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Biochemistry

Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells

(tomato cells/protein kinase/K-252a)

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ABSTRACT **Plant cells have an acute sense for pathogen-derived chemical stimuli, so-called elicitors, which induce the plant's defense response. To investigate the molecular basis of chemosensory transduction, elicitor-treated tomato cells were labeled with 1-min pulses of [^{32}P]phosphate. This technique revealed drastic changes in protein phosphorylation *in vivo* within minutes of stimulation. The protein kinase inhibitors K-252a and staurosporine completely prevented these elicitor-induced changes in protein phosphorylation. They also blocked two early biochemical responses to elicitors, extracellular alkalinization and biosynthesis of ethylene. The ability of K-252a, staurosporine, and benzoylated staurosporine derivatives to inhibit elicitor responses *in vivo* correlated with their ability to inhibit tomato microsomal protein kinase *in vitro*. When K-252a was given to elicited cells 1 min after the [^{32}P]phosphate, the radioactivity in certain newly labeled phosphoprotein bands disappeared again within minutes. This correlated with an arrest of alkalinization within minutes when K-252a was applied in midcourse of elicitation. These data show that phosphorylation of protein substrates by K-252a-sensitive protein kinases is essential for transduction of elicitor signals in plant cells and that continuous phosphorylation of these proteins is required to maintain the elicited state.**

Plant cells have a sensitive perception system for chemical signals derived from fungi, so-called elicitors. They react to elicitor stimulation with a concerted biochemical defense response (1), including changes in membrane properties (2), enhanced production of the stress hormone ethylene (3), and transcriptional activation of the genes encoding enzymes involved in phenylpropanoid metabolism (1, 4). There is evidence for elicitor-binding proteins in the plasma membrane (5) but the pathway for transduction of the elicitor signal is unknown (1, 6). Protein phosphorylation plays a central role for chemosensory transduction in bacteria (7) as well as in eukaryotic microorganisms and animals (8). In plant cells, too, protein kinases have been identified by biochemical methods (for review, see ref. 9) and homology probing (10–12), and changes in the phosphorylation status of specific proteins (13, 14) and protein kinase activities (15–17) have been observed upon treatment with elicitors or related chemicals. The functional significance of these changes, however, is unknown.

We recently found that K-252a, a known inhibitor of animal protein kinases (18), blocks two characteristic responses of tomato cells to elicitors—namely, induction of ethylene biosynthesis and phenylalanine ammonia-lyase (19). Control experiments showed that K-252a was a potent inhibitor of the histone III phosphorylating protein kinase in tomato microsomes and that it had little effect on protein synthesis or growth and metabolism (19). Here we use K-252a and related protein kinase inhibitors to establish a functional link between protein phosphorylation and signal transduction in the elicitor response. We show drastic changes in the pattern of protein phosphorylation within minutes of elicitation, and we demonstrate that K-252a prevents these changes and reverses them within minutes. We further show that K-252a blocks the earliest biochemical responses to elicitors with similar kinetics, even in midcourse of elicitation.

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MATERIALS AND METHODS

Plant Materials and Elicitors. Suspension cultures of tomato cells, line Msk8 (kindly supplied by M. Koornneef, Department of Genetics, Agricultural University, Wageningen, The Netherlands), were grown in a Murashige–Skoog type medium as described (20). Cells were used for experiments 7–10 days after subculture. An elicitor preparation (yeast extract-derived elicitor, YE) was obtained by dialysis and partial purification (20) from yeast extract (Difco).

Extracellular Alkalinization. Cells (*ca.* 0.2 g of fresh weight in a 2-ml suspension) were incubated in open vials on a shaker. The extracellular pH was continually registered with a glass pH electrode; in different experiments, the initial pH varied between 5.3 and 6.0. K-252a was added from stock solutions in dimethyl sulfoxide (DMSO) to yield a final concentration of 0.4 μM (with <0.5% DMSO). DMSO (up to 1%) had no effect on elicitor reactions (19).

Rate of Ethylene Production. Cells (0.2 g of fresh weight in 2 ml of medium) were enclosed in 5-ml syringes capped with a rubber septum and incubated on a shaker at room temperature. At 30-min intervals, gas samples (1 ml) were withdrawn and assayed for ethylene by gas chromatography (20). The syringe with the cells was quickly aerated with fresh air and recapped for the next incubation period.

Assay of Protein Kinase in Tomato Microsomes. Microsomal preparations were isolated as described (19) from tomato cell cultures not treated with elicitors. The protein kinase activity in microsome suspensions was assayed in the presence of various concentrations of K-252a, staurosporine, or its benzoylated derivatives (21) using histone III as substrate (19).

Pulse Labeling of Phosphoproteins. Aliquots of cells (70 mg in 0.3 ml) were added to carrier-free inorganic [^{32}P]phosphate (10 μCi; 1 Ci = 37 GBq) and incubated for 60 sec, if not otherwise stated, before adding 0.3 ml of 10% trichloroacetic acid containing 10 mM ATP and freezing immediately in liquid nitrogen. Samples were thawed in a sonicator bath and centrifuged for 2 min at 10,000 × *g*. Pellets were washed with 80% acetone and extracted with 100 μl of sample buffer (22) at 95°C for 5 min. After centrifugation, 30 μl of the supernatants (containing equal amounts of protein and on average 24 nCi of radioactive phosphorus) were subjected to SDS/PAGE (22) and autoradiography. Molecular mass standards were rabbit muscle phosphorylase b (97.4 kDa), bovine

Abbreviations: YE, yeast extract-derived elicitor; DMSO, dimethyl sulfoxide.

serum albumin (66.0 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20.1 kDa).

RESULTS

The effects of elicitors on ion flux changes across the plasma membrane, comprising increased influx of Ca^{2+} and H^+ and efflux of K^+, have been described and proposed to be part of the signal transduction chain (2), but evidence for this is lacking. We studied the most easily measurable consequence of these changes—namely, extracellular alkalinization—and found that elicitor treatment induced a rapid increase of the extracellular pH value after a lag time of 1–2 min (Fig. 1). This response was completely blocked by a brief pretreatment of the cells with K-252a (Fig. 1). When K-252a was added in midcourse of the elicitor response, the pH continued to increase for 20–40 sec before stopping abruptly (Fig. 1).

Similar characteristics of inhibition were observed for ethylene biosynthesis, a slower and more prolonged response of the cells to elicitor treatment. When added simultaneously with the elicitor, K-252a completely blocked induction of ethylene biosynthesis (Fig. 2). Addition of K-252a in midcourse of the elicitor response immediately prevented any further increase in the rate of ethylene production (Fig. 2).

K-252a is a glycosylated indole carbazole alkaloid related to staurosporine (18). Benzoylated derivatives of staurosporine have been used to test the specificity of protein kinase inhibition in animal tissues (21). We compared K-252a and staurosporine with two derivatives that were shown to be active and inactive as inhibitors of animal protein kinases

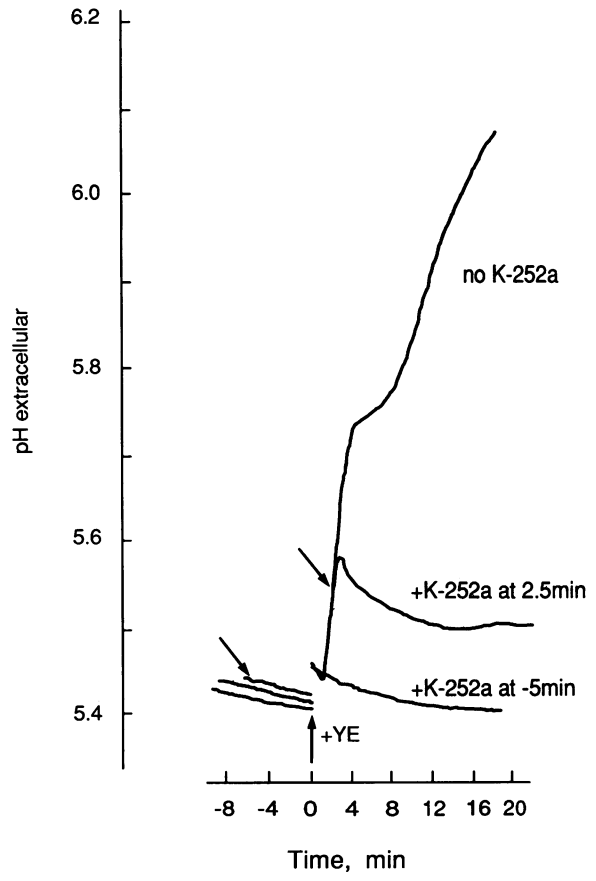

FIG. 1. Effect of K-252a on elicitor-induced extracellular alkalinization of tomato cells. Addition of elicitor (YE), 10 $\mu g \cdot ml^{-1}$, at zero time caused a small increase (*ca.* 0.05 pH unit) of extracellular pH, as shown by the discontinuity in the traces. Where indicated, samples received 0.4 μM K-252a at the times indicated by arrows.

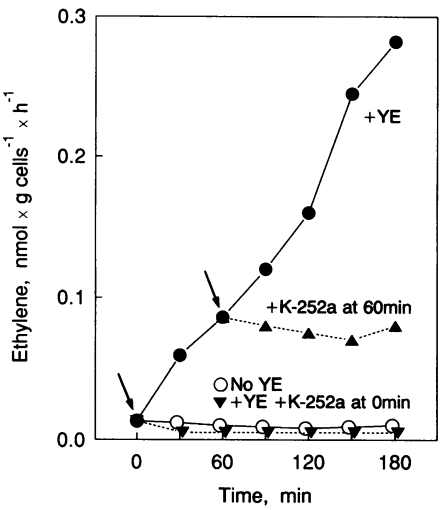

FIG. 2. Effect of K-252a on elicitor-induced ethylene biosynthesis in tomato cells. Cells were treated with elicitor (YE), 5 $\mu g \cdot ml^{-1}$, at zero time (closed symbols) or did not receive elicitor (controls, open symbols). Where indicated, samples received 0.4 μM K-252a at the times indicated by arrows.

(21). The dose–response curves for inhibition of elicitor-induced alkalinization (Fig. 3*A*) and ethylene biosynthesis (Fig. 3*B*) yielded ED_{50} values of *ca.* 50 nM for K-252a, *ca.* 200 nM for staurosporine, and *ca.* 1 μM for the active derivative CGP 41 251. No inhibition was observed at concentrations up to 100 μM for the inactive derivative CGP 42 700. When protein kinase activity was measured in tomato microsomes, a qualitatively similar pattern of inhibition was obtained with the same compounds (Fig. 3*C*).

These findings prompted us to look at protein phosphorylation in elicitor-stimulated cells. To label phosphoproteins, we took advantage of the rapid uptake of phosphate into plant cells. We found that regardless of the presence or absence of elicitor or K-252a, tomato cells took up >90% of added carrier-free [^{32}P]phosphate within 1 min and that about 0.8% of the added label was incorporated into the trichloroacetic acid-insoluble fraction within this period of rapid [^{32}P]phosphate influx. We therefore chose 1-min pulses of carrier-free [^{32}P]phosphate to study the process of protein phosphorylation *in vivo*. One-dimensional SDS/PAGE was sufficient to reveal drastic changes in the pattern of protein phosphorylation induced by elicitor: at least three new labeled phosphoproteins appeared between 0.8 and 3.3 min after elicitor treatment, with molecular masses of *ca.* 52, 28, and 22 kDa (Fig. 4). Other phosphoproteins, including a prominent one with a molecular mass of *ca.* 34 kDa, became visible after 5 min.

K-252a did not affect the basic pattern of pulse-labeled phosphoproteins in cells without elicitor but completely prevented the changes induced by the elicitor (Fig. 4). Similar results were obtained with staurosporine (unpublished data). These results demonstrate that rapid uptake or exchange of phosphate groups in specific polypeptides is an early consequence of elicitor action and that this process depends on protein kinases sensitive to staurosporine-type inhibitors.

To study the dynamics of the phosphate group exchange in these polypeptides, cells preincubated with elicitor for 6 min and prelabeled with [^{32}P]phosphate for 1 min were treated either with 500 nM K-252a or, as a control, with a corresponding amount of the solvent (Fig. 5). The bulk label in the phosphoproteins increased throughout both treatments. However, label in some of the bands induced in response to elicitor, including the ones with apparent molecular masses of 52, 28, and 22 kDa, increased little during the control treat-

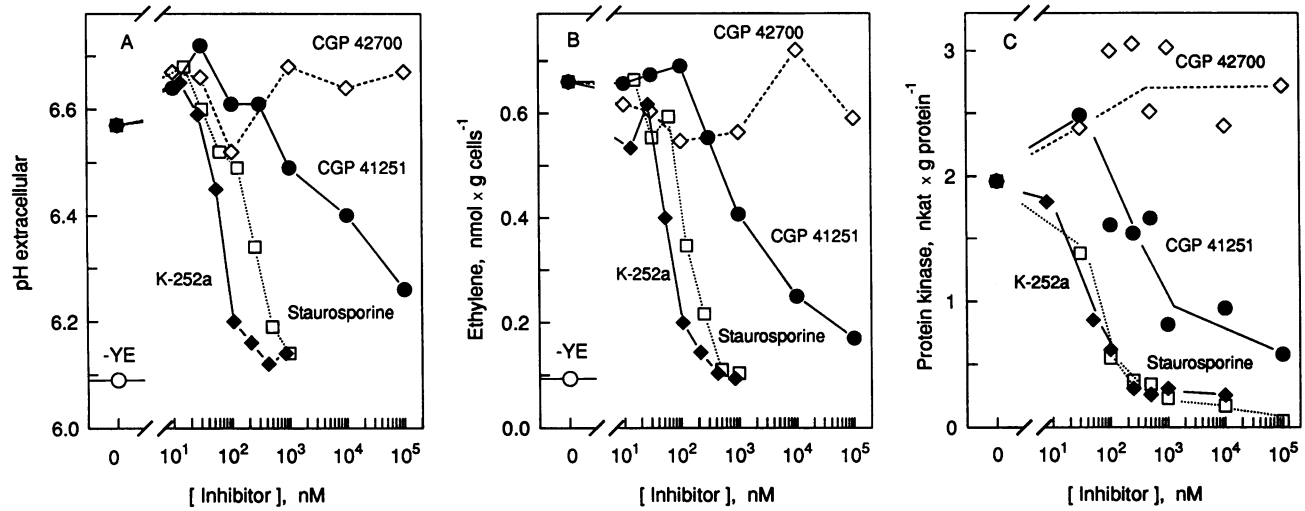

FIG. 3. Dose–response curves for the inhibition of elicitor-induced responses and microsomal protein kinase by K-252a (◆), staurosporine (□), and its benzoylated derivatives, CGP 41251 (●) and CGP 42700 (◇). (*A*) Extracellular pH in culture medium 18 min after treatment with elicitor. (*B*) Ethylene accumulation in the time between 20 and 120 min after elicitation. (*C*) Activity of protein kinase in isolated tomato microsomes. nkat, nmol of phosphoryl groups incorporated per sec.

ment. The same bands lost most radioactivity within 1–4 min after addition of K-252a, indicating that their phosphate groups are rapidly turning over and cannot be rephosphorylated in the presence of K-252a.

DISCUSSION

We have established a pulse-labeling technique to study *in vivo* protein phosphorylation in plant cells, based on their highly efficient phosphate transport system. It has been reported earlier that elicitors inhibit phosphate uptake in parsley cells (23). In our model system, most of the [^{32}P]phosphate was taken up into the cells within 1 min, regardless of the presence or absence of elicitor, and the γ-phosphate group of cellular ATP had its highest specific activity during this time (unpublished data). Thus, short pulses of radioactive phosphate are well suited to study the dynamics of ATP-dependent protein phosphorylation in plants.

Our technique reveals strong changes in the pattern of labeled phosphoproteins within minutes after elicitation. Three lines of correlative evidence indicate that at least some of these changes are involved in signal transduction.

(*i*) The initial changes in protein phosphorylation occur rapidly in the time between 1 and 2 min after addition of the elicitor. This correlates with the onset of alkalinization of the growth medium, an early sign of the elicitor response in the tomato cells employed. Alkalinization is probably an indicator of changes at the plasma membrane that might be involved in signal transduction (2, 6), and it precedes other typical early elicitor responses, such as induction of ethylene biosynthesis or induction of phenylalanine ammonia-lyase. Subsequent changes in protein phosphorylation, peaking 5–10 min after elicitation (Fig. 4) or even later, as those previously observed in other systems (13, 14), may be related more to the expression and modulation of the elicitor responses.

(*ii*) K-252a, a known inhibitor of protein kinases involved in signal transduction in animal systems (18), blocks the changes in protein phosphorylation (Fig. 4) as well as all early elicitor responses studied, including medium alkalinization (Fig. 1), ethylene biosynthesis (Fig. 2), and phenylalanine ammonia-lyase induction (19). In conjunction with the qualitatively similar effect of K-252a, staurosporine, and staurosporine derivatives on elicitor responses *in vivo* and on protein kinase activity *in vitro* (Fig. 3), this supports the notion that these staurosporine-type compounds act as specific protein kinase inhibitors also in plants. Furthermore, the results strongly suggest that the phosphorylation of protein

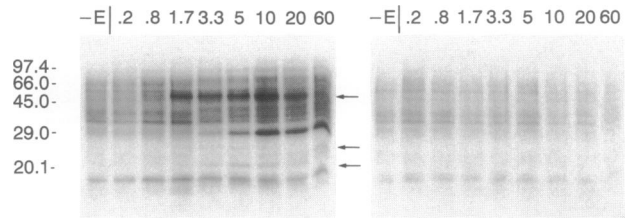

FIG. 4. SDS/PAGE of extracts from tomato cells pulse labeled *in vivo* with [^{32}P]phosphate for 60 sec after various times of elicitation (given in minutes above the autoradiograms). (*Left*) No pretreatment: addition of DMSO alone 5 min before the elicitor. (*Right*) Pretreatment with K-252a: addition of 0.5 μM K-252a 5 min before the elicitor. Lanes −E, no elicitor treatment. Arrows indicate bands that appear between 0.8 and 3.3 min of elicitor treatment. Molecular masses are indicated in kDa.

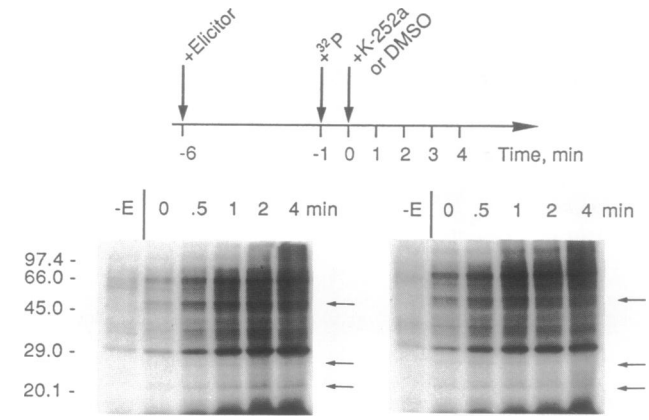

FIG. 5. SDS/PAGE of extracts from tomato cells labeled with [^{32}P]phosphate *in vivo*. Cells were labeled for 1 min in the absence of elicitor (−E) or for 1–5 min in its presence according to the scheme shown (*Upper*). One minute after the [^{32}P]phosphate labeling, the cells received either DMSO alone (control, *Lower Left*) or 500 nM K-252a in DMSO (*Lower Right*) for the time period stated above the autoradiograms. Arrows indicate labeled bands that appear in elicitor-pretreated cells and disappear upon K-252a treatment. Molecular masses are indicated in kDa.

substrates of K-252a-sensitive protein kinase(s) may be essential for signal transduction.

(*iii*) The radioactive phosphate groups disappear from the newly labeled phosphoproteins within minutes after addition of K-252a, even though bulk phosphoproteins continue to take up radioactive phosphate. This correlates well with the observation that K-252a blocks elicitor-induced medium alkalinization within 0.5–1.0 min when given in midcourse of elicitation. In combination, the two findings show that continuous protein phosphorylation is necessary for elicitation and that the phosphate groups in phosphoproteins important for signal transduction are turning over rapidly. Phosphoprotein phosphatases are an essential component of signal transduction mechanisms in vertebrate signal transduction pathways. These phosphatases, known to occur in plants (24), are probably responsible for the rapid reversion of the elicited state upon K-252a treatment.

In conclusion, our results provide strong, albeit indirect, evidence that rapid reversible phosphorylation of specific protein substrates is an essential component of signal transduction in elicitor-stimulated plant cells. Identification and characterization of the K-252a-sensitive protein kinases and their protein substrates are necessary to gain a better understanding of plant chemoperception.

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