natural analogues of tocopherols, α, β, γ, and δ, which have different bioactivities. α-tocopherol commonly accumulates in the leave of plants whereas γ-tocopherols are abundant in seeds. β- and δ-tocopherols are not very abundant in most plant species. In addition, it has been proposed that α-tocopherol participates in the detoxification of ROS together with the

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T. K. Hyun · T. Roitsch
Department for Plant Physiology, Institute for Plant Science, Karl-Franzens University Graz, Schubertstrasse 51, 8010 Graz, Austria

K. Kumar · K. P. Rao · A. K. Sinha (✉)
National Institute of Plant Genome Research, PB No. 10531, Aruna Asaf Ali Road, New Delhi 110067, India
e-mail: alok_sinha@nipgr.res.in

T. K. Hyun · K. Kumar · K. P. Rao · A. K. Sinha · T. Roitsch
Julius-von-Sachs-Institut for Biosicences, University Wuerzburg, Julius-von-Sachs-Platz 2, 97082 Wuerzburg, Germany

hydrophilic antioxidants, glutathione and ascorbate (Foryer and Noctor 2003). In addition to the antioxidant activity, tocopherols have been shown to be involved in regulating various physiological and developmental processes in plants. The tocopherol-deficient mutant *vte2* showed as defective in transfer cell wall development and photoassimilate transport at low temperatures (Maeda et al. 2006), reduced seed viability, and impaired seeding development (Sattler et al. 2004). In addition, the overexpression of tocopherol cyclase, a key enzyme that regulates the formation of the chromanol ring structure of the tocopherols (Stocker et al. 1996; Vidi et al. 2006), enhanced tolerance to drought stress and resulted in accumulation of tocopherol (Liu et al. 2008).

Mitogen-activated protein kinase (MAPK) cascades are conserved signaling modules found in all eukaryotic cells including plants, fungi, and animals. MAPKs form one component of a "three-kinase" module involving the upstream activators, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). The three-kinase modules are functionally interlinked and have been found to play a central role in signal transduction pathways in eukaryotic organisms. Genetic studies coupled with biochemical approaches showed that MAPKs are key signaling compounds which are regulated by biotic and abiotic stress-related signals, and are also involved in hormonal and developmental signaling in plants (Colcombet and Hirt 2008). Also, an increasing number of reports suggest that tocopherols are involved in the perception and transduction of extracellular stimuli in animal cells and modulate the activation of specific enzymes involved in signal transduction pathways. The tumor necrosis factor-α-induced activation of MAPKs like ERK 1/2 and p38 was inhibited by α-tocopherol in bronchial epithelial cell lines (Ekstrand-Hammarstroem et al. 2006). The similar effect of α-tocopherol on phorbol myristate acetate (PMA)-induced ERK 1/2 activation in smooth muscle cells has been reported (Clément et al. 2002). In addition, α-tocopherol induced the activity of phosphoserine–threonine phosphatase (PP2A) and phosphotyrosine phosphatases (PTP) Zingg (2007). Taken together, these findings from the animal system suggest that α-tocopherol has various non-antioxidant functions. However, in plants, most studies on the physiological role of tocopherols and the underlying regulatory mechanism focus on their antioxidant property, while a possible non-antioxidant role in plants is still very poorly understood.

Here, we investigated for the first time the effect of α-tocopherol on the activation of stimuli-induced MAPKs in plants. In a tobacco suspension of culture cells and leaves, pretreatment with α-tocopherol interferes with stress-induced MAPK activation, resulting in lower MAPK activity. Comparison of the effect of α-tocopherol analogue, α-tocopherol phosphate, with the effect of α-tocopherol per se revealed that a small modification of α-tocopherol caused a change of physiological function, and the inactivation of elicitor-induced MAPKs might be unrelated to the antioxidant property of α-tocopherol. Our results support the suggestion that α-tocopherol plays a role in the regulation of signal transduction pathways in response to stress-related stimuli in plants.

Materials and methods

Treatment of BY2 cells and tobacco

BY2 cells were sub-cultured for 1 week in MS medium supplemented with 2 g l^{-1} MES, 100 mg l^{-1} inositol, 30 g l^{-1} sucrose, 200 mg l^{-1} KH_2PO_4, thiamine, and 2,4-D. Five-day-old BY2 cells were treated with 50 μM of α-tocopherol, α-tocopherol phosphate and/or 100 μg ml^{-1} of E-Fol. At the time course, 10 ml of cells were harvested by filtration. Fourth or fifth leaves of *Nicotiana benthamiana* were infiltrated with 300 μl of 50 μM solution of α-tocopherol by using a sterile 1-ml needleless syringe. A sample of 10 mg ml^{-1} of stock solution α-tocopherol was prepared in acetonitrile and diluted with water. Mock treatments were performed by infiltration of H_2O as control. After 1- and 5-h treatments with α-tocopherol, the leaf of tobacco was lightly pressed 3 times by a gloved hand. The samples were quickly frozen in liquid nitrogen and stored at −80°C until analysis.

Protein extraction

Proteins were extracted by grinding samples in extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-Glycerophophate, 10% glycerol, 0.1 mM PMSF, and 1 mM Benzamidin). After centrifugation at 20,000*g* for 10 min, supernatants were transferred into new tubes, and stored at −80°C. The concentration of protein extracts was determined according to the Bradford method (Bradford 1976) with bovine serum albumin as a standard.

MAPK assays and inhibitor study

In-solution assay and in-gel kinase assay were performed as described by Zhang and Klessig (1997) and Link et al. (2002). To investigate the inhibition effect of tocopherol, 1 mg/ml α-tocopherol in acetonitril was diluted in water to a final concentration of 50 μM in the in-solution assay.

Results

α-tocopherol does not induce the activation of MAPKs

Elicitor-treated cell cultures have been successfully used to study plant defense reactions including signal transduction by MAPKs. Our previous studies showed that an elicitor preparation of the wilt-inducing fungus *Fusarium oxysporum lycopersici*, referred to as E-Fol, resulted in strong MAPK activation in photo-autotrophic cultures of tomato and *C. rubrum* (Ehness et al. 1997; Link et al. 2002). To examine whether α-tocopherol and α-tocopherol phosphate (TPh), a phosphoric acid ester of α-tocopherol, are able to induce activation of MAPKs, BY2 suspension culture cells were treated with a low concentration of 50 μM of α-tocopherol, TPh or 100 μg ml^{-1} of E-Fol elicitor as positive control. MAPK activity was analyzed by in-solution assay using myelin basic protein (MBP) as an artificial substrate. E-Fol induced the rapid and transient activation of MBP phosphorylating protein kinases in the BY2 suspension culture cells, with a maximum activity at 10 min. Similarly, protein kinases were transiently activated 5 min after treatment with 50 μM of TPh and were found to be deactivated approximately 30 min after treatment (Fig. 1). In contrast, α-tocopherol did not induce the activation of the MBP phosphorylating activity in the BY2 cell culture (Fig. 1). Slight activation of protein kinase observed for α-tocopherol treatment within 5 min was also observed for treatment of acetonitrile, the solvent used for α-tocopherol (data not shown).

α-tocopherol inhibits the activation of elicitor-induced MAPKs

To assess the role of α-tocopherol in stress-induced signal transduction pathways, we studied the effect of α-tocopherol pretreatment on elicitor-induced MAPK activation. For this purpose, BY2 cells were pre-incubated with 50 μM of α-tocopherol for different time periods ranging from 20 to 300 min. Pre-incubated BY2 cells were then challenged by addition of the E-Fol elicitor to induce the activation of MAPKs and MAPK activity which was analyzed both by in-solution and in-gel kinase assay. α-tocopherol was shown to inhibit the activation of MAPKs induced by E-Fol (Fig. 2a). Pre-incubation of BY2 cells with α-tocopherol for 20 and 40 min resulted in a reduction of MAPK activity with more pronounced effects at later time poinst, whereas the treatment of E-Fol together with α-tocopherol did not change the activation pattern of elicitor-induced MAPK activation (Fig. 2b). Interestingly, the inhibitory effect on MAPK activation was transient and dependent on the pre-incubation time with α-tocopherol. The inhibitory effect was reduced with increasing pre-incubation times of 60 and 300 min. MAPK activity was also analyzed by in-gel kinase assay using crude extracts from BY2 cells treated with E-Fol and BY2 cells which were pre-incubated in the presence of α-tocopherol for 40 min and then stimulated by E-Fol. The strong activation of MAPKs observed upon stimulation of E-Fol was also found to be inhibited in the in-gel kinase assay when the BY2 cells were pre-incubated with α-tocopherol (Fig. 2c), supporting the suggestion that the inhibited MBP phosphorylating activity corresponds to MAPK activity. To investigate whether inhibitory effect of α-tocopherol on MAPK activation is mediated by an anti-oxidant function, the BY2 cells were pre-incubated with 50 μM of TPh or ascorbate. No inhibition of the activity of MAPKs was observed after different pre-incubation times with TPh or ascorbate before elicitation with 100 μg ml^{-1} of E-Fol (Fig. 2a), indicating that the inactivation of stimuli-induced MAPKs by α-tocopherol might be independent of antioxidant pathways.

To analyze the α-tocopherol effect on stress induced MAPK activity in-planta, 300 μl of a 50-μM stock solution of α-tocopherol were infiltrated into mature tobacco leaves. After 1 and 5 h pre-incubation in the presence of tocopherol, activation of MAPKs was induced by mechanical wounding. The scratched leaves were sampled 5 min post-wounding and analyzed for MAPK activation. The MAPK activity was strongly induced by wounding in the tobacco control leaves infiltrated with H_2O while the induction if MAPK activity in the leaves pre-infiltrated with α-tocopherol was greatly reduced (Fig. 2d). Wounding alone leads to fast and transient activation of MAPK in tobacco leaves (Supplementary Fig. 1).

The results obtained both with elicitor-treated suspension culture cells and wounded leaves strongly indicated that accumulation of α-tocopherol can transiently inhibit the stimuli-induced signal transduction pathway mediated by MAPKs.

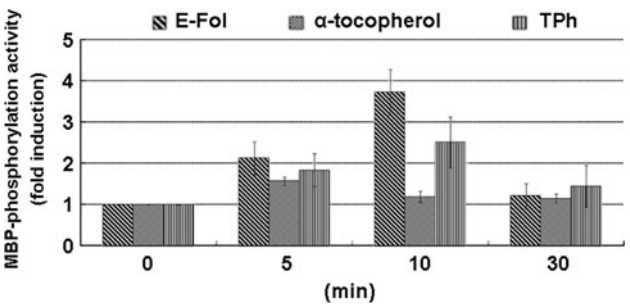

Fig. 1 Rapid and transient activation of MAPKs by E-Fol but not by α-tocopherol. The MBP protein kinase activity was analyzed by in-solution assay. BY2 cells were treated with 100 μg ml^{-1} of E-Fol elicitor, 50 μM α-tocopherol phosphate (TPh) or α-tocopherol. MBP phosphorylation activity is shown as the fold induction of activity, with the activity from the non-treated sample (time point '0') set equal to 1. *Bars* mean ± SD of three replicates

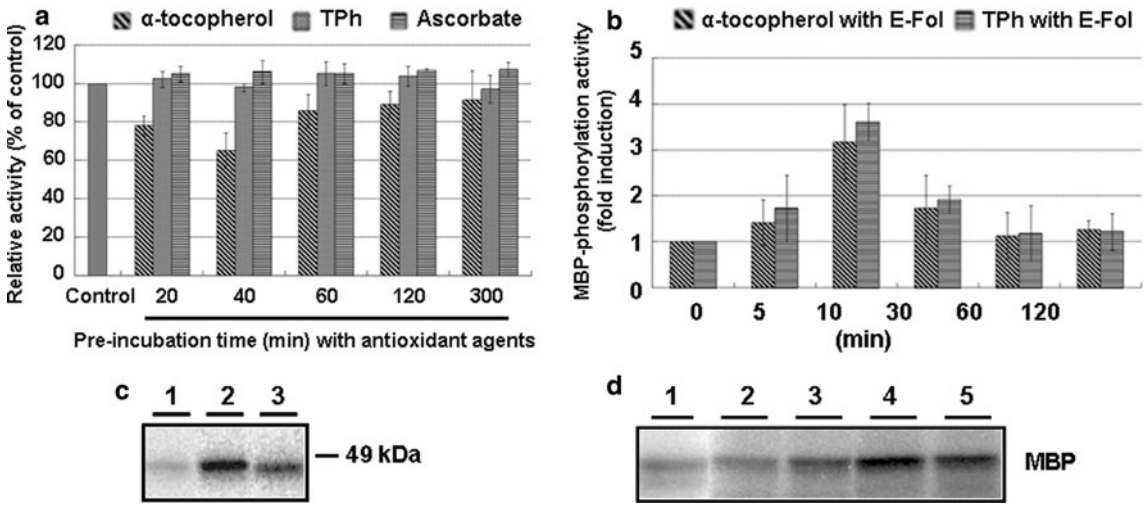

Fig. 2 Effect of α-tocopherol on elicitor-induced MAPK activity. **a** Pre-incubation of BY2 cells with α-tocopherol resulted in a reduction of MAPK activity. BY2 cells were pretreated with 50 μM of *α-tocopherol*, α-tocopherol phosphate (*TPh*) or *ascorbate* for 20, 40, 60, 120 and 300 min, and then treated with E-Fol for 10 min. The activity was quantified as described in Fig. 1 and is depicted as percentage of control (activity of stimuli-induced MAPK without the addition of agents). *Bars* mean ± SD of three replicates. **b** The activation pattern of E-Fol-induced MAPK did not change in the presence of α-tocopherol or TPh. BY2 cells were treated with 100 μg ml^{-1} of E-Fol elicitor together with 50 μM TPh or 50 μM α-tocopherol. *Bars* mean ± SD of three replicates. **c** MAPK activity was analyzed by in-gel kinase assay. BY2 cells were pretreated with 50 μM α-tocopherol (*lane 3*) or mock (*lane 2*) for 40 min, and then treated with E-Fol for 10 min. Cells without any treatment were considered as control (*lane 1*). **d** Wounding-induced MAPK activities were reduced by pre-incubation with α-tocopherol in tobacco plants. Tobacco leaves were infiltrated with 50 μM of α-tocopherol, pretreated for 1 h (*lane 2*) and 5 h (*lane 3*), then wounded and further incubated for 5 min. *Lanes 4* and *5* indicate the treatment with mock for 1 and 5 h, respectively, then wounded, and further incubated for 5 min. Cells without any treatment were considered as control (*lane 1*). The MBP protein kinase activity was analyzed by in-solution assay. All experiments were repeated at least three times with similar results

α-tocopherol neither affects MAPKs directly nor induces any MAPKs inhibitory or inactivating component

To investigate whether α-tocopherol directly inhibits MAPK activity, the crude extract from BY2 cells treated with E-Fol was incubated with reaction buffer containing 50 μM of α-tocopherol or 1 μM of straurosporine, a non-specific inhibitor of many Ser/Thr protein kinases. Staurosporine strongly inhibited the MAPK activity, but α-tocopherol did not show any inhibitory effect on MAPK activity (Fig. 3a). This finding suggests that the reduction of MAPK activity by α-tocopherol is an indirect effect on stimuli-induced signal transduction pathways.

The indirect effect of α-tocopherol on MBP-phosphorylation activity prompted us to investigate whether the inhibitory effect of α-tocopherol is mediated by an increased activity or level of a MAPK inhibitory component such as phosphatases. A sample of 20 μg of crude protein extract from BY2 cells treated with E-Fol was incubated at room temperature for 20 min with the same amount of crude extract prepared from α-tocopherol pre-incubated samples or extraction buffer alone. The E-Fol-activated MAPK was not inhibited by incubation with crude extracts from α-tocopherol pre-incubated plant samples, ruling out the induction of any MAPK inhibitory

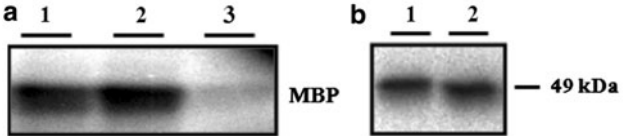

Fig. 3 α-Tocopherol does not inhibit the activity of MAPKs, and does not induce any MAPK inhibitory or inactivating component. **a** α-tocopherol does not inhibit the activity of MAPKs. A sample of 5 μl of crude extract from E-Fol treated cells was incubated in vitro in a reaction buffer containing 50 μM of α-tocopherol (*lane 2*), 10 μM of staurosprine (*lane 3*) or mock (*lane 1*) at room temperature (RT) for 20 min. The MBP-phosphorylation activity was analyzed by in-solution assay. **b** α-tocopherol does not induce any MAPK inhibitory or inactivating component. Crude extract from E-Fol-treated cells was incubated with the same amount of crude extract from α-tocopherol-treated samples (*lane 2*) or extraction buffer alone (*lane 1*) at RT for 20 min. The activity of MAPKs was analyzed by in-gel kinase assay. All experiments were repeated at least three times with similar results

or inactivating component by α-tocopherol (Fig. 3b). These data strongly indicate that the α-tocopherol negatively regulates an upstream component of the signaling pathway leading to elicitor-dependent MAPKs activation.

Discussion

Tocopherols are lipophilic antioxidants that are synthesized exclusively in photosynthetic organisms. In higher plants,

both the level and ratio of α- and γ-tocopherol are under spatial and temporal control (Sattler et al. 2006). A number of studies suggest that the accumulation of α-tocopherol is related to stress tolerance, and a reduction is associated with stress susceptibility (Gossett et al. 1994; Leipner et al. 1999; Munné-Bosch and Alegre 2000). However, knock-down of γ-tocopherol methyltransferase, which catalyzes the conversion of γ-tocopherol into α-tocopherol, causes an accumulation of γ-tocopherol instead of α-tocopherol, and results in increased tolerance to osmotic stress and decreased tolerance to salt stress (Abbasi et al. 2007). These findings indicate the complexity of interplay between tocopherol including α- and γ-tocopherols and various stress responses, and suggest that tocopherol may interfere with the complex signaling pathways which are involved in plant responses to stimuli. However, tocopherol function in regulating stress-induced signaling pathways in plants is still largely unknown.

Here, we investigated the function of α-tocopherol in stress stimuli-induced signal transduction pathways mediated by MAPKs both in tobacco suspension culture cells and leaves. Pre-incubation with a low concentration of α-tocopherol resulted in strong inhibition of stimuli-induced MAPK activation (Fig. 2b, d). The α-tocopherol effect on the activation of elicitor-induced MAPKs was transient. In the BY2 cells, 40 min pre-incubation period proved to have the strongest effect, while shorter or longer pre-incubation times such as 20 or 300 min with α-tocopherol before E-Fol stimulation yielded less inhibition of activation of MAPKs. Furthermore, control experiments reveal that the observed inhibition of MAPK activity is neither a direct effect of α-tocopherol nor due to the activation of an inhibitory or inactivating compound such as a phosphatase during the stress-response in plants (Fig. 3a, b).

In mammalian cells, vitamin E, including α-tocopherol, has been shown to have a direct and also indirect effect on several enzymes involved in intercellular signaling. α-tocopherol has been shown to inhibit PKC activity and extracellular signal-regulated kinase (ERK) activation in smooth muscle cells (Clément et al. 1997), and activation of ERK, p38 and NF-kB in lung epithelial cells (Ekstrand-Hammarstroem et al. 2006). A similar inhibitory effect on ERK-1 activation has been shown in human erythroleukemia cells (HEL) but not another cell lines like human monocyte tumor cell line (U937) (Breyer and Azzi 2001). Vitamin E differentially affected the activation of distinct MAPK in response to copper treatment (Mattie et al. 2008). Contrary to the above effects, α-tocopherol induces the activation of ERK for increasing survival in response to H_2O_2 (Numakawa et al. 2006). These findings indicate that the effect of α-tocopherol on signaling pathways in animals is variable and determined by the type and degree of the stress stimulus, and by cell type and developmental stage.

Reactive oxygen species or the resulting lipid peroxidation products may act as a second messenger in eukaryotes to induce MAPKs (Thoma et al. 2003; Matsuzawa and Ichijo 2008). Even the early signaling events induced by elicitors, like activation of MAPKs, regulate and generate directly or indirectly the diverse signaling molecules including Ca^{+}, nitric oxide and ROS which interconnect a lot of branch pathways (Garcia-Brugger et al. 2006). This finding indicates that the early stage of activation MAPKs by elicitors is not mediated by ROS and NO production (Besson-Bard et al. 2008; Dahan et al. 2009). In tomato, an elicitor preparation leads to MAPKs activation, and results in the accumulation of H_2O_2 and induction of defence gene expression (Link et al. 2002), whereas the exogenous application of H_2O_2 or NO in tobacco and *Arabidopsis* cells activates MAPKs (Clarke et al. 2000; Kumar and Klessig 2000; Pitzschke and Hirt 2006). These findings suggest that the signaling pathway leading to H_2O_2 might be separate from the early response pathway mediated by elicitor-induced MAPKs, and the H_2O_2 generated can then feed into the MAPK pathway (Yang et al. 2001). The data reported in the present study indicate that α-tocopherol may negatively regulate the early stage of activation of MAPK cascades by elicitors in higher plants. The observation that α-tocopherol phosphate, though having a similar structure and anti-oxidant activity as α-tocopherol (Rezk et al. 2004), does not affect elicitor-dependent MAPK activation further proves the specificity of the inhibitory effect of α-tocopherol as being unrelated to the anti-oxidant potential (Fig. 2c).

The inhibitory effect of α-tocopherol on stress-induced signal pathways raises the question of the molecular mechanism of α-tocopherol inhibition of MAPK activity. Our data suggest that α-tocopherol directly or indirectly interferes with upstream signaling components such as MAPKKs and MAPKKKs, or even upstream of the MAPK cascade, subsequently resulting in reduced activation of MAPKs. It is neither inhibiting activated MAPKs directly in an in-solution assay (Fig. 3a) nor inducing any inhibitory or inactivating component in the treated cells (Fig. 3b). In contrast, in mammalian cells, α-tocopherol induces protein phosphatase activity. For example, α-tocopherol up-regulates the activity of phospho-serine/threonine phosphatase 2A (PP2A) in mouse BV-2 microglial cells (Egger et al. 2001), and phospho-tyrosine phosphatase (PTP) in vascular smooth muscle cells (Frank et al. 2000) and human neutrophils (Chan et al. 2001). These data suggest that the effect on the inactivation of MAPKs by α-tocopherol in plants could be due to down-regulation of upstream signaling events via activation of protein phosphatases. Taken together, we conclude that inhibition of MAPK activity by α-tocopherol is not due to a direct interaction, but rather the result of interference with epistatic components in the signal transduction pathways.

Our study demonstrates for the first time that α-tocopherol may negatively interfere with stress signaling in plants, and suggests that the inhibitory effect on MAPK activation might be related to a novel non-antioxidant function for α-tocopherol. An important issue to be addressed in the future will be to elucidate the molecular target of α-tocopherol action within the complex plant stress signaling network.

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