Signal transduction during defense response and source-sink transition in tomato

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ABBREVIATIONS

ERF or EREBP	Ethylene responsive element binding factor / protein
ESI-MS	Electrospray ionisation mass spectroscopy
HR	Hypersensitive response
LRR	Leucine rich repeat
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MAP Kinase	Mitogen activated protein kinase
MAPK Kinase	Mitogen activated protein kinase kinase
MAPKK Kinase	Mitogen activated protein kinase kinase kinase
MEKK	MAPKK kinase (*Arabidopsis* nomenclature)
MKK	MAPK kinase (*Arabidopsis* nomenclature)
MMK	Medicago MAP kinase
MPK	Mitogen activated protein kinase (*Arabidopsis* nomenclature)
PAL	Phenylalanine ammonia-lyase
PR	Pathogenesis related
Ser, S	Serine
SIPK	Salicylic acid induced protein kinase
Thr, T	Threonine
TMV	Tobacco mosaic virus
Tyr, Y	Tyrosine
WIPK	Wounding induced protein kinase

SUMMARY

Plants have evolved an elaborate system to cope with a variety of biotic and abiotic stresses. Typically, under stress conditions an appropriate defense response is invoked which is accompanied by changes in the metabolic status of the plant. Photosynthesis is downregulated and sucrose is imported into the tissue, which provides a faster and more constant flux of energy and carbon skeletons to perform the defense response. Interestingly, these processes are co-ordinately regulated and the signal transduction chains underlying these cellular programs appear to share at least some common elements. Both the induction of sink metabolism and defense response is dependent on signal transduction pathways involving protein phosphorylation. Furthermore, regulation of extracellular invertase (INV) and phenylalanine ammonia lyase (PAL) which are markers for sink metabolism and defense response is preceded by the transient activation of MAP kinases.

In depth analysis of MAP kinase activation by partial purification led to the discovery that, depending on the stimulus, different subsets of MAP kinases are activated. This differential MAPK activation is likely to possess a signal encoding function. In addition, the partial purification of MAP kinases was found to be suitable to address specific cellular functions to individual MAP kinase isoenzymes. By this way, LpWIPK was identified as the major MAP kinase activity induced after stimulation of tomato cells with different elicitors. LpWIPK is thus considered as a key regulator of defense response together with sink induction in tomato. A study using nonmetabolisable sucrose analogs revealed that the regulation of photosynthesis is not directly coupled to this signal transduction pathway since it is independent of MAP kinase activation. Nonetheless, downregulation is induced by the same stimuli that induce the defense response and sink metabolism and it will therefore be interesting to uncover the branch points of this signalling network in the future.

MAP kinases are not only central components regulating the response to biotic stresses. In addition to e.g. pathogens, MAP kinases are as well involved in signal transduction events invoked by abiotic stresses like cold and drought. In a recent study,

we could show that a MAP kinase is activated by heat stress, under conditions a plant will encounter in nature. This previously unknown MAP kinase is able to specifically recognise the heat stress transcription factor HsfA3 as a substrate, which supports a role of this MAP kinase in the regulation of the heat stress response. Moreover, the observation that HsfA3 is phosphorylated by the heat activated MAP kinase *in vitro* provides a promising basis to identify HsfA3 as the first physiological substrate of a plant MAP kinase.

Intracellular protons have been implicated in the signal transduction of defense related signals. In a study using *Chenopodium rubrum* cells, we could show that cytosolic changes in pH values do not precede the regulation of the marker genes INV and PAL. Depending on the stimulus applied, cytosolic acidification or alkalinisation can be observed, which excludes a role for protons as signals in this pathway. Together with the concomitant changes of the pH value of the extracellular space, these variations can thus be considered as terminal part of the defense response itself rather than as a second messenger.

WRKY transcription factors have only recently been identified as indirect targets of a central plant MAP kinase cascade. In addition, the identification of cognate binding sites in the promoters of INV and PAL supports a role for these proteins in the co-ordinate regulation of defense response and sink induction. A novel elicitor responsive WRKY transcription factor, LpWRKY1, was cloned from tomato and characterised with respect to its posttranslational modification. This immediate early transcription factor is transiently induced upon pathogen attack and the induction is dependent on phosphorylation. Furthermore, it was shown for the first time with respect to WRKY transcription factors, that LpWRKY1 is phosphorylated *in vivo*. Analysis of the role of this phosphorylation by *in gel* assays using recombinant WRKY protein as the substrate revealed two protein kinases that are transiently activated during the defense response to phosphorylate LpWRKY1. This data demonstrates that WRKY proteins require phosphorylation to modulate their DNA binding or transactivating activity.

ZUSAMMENFASSUNG

Pflanzen haben ein aufwendiges System entwickelt um auf verschiedene Umweltreize zu reagieren. Meist wird unter Stressbedingungen ein passendes Abwehrprogramm aktiviert. Gleichzeitig wird die Photosynthese im jeweils betroffenen Gewebe abgeschaltet und stattdessen Saccharose importiert. Dieser Source-Sink Übergang stellt sicher, dass Energie und Kohlenstoffbausteine für die Abwehr schnell zur Verfügung stehen. Diese beiden Prozesse sind koordiniert reguliert und die zugrundeliegenden Singnaltransduktionswege scheinen wenigstens einige Komponenten gemeinsam zu haben. Sowohl die Induktion des Sink Metabolismus (gemessen an der Induktion der extrazellulären Invertase, INV) als auch des Abwehrprogramms (gemessen an der Induktion der Phenylalanin Ammonia-Lyase, PAL) sind von Phosphorylierungen abhängig. Außerdem geht der Induktion beider Gene die transiente Aktivierung von MAP Kinasen voran. Eine genauere Analyse der MAP Kinase Aktivierung durch partielle Reinigung zeigte, dass abhängig vom Stimulus mehrere MAP Kinasen aktiviert werden. Diese differentielle MAP Kinase Aktivierung stellt somit eine Möglichkeit der Signalcodierung dar. Die partielle Reinigung der MAP Kinasen wurde auch verwendet, um einzelnen MAP Kinase Isoenzymen spezifische zelluläre Funktionen zuzuweisen. Dadurch konnte LpWIPK (wound induced protein kinase) als Hauptaktivität nach Stimulation on Tomatenzellen mit Elicitoren bestimmt werden. LpWIPK könnte also sowohl die Pathogenabwehr als auch den source-sink Übergang gleichzeitig steuern. Allerdings ist die Regulation der Photosynthese unabhängig von diesem Signaltransduktionsweg. Eine Untersuchung mit nichtmetabolisierbaren Saccharose-Analoga zeigte, dass die Regulation der Photosynthese unabhängig von einer MAP Kinase Aktivierung stattfindet. Das abschalten der Photosynthese wird durch die gleichen Stimuli hervorgerufen, die auch Pathogenabwehr und Sink Metabolismus induzieren. Eine Interaktion der beiden Signaltransduktionswege ist somit wahrscheinlich.

MAP Kinases spielen nicht nur bei der Antwort auf biotischen Stress eine wichtige Rolle. Zusätzlich werden MAP Kinasen auch durch abiotischen Stress wie

Kälte und Dürre aktiviert. Kürzlich konnten wir zeigen, dass Hitzestress unter natürlichen Bedingungen ebenfalls eine MAP Kinase aktiviert. Diese bisher unbekannte MAP Kinase akzeptiert den Hitzestress Transkriptionsfaktor HsfA3 als *in vitro* Substrat. Dies unterstützt die Vermutung, dass diese MAP Kinase eine Rolle in der Regulation der Hitzestress Antwort spielt. Außerdem stellt die Beobachtung, dass die hitzeaktivierte MAP Kinase HsfA3 *in vitro* phosphoryliert eine vielversprechende Ausgangssituation dar, um HsfA3 als erstes physiologisches MAP Kinase Substrat in Pflanzen zu identifizieren.

Intrazelluläre pH Änderungen wurden als Komponenten der Signaltransduktion für die Pathogenabwehr diskutiert. In Zellen von *Chenopodium rubrum* konnten wir zeigen, dass solche Änderungen nicht in Zusammenhang mit der Induktion der beiden Markergene INV und PAL stehen. Die Änderung des intrazellulären pH Wertes erfolgt nach der Induktion der Markergene, und kann je nach Stimulus Alkalisierung oder Ansäuerung zur Folge haben. Diese Beobachtungen schließen eine Rolle der pH Änderungen in der Signaltransduktion von Stressreizen aus. Die gefundenen pH Wert Änderungen sind somit eher als Teil der Pathogenabwehr und nicht als vorgelagerte Komponente zu verstehen.

WRKY Transkriptionsfaktoren wurden erst kürzlich als indirekte Ziele einer MAP Kinase Kaskade beschrieben. Außerdem finden sich in den Promotoren von INV und PAL Bindestellen für WRKY Transkriptionsfaktoren, was eine Beteiligung dieser Proteine an der koordinierten Regulation von Abwehr und Sink Metabolismus wahrscheinlich macht. Ein neuer WRKY Transkriptionsfaktor, LpWRKY1 wurde aus Tomate kloniert und in Hinblick auf mögliche posttranslationelle Modifikationen charakterisiert. Dieser Trankriptionsfaktor zählt zu den schnellen frühen Genen und wird in Antwort auf Elicitoren transient induziert. Die Induktion des Faktors in abhängig von Phosphorylierungen. Es konnte weiterhin erstmalig gezeigt werden, dass LpWRKY1 *in vivo* phosphoryliert wird. Eine weitere Analyse durch *in gel* Kinase Tests mit rekombinantem LpWRKY1 als Substrat zeigte, dass zwei Proteinkinasen während der Abwehrantwort transient aktiviert werden und LpWRKY1 phosphorylieren. Diese Daten legen nahe, dass die DNA Bindeaktivität oder die Transaktivierungsaktivität von WRKY Transkriptionsfaktoren durch Phosphorylierung gesteuert wird.

INTRODUCTION

Cells constantly sense their surrounding to obtain information that allows a rapid and adequate adjustment of the cells metabolism and biosynthetic activity to the current needs determined by the environment. The ability to receive information, to score it and to take appropriate actions is the basis for the survival of unicellular organisms in an ever-changing environment. In addition, the capability to receive signals and information from other cells and to react accordingly formed the basis for single cells to arrange into organs and organisms during evolution. The information provided can be complex: it ranges from the current pH value to temperature or light and from the availability and type of nutrients to hormones and growth factors. In most cases, the information is perceived by intra or extracellular receptors, which generate signals that finally result in a change of gene expression. However, the route such a signal takes in a cell is not linear. The information a cell obtains has to be integrated to result in an output that adequately fits the cells needs which is reflected by the fact that the same information might result in different outcomes depending on the cell type.

One prominent and central component that receives information from a membrane-bound receptor to transduce it to such components that regulate gene expression or other cellular changes is the MAP kinase module. MAP kinase modules are conserved from yeast to human and plants and the basic assembly consists of three protein kinases that determine the activity of a MAP kinase. MAP kinases are activated by dual phosphorylation of the Thr and Tyr residues in the tripeptide motif T-X-Y which is located in the activation loop (T-loop) between the subdomains VII and VIII. The activating phosphorylation of a MAP kinase is mediated by a MAPK kinase (MAPKK or MEK), which in turn is activated by phosphorylation by a MAPKK kinase (MAPKKK). The activity of the MAPKK kinase is determined either by

MAPKKK kinases (MAPKKKK) or by small GTP binding proteins which link the MAP kinase module to the receptor. The MAP kinase module thus creates a directional flow of information in which specificity is ensured by the interaction of the individual components of the module. However, the MAP kinase module also offers the possibility to integrate and differentiate information since some components of MAP kinase modules are interchangeable. It has been shown that some MAPK kinases and MAPKK kinases recognise and activate more than one specific substrate *in vivo* and can thus create crosstalk between individual MAP kinase cascades (Widmann et al., 1999; Zhang and Klessig, 2001). The recent completion of the *Arabidopsis* genome sequencing project revealed the existence 20 MAPKs which are subdivided into six groups (Zhang and Klessig, 2001), 10 MAPKKs and 60 MAPKKKs (Jonak et al., 2002) and similar numbers can be expected for other plant species. However, specific functions have been assigned to only few of these tentative sequences and only one MAP kinase cascade has been completely elucidated *in vivo* (Asai et al., 2002).

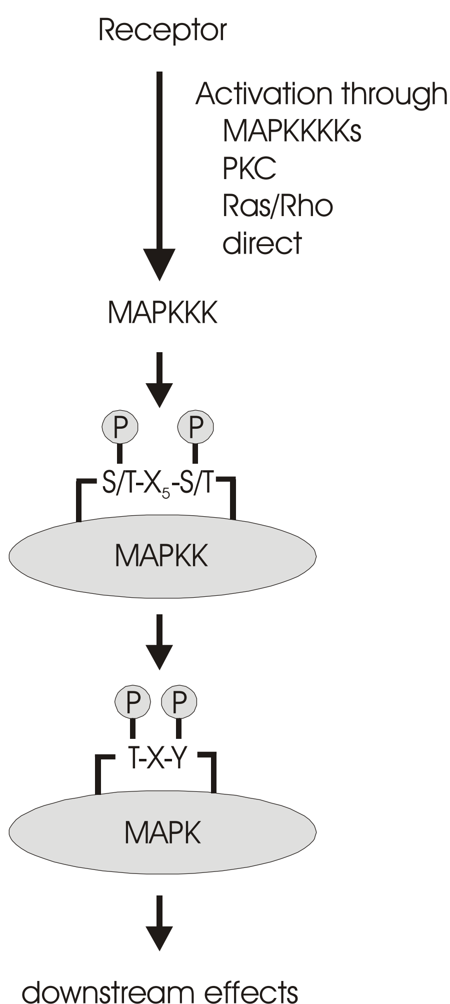

Figure 1: Schematic flow diagram of a MAP kinase pathway.
For specific information please refer to the text.

MAP KINASES IN PLANT DEFENSE SIGNALLING

Plants have evolved an elaborate system to cope with various biotic and abiotic stresses. As the picture emerged during the last ten years of research, it became clear that MAP kinases play a central role in the plants ability to defend themselves against pathogens (Hirt, 2000; Zhang and Klessig, 2001) and such severe stresses like wounding (Seo et al., 1995; Usami et al., 1995; Seo and Ohashi, 2000) and to adapt to environmental changes like UV-light stress (Stratmann et al., 2000; Kabuyaama et al., 2001), temperature stress (Jonak et al., 1996; Link et al., 2002a; Sangwan et al., 2002), salinity (Munnik et al., 1999), drought (Jonak et al., 1996) or ozone (Samuel et al., 2000). Adding up to this complex set of stimuli are endogenous signals like systemin (Stratmann and Ryan, 1997), salicylic acid (Zhang and Klessig, 1997) and phytohormones (Kovtun et al., 1998; Burnett et al., 2000; Mockaitis and Howell, 2000) that result in the activation of MAP kinases. To date, a complex picture has emerged that describes the concerted roles of different MAP kinases in the plants defence response.

Tools to study MAP Kinases

An intrinsic feature of MAP kinases is the possibility to assay their activity in *in gel* kinase assays. These assays involve de- and renaturation cycles with which other protein kinases apparently cannot be reconstituted. The *in gel* kinase assay thus provides a tool for an initial discrimination of kinase identity.

This assay not only provides information about the *in vivo* activity of a MAP kinase but also gives information on the molecular weight of the MAP kinase assayed. The identity of an individual MAP kinase, however cannot be unequivocally determined by *in gel* assays. Hence, immunoprecipitation using specific antibodies followed by an immunocomplex assay have to be used to assign a specific activity to a MAP kinase isoenzyme. However, due to the high degree of conservation of MAP

kinases, the generation of antibodies specific for a single MAP kinase isoenzyme is a major task and antibodies are available against only a few MAP kinases. In fact, this approach has proven to be a powerful tool to study MAP kinases but, nevertheless, it is limited to previously *known* MAP kinases. We have therefore developed a nonbiased biochemical approach to identify unknown MAP kinases that puts no restriction on the possibility to assay the activity after treatment of the cells (Hofmann et al., 1999; Link et al., 2002b). The technique employs the partial purification of MAP kinases by anion exchange chromatography which is able to separate individual isoenzymes as immunoprecipitation would, however the detection of activities is not limited to known MAP kinases. This technique will be combined with peptide sequencing using ESI/MS-MS (electrospray ionisation mass spectroscopy) and thus will provide a tool to identify the roles of yet unknown MAP kinases. The rapid advances in the development of transient protoplast expression systems (Sheen, 2001) made it possible to assemble an entire MAP kinase cascade *in vivo* (Asai et al., 2002) and thus provides a versatile tool to study the assembly and function of MAP kinase cascades *in vivo*.

Regulation of MAP kinase cascades

One of the earliest reports of an involvement of a MAP kinase in a plants stress response was the induction of WIPK (wounding induced protein kinase) transcripts by wounding (Seo et al., 1995). Other studies have later demonstrated that this MAP kinase transcript is as well induced by a number of elicitors and pathogens (Zhang and Klessig, 1998; Romeis et al., 1999). The same mode of regulation also applies to the WIPK homologs belonging to MAPK subfamily I (Zhang and Klessig, 2001) in other species: MMK4 (SAMK) from alfalfa (Jonak et al., 1996), MPK3 from *Arabidopsis (Mizoguchi et al., 1996)*, and LpWIPK (A. Hoffmann and T. Roitsch, unpublished) are as well induced by wounding and other stimuli. Interestingly only

members of subfamily V (MsTDY1 and OsBWMK1) have also been reported to be transcriptionally regulated upon stress treatment (He et al., 1999; Schoenbeck et al., 1999). However, full activation of MAP kinases requires the phosphorylation of both the T and the Y residue in the T-X-Y motif by a MAPK kinase which increases the MAPK´s activity approximately 1000 fold (Widmann et al., 1999). This dual regulation is especially well studied for the tobacco MAP kinase WIPK and might serve as a possibility for signal-coding or priming. In the case of wounding, WIPK transcripts are induced, whereas only little *de novo* WIPK protein biosynthesis occurs and the activity of WIPK rises only marginally. In contrast, in response to *Avr*9, a disease resistance protein from *Pseudomonas syringae*, WIPK transcripts are strongly induced without *de novo* protein biosynthesis but a strong increase in WIPK activity. Yet another mode of regulation applies for WIPK´s response to a cell wall derived elicitor from *Phytophthora*. In this case, both transcription and translation of WIPK are strongly enhanced without greatly affecting WIPK activity. Only in the case of TMV (tobacco mosaic virus) infection and purified elicitins, WIPK transcription, translation and activation are co-ordinately regulated (Zhang and Klessig, 2000). However, this complex mode of dual regulation does not apply to all MAP kinases. In the case of SIPK (salicylic acid induced protein kinase), a well studied member of subfamily II, transcriptional regulation could not be observed albeit strong increases in activity in response to various stress related stimuli (Zhang and Klessig, 2000). The effect that a MAP kinase takes on cellular function depends on the duration and degree of activation (Widmann et al., 1999) and since MAP kinase cascades are generally only transiently activated, mechanisms have to exist that counteract MAPK activation. Since treatment of cells with the protein biosynthesis inhibitor cycloheximide or the transcriptional inhibitor α–amanitine leads to the sustained activation of MAP kinases after treatment with an elicitor it was speculated that the negative regulator of MAP kinase activity is transcriptionally induced upon stress. Recently, a Ser/Thr phosphatase, MP2C and a Tyr phosphatase, PTP1 have been described that are rapidly induced upon stresses

(Meskiene et al., 1998). However, the exact role of these phosphatases in the regulation of MAP kinase activity *in vivo* still remains to be demonstrated.

Another mode of regulation that ensures the specificity of the response is mediated by scaffold proteins that act via docking domains (Tanoue et al., 2001). Scaffold proteins have been described in yeast and animal cells (Whitmarsh and Davis, 1998) and are believed to play a similar role in plants. The MAPKK SIMKK from Alfalfa was identified in a yeast two hybrid screen using the MAPK SIMK as a bait (Kiegerl et al., 2000). Functional studies then demonstrated that SIMKK is an upstream activator of SIMK under salt stress but activates both SIMK and MMK3 after treatment with elicitor (Cardinale et al., 2002). This MAPKK thus appears to act in two independent cascades, a mode of regulation that is described in yeast for a MAPKKK. The MAPKKK STE11 functions in both the osmotically induced as well as in the pheromone induced MAP kinase cascade. Crosstalk is in this case prevented by the scaffold protein STE5 that tethers STE11 to the cascade with pheromone specific components. Under osmotic stress conditions, STE11 is bound to the cascade specific for osmotic stress by the MAPKK PBS2 that acts at the same time as a scaffold protein (Whitmarsh and Davis, 1998).

MAP kinase cascades regulating the plants defence response

MAP kinase cascades can regulate defence response associated changes of a cells activity both positively and negatively. A complex picture of interactions between different components of individual MAP kinase cascades and regulatory proteins like MAP kinase phosphatases has emerged. However, to date only one single complete MAP kinase cascade has been identified based on *in vivo* data (Asai et al., 2002). In *Arabidopsis*, flagellin, the main protein constituting the bacterial flagella induces a rapid defense response via a specific receptor interaction of flagellin with the flagellin receptor FLS2, an LRR (leucine rich repeat) receptor kinase (Gomez-

Gomez and Boller, 2000). The signal transduction chain downstream of the FLS2 receptor utilises the MAPKKK MEKK1 which activates the MAPKKs MKK4 and MKK5. The end of this MAP kinase module is formed by the two MAP kinases MPK3 and MPK6 which in turn indirectly trigger the transcriptional activation of two WRKY transcription factors and of the receptor kinase FRK1 (Asai et al., 2002). Strikingly, this MAP kinase cascade appears to be assembled redundantly. Both the positions of the MAPKK as well as the position of the MAPKs are doubly filled, which might simply reflect the importance of this signal transduction chain for the plants defence response. This view is backed by the lack of corresponding mutant phenotypes. However, this redundancy might as well offer the possibility for an extensive crosstalk with other MAP kinase cascades since some components of the pathway are additionally involved in other pathways. The H_2O_2 activated MAP kinase pathway also utilises both the MPKs 3 and 6 but employs the MAPKKK ANP1 to activate them (Kovtun et al., 2000). Interestingly, the H_2O_2 pathway, although activating the same MAPKs as the flagellin pathway invokes expression of different target genes as after treatment with fagellin (Asai et al., 2002). The question that remains here is how specificity of MAP kinase action is generated. This might be achieved by MAP kinase independent pathways or by a signal-encoding function of the MAP kinase activity-profile itself. Recently, parts of a MAP kinase pathway have been described for tobacco cells that are the orthologs to MKK4/5 and MEK3/6 but which are much better characterised (Yang et al., 2001). In this study, it is demonstrated that NtMEK2, the ortholog to MKK4/5 is a direct upstream component of the two MAP kinases SIPK and WIPK (the orthologs to MEK 3 and 6, respectively). This MAP kinase cascade functions as an inducer of the hypersensitive response cell death after treatment of tobacco cells with different elicitors (Desikan et al., 1999; Suzuki et al., 1999). It thus appears that the flagellin pathway described by Asai et al. is a central MAP kinase module mediating the plants response to various elicitors.

An interesting difference in the activation modes of WIPK and SIPK becomes evident when the activation patterns that result in the hypersensitive response cell death are compared to elicitors that also activate these two MAP kinases but do not result in cell death. Both MAP kinases are activated by a variety of biotic and abiotic stresses such as salt stress or purified elicitors. These stimuli do not induce the hypersensitive response and SIPK is transiently activated. WIPK, however is activated to a much lesser extend (Romeis et al., 1999; Cardinale et al., 2000; Ichimura et al., 2000). However, those stimuli that induce the hypersensitive response like TMV infection or elicitins from *Phytophthora* result in a prolonged activation of SIPK which is followed by a delayed but sustained activation of WIPK (Zhang and Klessig, 1998; Suzuki et al., 1999; Zhang et al., 2000). It is now tempting to speculate whether these differential modes of activation represent a signal coding function and the elucidation of upstream components which might be MAPKKs or additional crosstalking pathways will certainly provide valuable clues to understand signal transmission and specificity by MAP kinases.

Targets of MAP kinases

No direct targets, i.e. proteins recognised as substrates of MAP kinases in plants are known to date, although it is anticipated that substrates are mainly transcription factors or other cytosolic proteins as it is suggested from data obtained in animal and yeast systems. However, only very recently it was demonstrated that the heat activated MAP kinase from tomato is able to phosphorylate the heat shock factor HsfA3 *in vitro* (Link et al., 2002a) which might be the first *in vivo* substrate of a plant MAP kinase.

Some interesting insight into MAP kinase dependent cellular responses comes from studies that involved the expression of constitutively active mutants of MAPKKs or MAPKKKs. Overexpression of a constitutively active mutant isoform of NtMEK2, an upstream activator of SIPK and WIPK in tobacco cells, results in the

induction of the salicylic acid dependent hypersensitive response (HR) cell death (Yang et al., 2001). HR is preceded by the activation of SIPK and WIPK and the activation of these two MAP kinases is linked to the induction of genes encoding PAL (phenylalanine-ammonia lyase) and HMGR (hydroxymethyl glutaryl-CoA reductase). Since SIPK and WIPK are the orthologs of the *Arabidopsis* MAPKs MPK3 and MPK6, it is conceivable that these two MAP kinases also indirectly regulate the expression of the WRKY22/29 genes as it was demonstrated for the flagellin pathway (Asai et al., 2002). A pathway that is an apparent negative regulator of pathogen resistance was described during the analysis of the *Arabidopsis* mpk4 mutant. The plants are dwarfed and show constitutively high levels of salicylic acid (Petersen et al., 2000). This indicates that MPK4 is a upstream repressor of SA dependent expression of PR (pathogenesis related) genes. This study also demonstrated that MPK4 is required for a functional jasmonate dependent gene expression. Although MAP kinase activation by jasmonate could not be demonstrated, the mpk4 mutant is unable to perform the jasmonate dependent induction of the genes encoding PDF1.2 (plant defensin1.2) and THI2.1 (thionin2.1). Somewhat redundant targets have been described for WIPK: plants overexpressing WIPK show increased jasmonate levels and reduced salicylate levels. The reverse situation is the case for plants silenced for the WIPK gene (Seo et al., 1995).

Signal integration by MAP kinase cascades

MAP kinase modules clearly serve as integration and divergence points for signals leading to alterations in gene expression or other cellular responses. The fact that 20 MAP kinases encoded in the *Arabidopsis* genome face potential activation by up to 60 MAPKKKs through only 10 MAPKKs suggests a high degree of flexibility considering the composition of MAP kinase cascades. It is this flexibility, together

with crosstalk on the level of MAPKKs that leads to a specific output. Even on the level of the MAP kinases, interactions allowing the modulation of the output appear to be probable.

As it was described above, MAPKKs that are able to differentially activate alternative substrates depending on the stimulus serve as points of signal divergence. However, it remains to be elucidated how changes in substrate specificity are regulated in detail. Another prominent feature of MAP kinase cascades that became obvious only recently is the fact that after elicitation with most stress related stimuli more than one MAP kinase becomes activated (Cardinale et al., 2000; Link et al., 2002b).

TRANSCRIPTION FACTORS REGULATING THE PLANT DEFENSE RESPONSE

As it was outlined above, a typical signal transduction cascade connects a signal derived from a receptor to gene regulation. Gene regulation is mediated by transcription factors which can be considered as the final recipients of information channelled through a signal transduction cascade. The fact that the *Arabidopsis* genome encodes more than 1500 transcription factors, i.e. approximately 6% of the genome (Riechmann et al., 2000) underlines the fact that plants devote a large proportion of resources to transcriptional control and demonstrates the importance of this means of regulation.

In the case of plant MAP kinase cascades, the endpoints of the cascade are believed to be mainly transcription factors and it has been demonstrated *in vivo*, that an *Arabidopsis* MAP kinase cascade directly regulates the induction of two WRKY transcription factors under pathogen attack (Asai et al., 2002). Transcription factors typically are composed of several different domains: first, a DNA binding domain allows the sequence specific recognition of cis-acting elements and binding of DNA; secondly, a transactivation or transrepression domain mediates the activation or

repression of the target gene (Liu et al., 1999). However, a single factor might be activating for one and repressing for another target gene (McCarty et al., 1991; Hoecker et al., 1995). A transcription factor bound to a promoter element does not directly influence the transcriptional machinery. The activation of the target gene is brought about by multiple protein-protein interactions, which lead to modifications on histones and the recruitment of the RNA-polymerase machinery. In addition, transcription factors are subject to various mechanisms that control their activation or repression potential. These mechanisms include phosphorylation, dimerisation or heteromerisation and the regulation of nuclear import.

Transcription factors in plants are a rather diverse functional family and share remarkable similarities with their homologs in animal systems. The plant transcription factor multigene families can be subdivided regarding the domain mediating DNA binding. Commonly encountered motifs are zinc fingers, in which a DNA binding domain is formed by the complexation of a Zn^{2+} ion by several cysteine and histidine residues. bHLH domains are formed by a basic region with a helix-loop-helix motif, whereas the MYB domain contains up to three repetitions of the helix-turn-helix motif. bZIP factors are characterised by a basic region involved in DNA binding and a leucine zipper mediating protein-protein interaction. The MADS box is formed by a long α-helix flanked by to β-sheets and the homeobox is formed by three to four α-helices. Of course, these domains only describe the principle modes of protein-DNA interaction and a large number of variations of the motifs described above have been found, some of which are restricted in occurrence to the plant kingdom.

An overview on the role of transcription factors in the plants defense response

During a defense response, several waves of gene activation, and to a lesser extend also gene repression occur (Cutt and Klessig, 1992). This cascade is started by the activation of early transcription factors that apart from defense-related genes activate additional transcription factors that set the next wave into action. This mode of regulation allows a constant adaptation to the environment which is exemplified by the dual regulation of the acyl-CoA oxidase promoter (Logemann and Hahlbrock, 2002). The gene is induced by UV-light stress but transcription is shut off by concomitant pathogen attack. This regulation apparently acts through a single cis-acting element and is believed to downregulate UV-protection in favour of the more urgently needed pathogen defense. Such an overlapping regulatory mode might as well apply to other genes. During a defense response, not only defense related genes but also signal transduction components like receptor kinases (Du and Chen, 2000) are activated. It can thus be speculated that the waves of gene activation shape the response to more precisely fit the current need by a constant feedback that determines the next gene activation program.

As it was mentioned above, the role of transcription factors in the plants stress response is diverse. However, some prominent groups of factors have emerged that are mainly involved in the stress response. The ERF (ethylene responsive element binding factor) proteins are a subfamily of the Apetala 2/ ethylene responsive element binding protein (AP2/EREBP) family with 124 members in *Arabidopsis* (Riechmann et al., 2000). Transcripts of specific family members are upregulated in response to jasmonic acid, salicylic acid, wounding, pathogen attack and abiotic cues like cold and drought (Singh et al., 2002). Interestingly, loss-of-function phenotypes have not been reported which points towards a considerable functional overlap of these factors. This functional redundancy might be a common feature for these large families of transcription factors since the same is true for the WRKY superfamily (P. Rushton, pers. Comm.)

and is sustained by the interchangeable function of WRKY22 and WRKY29 in the flagellin MAP kinase cascade (Asai et al., 2002). However, overexpression of some of these factors conferred increased pathogen resistance, albeit the regulation appears to be complex. Overexpression of the *Arabidopsis* ERF1 conferred enhanced resistance to the attack of necrotrophic fungi, but at the same time made the plants more susceptible to infection by *Pseudomonas syringae* (Berrocal-Lobo et al., 2002). ERF proteins have also been used to engineer stress resistant plants: using a stress induced promoter system to overexpress the *Arabidopsis* DREB1A, plants were more resistant to freezing, drought and salinity (Kasuga et al., 1999).

bZIP transcription factors constitute a family of 75 members in *Arabidopsis* (Jacoby et al., 2002) and some family members have been implicated to act in the regulation of UV-light, salt and drought stress in an abscissic acid dependent pathway (Kang et al., 2002). Other family members regulate the PR1 and GST6 gene (glutathion-S-transferase 6), possibly by interaction with the transcriptional co-activator NPR1 (non-expressor of PR1 (Lebel et al., 1998; Zhang et al., 1999)) that is a direct target of WRKY proteins which will be discussed in the next section. The 125 members of the *Arabidopsis* MYB familiy are the least characterised major group of transcription factors. However, some family members regulate phenylpropanoid and anthocyanine metabolism and function in response to UV stress (Borevitz et al., 2000; Jin et al., 2000).

WRKY transcription factors

WRKY transcription factors constitute a large superfamily with approximately 70 members in *Arabidopsis*. Identified initially as regulators of the pathogenesis related PR genes in parsley (Rushton et al., 1996), WRKY transcription factors have now additionally been associated with regulatory roles regarding carbohydrate metabolism (Kim et al., 1996; Willmott et al., 1998), senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001) and development (Johnson et al.,

2002), dormancy and cold stress, wounding (Hara et al., 2000b), virus infection and salicylic acid signalling (Wang et al., 1998; Yang et al., 1999).

WRKY proteins are plant specific zinc finger transcription factors. The zinc finger domain is preceded by the name giving amino acid stretch WRKYGQK which appears to play a central role in DNA recognition and/or binding (Maeo et al., 2001). This 60 amino acid domain is called the WRKY domain. Based on their WRKY domains, WRKY proteins can be classified into three subgroups. Proteins containing two WRKY domains fall into class I, whereas those proteins carrying only one WRKY domain with a C_2H_2 zinc finger constitute class II proteins which are further subdivided, based on sequence similarities, into class IIa to class IIe. Proteins carrying one WRKY domain with a C_2HC zinc finger were classified into class III. WRKY proteins appear to act as multimeric complexes (Yang et al., 1999) and some WRKY proteins contain an additional leucine zipper domain possibly mediating dimerisation (Eulgem et al., 2000). Indeed it has been demonstrated, for PcWRKY4 and PcWRKY5, which both possess leucine zipper domains that a nonfunctional dimerisation domain drastically diminishes their transactivating activity (Cormack et al., 2002). WRKY proteins recognise a cis acting element known as the W box which carries the core motif (T)(T)TGAC(T/C) (Ishiguro and Nakamura, 1994; Rushton et al., 1995; de Pater et al., 1996; Rushton et al., 1996). W boxes have been found in the promoters of a number of defense related genes including WRKY genes themselves. In fact, it appears to be a common feature of WRKY proteins to be able to recognise and bind their own promoter, creating a positive (Eulgem et al., 1999) or negative (Robatzek and Somssich, 2002) feedback loop. The induction of WRKY genes after stimulation is often extremely rapid and transient and is independent from *de novo* protein biosynthesis (Rushton et al., 1996; Hara et al., 2000a; Hofmann et al., 2002). This expression behaviour grades WRKYs as immediate early genes possibly being key regulators of the plant defense response (Eulgem et al., 2000). According to their role as transcriptional regulators, nuclear localisation of WRKY proteins has been demonstrated (Eulgem et al., 1999; Hara et

al., 2000a; Hara et al., 2000b; Robatzek and Somssich, 2001; Yoda et al., 2002). In addition, induction and DNA binding of WRKYs is phosphorylation dependent. It has been demonstrated, that the induction of LpWRKY1 can be abolished by the kinase inhibitor staurosporine and that LpWRKY1 is phosphorylated *in vivo* (Hofmann et al., 2002). The exact role of this phosphorylation remains to be elucidated, however, it has been shown that treatment of nuclear extracts containing WRKY proteins with a phosphatase abolishes DNA binding (Fukuda, 1997). Phosphorylation of WRKYs thus appears to modulate their DNA binding activity.

Targets of WRKY transcription factors

The involvement of WRKY transcription factors is best characterised for their role in senescence and pathogen response. Strikingly, both these central cellular programs appear to be closely connected (Quirino et al., 1999; Robatzek and Somssich, 2002). Several WRKY proteins are upregulated during leaf senescence in *Arabidopsis* (WRKY 3, 4, 6, 7, 11) of which AtWRKY6 shows the strongest association with leaf senescence (Robatzek and Somssich, 2001). AtWRKY6 appears to be sink specific, since transcripts are only found in roots and flowers but not in young or mature leaves. In addition, AtWRKY6 is wound inducible, responds to the infection with virulent and avirulent bacteria and AtWRKY6 protein accumulates after treatment with flagellin. AtWRKY6 is capable of regulating its own promoter activity. However, the regulation on the AtWRKY6 promoter is negative, which is so far unique for WRKY proteins. Interestingly, AtWRKY6 is at the same time capable of positive regulation of the PR1 gene and of a SIRK, a receptor kinase specifically expressed during leaf senescence (Robatzek and Somssich, 2002). This dual effect on different promoters by AtWRKY6 offers the possibility to autoregulate the transient induction which is often observed for a number of PR proteins after treatment with elicitors. However, since the expression of AtWRKY6 is not transient during senescence, additional mechanisms have to exist that

counteract AtWRKY6 downregulation, possibly by constantly triggering its expression by a persisting stimulus. A similar phenotype is observed by the overexpression of AtWRKY18. The gene confers an increased resistance against *Pseudomonas syringae* infection, however maximum impact on resistance can only be observed at late stages of plant development (Chen and Chen, 2002). Another WRKY protein involved in development is TTG2 (transparent testa glabra 2) which appears to have a central regulatory function during trichome morphogenesis (Johnson et al., 2002).

WRKY proteins have been shown to be involved in the regulation of PR proteins upon pathogen infection (Rushton et al., 1996; Eulgem et al., 1999; Robatzek and Somssich, 2002) and of NPR1 (Yu et al., 2001). NPR1 is an early key regulator of the systemic acquired resistance which possibly functions as a transcriptional coactivator for further pathogen dependent gene regulations (Cao et al., 1998). Only very recently, it was demonstrated, that WRKY proteins might also play a central role in the gene-for-gene recognition of pathogens (Deslandes et al., 2002). The RRS1-R gene was identified as an *R* gene recognising an *avr* gene from *Ralstonia solanacearum*. Analysis of the RRS1 gene product revealed that it is composed of a LRR domain mediating the interaction with the *avr* gene and a class III WRKY domain. These results point towards a central function of WRKYs in plant pathogen interaction since binding of the *avr* gene might activate the WRKY domain to regulate the onset of defense response (Lahaye, 2002). WRKY proteins not only regulate genes which are directly associated with pathogen defense. The recent discovery that WRKY proteins regulate a set of receptor kinases that are believed to participate in pathogen recognition demonstrates that WRKYs might in addition act on the modulation of signal transduction pathways upon pathogen infection (Du and Chen, 2000). In addition to a role in the defense response, WRKY proteins were also found to be involved in the regulation of primary metabolisms. SPF1, a WRKY factor from sweet potato was reported to mediate the sucrose and polygalacturonic

acid induced expression of sporamin and β-amylase (Ishiguro and Nakamura, 1994). A gene encoding α-amylase from wild-oat is also highly likely regulated by a WRKY transcription factor (Rushton et al., 1995)

SCOPE OF WORK

Upon pathogen infection, plant cells undergo fundamental changes in gene expression that initiate a defense response by the expression of factors that directly interfere with e.g. bacterial or fungal growth. However, in addition both primary and secondary metabolism of the cell is reprogrammed to produce other secondary metabolites that interfere with the pathogen and to provide compounds used for cell wall strengthening. The primary metabolism is in the case of source cells, like those found in leaves, drastically changed towards sink metabolism: the cells shut down photosynthesis and start with the net import of the transport sugar sucrose. This source-sink transition guarantees the fast availability of energy and carbon skeletons to perform the defense response. Central to this source-sink transition is the enzyme extracellular invertase (Roitsch et al., 2002) which irreversibly cleaves sucrose to yield the hexoses glucose and fructose which can then be taken up by the cell. Since extracellular invertase is the key step for the export of sucrose from the extracellular space, the activity of this enzyme directs sucrose mass flow to areas of consumption.

Extracellular invertase is a key enzyme for the supply of energy to sink organs. Plants silenced in a pollen specific isoform of extracellular invertase are male-sterile due to a defect in pollen grain filling (Goetz et al., 2001). The regulation of extracellular invertase is complex: the gene is regulated by all phytohormones and other endogenous signals like SA and jasmonates, is expressed dependent on development and underlies circadian regulation. In addition, extracellular invertase is regulated by various biotic and abiotic stimuli, ranging from wounding to pathogen

attack. Regulation of extracellular invertase activity appears to mainly take place on the transcriptional level (Roitsch et al., 2000; Roitsch et al., 2002). Regulation on the protein level has been described to be mediated by an interacting factor called the invertase inhibitor (Greiner et al., 1998) but posttranslational modifications mediating invertase activity are not known.

We have previously described the co-ordinate regulation of defense response, induction of extracellular invertase and downregulation of photosynthesis (Ehness et al., 1997) upon treatment with various elicitors and carbohydrates. This co-ordinated regulation is achieved by signal transduction pathways that, at the least, share some common components. Most prominently, induction of extracellular invertase and defense response (measured by the induction of phenylalanine-ammonia lyase) is preceded by the activation of MAP kinases and can be blocked by the kinase inhibitor staurosporine (Ehness et al., 1997; Sinha et al., 2002). It was one task of this work to shed further light on signalling events associated with the defense response and source-sink transition with a special emphasis on MAP kinases (chapters 1-3). To perform a causal analysis of MAP kinase action in the co-ordinated regulation of defense response and source-sink transition, a biochemical method was developed that allows the identification of the associated MAP kinases. The application of this purification technique in the analysis of MAP kinase activation yielded insights into the complex patterns of MAP kinase activation during a defense response (chapter 4). To date, direct substrates of MAP kinases are unknown. However, information on substrates of MAP kinases would greatly facilitate the characterisation of their modes of action. The characterisation of MAP kinases in stress responses was therefore as well carried out to gain information on possible substrates. The discovery of a novel heat activated MAP kinase activity in tomato allowed us to demonstrate, in analogy to yeast and animal systems, that this MAP kinase directly phosphorylates a heat stress transcription factor (chapter 5). As it was outlined above, the recently identified WRKY transcription factors appear to play a central role during defense response. However, WRKY proteins have as well

been reported to regulate genes associated with primary metabolism (Ojalvo et al., 1987; Ishiguro and Nakamura, 1994; Rushton et al., 1995) which could imply a possible link between defense response and metabolism. Identification of cognate binding sites for WRKY proteins in the promoter of the extracellular invertase Lin6 (R. Proels and T. Roitsch, unpublished) in addition pointed towards a role of these proteins in source-sink transition. Chapter 6 thus describes the cloning and the characterisation of posttranscriptional modifications of a novel WRKY transcription factor from tomato. The gene is induced transiently after treatment with elicitors and can be considered as an immediate early gene since no *de novo* protein biosynthesis is required for its expression. In addition, it is shown that this WRKY protein is modified *in vivo* by phoshorylation.

The present thesis consists of five publications and one submitted manuscript which are arranged into six independent chapters. Each of the chapters is preceded by individual, specialised introductions for the specific topic. Likewise, each chapter is discussed separately and generalised remarks, which assemble the chapters into the scope of the work group, follow at the end of the thesis.

Intracellular protons are not involved in Elicitor Dependent Regulation of mRNAs for Defence Related Enzymes in *Chenopodium rubrum*

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Intracellular Protons are not Involved in Elicitor Dependent Regulation of mRNAs for Defence Related Enzymes in *Chenopodium rubrum.*

Protons have been implicated in the signal transduction of elicitor-generated defence reactions. To address the possible function of cytosolic acidification we determined both intracellular and extracellular pH values and phenylammonia-lyase (PAL) and extracellular invertase (CIN1) mRNA levels of photoautotrophic suspension culture *Chenopodium rubrum* cells in response to a number of stimuli known to elicit defence responses. The results of these experiments show that there is no correlation between persistent pH-shifts and the induction of the defence related mRNAs. Treatment with the fungal elicitor chitosan led to intracellular acidification, extracellular alkalisation, and a rise of both PAL and CIN1 mRNA levels. However, when these pH shifts are reversed by treatment with fusicoccin, a known activator of the plasmamembrane ATPase, both PAL and CIN1 mRNA levels are also elevated. A comparison between the kinetics of intracellular acidification and the rise in PAL and CIN1 mRNA levels in response to chitosan shows that the activation of these genes precedes intracellular acidification by 1h, indicating that protons are not involved in the analysed signal transduction pathway leading to the induction of PAL and CIN1 mRNAs.

Introduction

Protection against pathogen infection in plant cells involves the activation of various defence responses such as the induction of the phenylpropanoid pathway and the activation of cell wall reinforcement. These responses are triggered by exogenous or endogenous elicitors that also induce a set of rapid responses such as changes in membrane properties, net calcium uptake and oxidative burst. Elicitor-induced extracellular alkalisation (Hagendoorn et al., 1991; Fukuda, 1996; Rouet-Mayer et al., 1997) and intracellular acidification (Mathieu et al., 1994; Ojalvo et al., 1987) have also been observed as a result of elicitation and it has been suggested that protons may act as second messengers in the transduction of the elicitor-induced signal (Ojalvo et al., 1987; Hagendoorn et al., 1991; Mathieu et al., 1994; Lapous et al., 1998). Since treatment with an elicitor has been shown to be accompanied by extracellular alkalisation, the activation of defence responses was verified by determining the extracellular pH in some studies (Felix et al., 1994; MacKintosh et al., 1994). Although ionic responses that lead to an acidification of the cytoplasm and an alkalisation of the culture medium have been observed in different species, it remains unclear whether protons act as second messengers or whether the effect of specific elicitors on pH-values is independent of signal-transduction events. It has been reported that elicitor-treatment results in an alkalisation of both the cytoplasm and culture medium in *Morinda* cells (Hagendoorn et al., 1994) and that no changes in cytoplasmic pH occur in soybean cells after elicitor treatment (Horn et al., 1992). Furthermore, the kinetics of intracellular pH changes is not always of a transient nature as would be expected for a second messenger (Hagendoorn et al., 1994; Kneusel et al., 1989).

We have reported previously (Ehness et al., 1997) that treatment both with stress related stimuli, such as the fungal elicitor chitosan and wounding, and glucose, as a metabolic stimulus, leads to the activation of different signalling pathways that are ultimately integrated to co-ordinately regulate source and sink metabolism and defence responses. These signalling pathways involve protein kinases which phosphorylate myelin basic protein as a substrate. Since protons have been discussed as second messengers in defence related reactions, the possible role of intracellular acidification in the underlying elicitor activated signal transduction pathway has been analysed. Photoautotrophic suspension cultured *Chenopodium rubrum* cells were treated with a variety of elicitors and the activation of defence responses was verified by Northern blot analysis of PAL (EC 4.3.1.5) and extracellular invertase CIN1 (EC 3.2.1.26) as representative enzymes. PAL catalyses the deamination of L-phenylalanine, the first step of the phenylpropanoid pathway which leads to diverse defence related products. Extracellular invertase is the key enzyme for providing carbohydrates to sink

tissues via apoplastic cleavage of sucrose (Roitsch and Tanner, 1996) and thus links the pathogen response to an increased carbohydrate supply (Sturm and Chrispeels, 1990; Ehness et al., 1997). Extracellular invertases were shown to be induced by pathogen infection, wounding and other stress related stimuli in different species (Sturm and Chrispeels, 1990; Ehness et al., 1997).

To monitor variations in intracellular pH, we used the distribution of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO). The use of weak acids as pH markers has been shown previously to be a convenient and reliable alternative to ^{31}P-NMR measurements in plant suspension culture cells (Mathieu et al., 1996). The comparison of the data obtained by both methods indicates that the distribution of a weak acid reflects the cytoplasmic pH rather than the overall proton concentration of all organelles.

Although the widely used fungal elicitor chitosan results in intacellular acidification and extracellular alkalisation in *C. rubrum*, which is assumed to be a typical early elicitor response (Felix et al., 1993; Mathieu et al., 1996), the results presented in this study demonstrate that the treatment with other functional elicitors differentially affects intracellular and extracellular pH values. Even when extracellular and intracellular pH values are perturbed by fusicoccin, both mRNA levels for PAL and CIN1 are elevated. Furthermore, time course experiments show that intracellular acidification induced by chitosan does not precede the induction of PAL and CIN1 mRNA induction.

Materials and Methods

Cell Culture

A photoautotrophic cell line of *Chenopodium rubrum* was maintained as described by Ehness et al. (1997).

Treatment with Stimuli

Cells were treated with 0.1 % chitosan (Roth, Karlsruhe, Germany), 100 mM D-glucose, 0.2 % polygalacturonic acid (added as powder), 0.5 mM benzoic acid, 10 µM fusicoccin (both dissolved in ethanol), 5 ng x ml^{-1} xylanase, and 1.5 µg x ml^{-1} pectolyase (both dissolved in water). The inhibitors cycloheximide (5 µg x ml^{-1}) and staurosporine (2 µM, Boehringer Mannheim, Germany) were dissolved in water and DMSO, respectively. Control experiments showed that the solvents, in the concentrations used, have no effect on pH or mRNA levels (data not shown). For the treatment with stimuli, the number of cultures required were pooled and redistributed into the culture flasks to rule out differences between the individual cultures. Cells were incubated with the stimulus for about 15 h, except for experiments involving the treatment with cycloheximide, staurosporine, and fusicoccin, where the cells were incubated for 4 h.

Measurement of intracellular and extracellular pH

After the incubation with the elicitor, two aliquots were removed for determination of intracellular pH. The remainder of the culture was harvested by centrifugation and the cells were frozen in liquid nitrogen for RNA extraction. Extracellular pH was determined in

the supernatant with a glass pH electrode. Intracellular pH was measured by equilibration of ^{14}C-labelled 5,5-dimethyl-2,4-oxazolidinedione (DMO, L`Allemain et al., 1984). 1mL of cell culture was washed in 500µL of 0.1M KPO_4 buffer, pH 6, and resuspended in 760µL of the same buffer. Samples were incubated with 1mM unlabeled DMO and 10^5 cpm ^{14}C-labeled DMO at 26°C for 10 min. Cells were separated from the supernatant by filtration through 0.8-µm nitrocellulose filters. The radioactivity of the filtrate and of the filters with the cells was determined in a liquid scintillation counter. The intracellular pH-value was calculated according to the Henderson-Hasselbalch equation. The formula $6.3 + \log_{10}$ $[(cpm_{cells} \times 760)(cpm_{filtrate} \times 160)^{-1}]$ is based on the pKa of DMO (6.3), the volume of the cells (166 µL), and the volume of the filtrate (760 µL).

Extraction of total RNA and Northern blot analysis

Total RNA was extracted as described previously (Ehness et al., 1997). RNA (30 µg) was blotted onto nitrocellulose and probed with homologous fragments of PAL (Ehness et al., 1997) and CIN1 (Roitsch et al., 1995).

Results

To investigate the role of protons in the transduction of pathogen-generated signals that result in the activation of the phenylpropanoid pathway and sink metabolism, photoautotrophically grown *C. rubrum* cells were elicited with different stimuli. After the cells were incubated with the individual stimulus, intracellular and extracellular pH (pH_i and pH_e, respectively) was determined. Total RNA was extracted from cells of the same culture flask for northern-blot analysis. The elicitors used in this study are known potent activators of defence responses. Pectolyase (Atkinson et al., 1986; Mathieu et al., 1994) and xylanase (Lotan and Fluhr, 1990; Grosskopf et al., 1990) are enzymes that are secreted by some plant pathogens. Another group of elicitors used are cell-wall polysaccharides, such as the plant derived polygalacturonic acid (Ryan, 1988; Otho et al., 1992) and the fungus derived chitosan (Köhle et al., 1985; Ryan, 1988; Ehness et al., 1997) that both have been shown to activate defence responses. The metabolic stimulus glucose and benzoic acid, a precursor of salicylic acid, have been shown in a previous work to increase CIN1 as well as PAL mRNA levels (Ehness et al., 1997).

The results obtained in these experiments are shown in Fig. 1. Fig. 1A shows the data for the intracellular pH (open bars) and extracellular pH (filled bars) for representative experiments. Positive pH values refer to an alkalisation relative to an untreated control culture, negative values refer to an acidification, whereby changes of more than 0.1 pH values are considered to be significant due to the corresponding mean error deviation. Fig. 1B shows the corresponding northern-blot that was probed for mRNA levels of PAL and CIN1.

As shown in Fig. 1A, chitosan induces intracellular acidification and extracellular

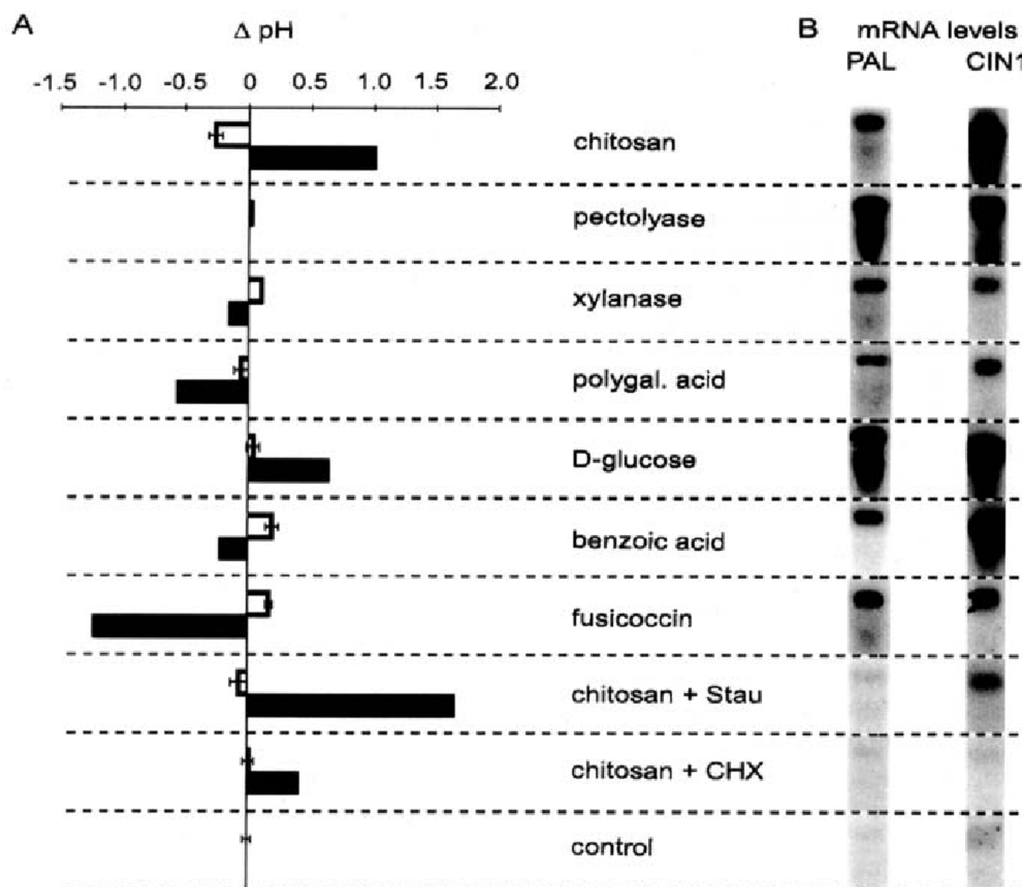

Fig. 1: Effect of various elicitors on mRNA levels of PAL and CIN1 and on intracellular and extracellular pH. Cells were treated with the substances indicated for 15 h (except for experiments involving the treatment with fusicoccin, staurosporine and cycloheximide which were incubated for 4 h), then one aliquot of cells was removed for the determination of the extracellular and intracellular pH. From the remaining cells, total RNA was extracted and subjected to northern-blot analysis with PAL and CIN1 cDNAs as homologous probes. **A:** The effect of elicitors on extracellular (filled bars) and intracellular (open bars) pH. The data is given as the difference of an individual pH value in comparison to an untreated control culture which was set to zero. Thus, positive values represent alkalisation, negative values represent acidification. **B:** Corresponding Northern-blot analysis of the cells treated with the substances indicated.

alkalisation when added to *C. rubrum* cells and a rise in the concentration of mRNAs for CIN1 and PAL mRNAs. This is a pattern that is considered to be a common early result of elicitor treatment (Felix et al., 1993; Mathieu et al., 1996). Hence, as the system used here responded as it was described for other species, other known elicitors were used to assess the possible role of protons as second messengers in the analysed defence related signal transduction pathways of *C. rubrum*. The northern blot analysis in Fig. 1B shows that stimulation with pectolyase, xylanase and polygalacturonic acid results in an increase of both PAL and CIN1 mRNAs. Transcription of these genes is also induced by glucose, benzoic acid and fusicoccin. In contrast, the changes in intracellular and extracellular

proton concentrations induced by the stimuli tested, however, are not homogenous and are different from the changes induced by chitosan. Pectolyase does not induce any alterations in pH_i or pH_e, and xylanase and benzoic acid induce pH changes reversed to those observed with chitosan (alkalisation of pH_i and acidification of pH_e). In contrast, polygalacturonic acid does not induce changes of pH_i but leads to a significant acidification of the culture media. Finally, glucose, as well as polygalacturonic acid, does not induce a change of the intracellular pH while leading to an alkalisation of the culture media.

It has been reported that the effect of fungal elicitors is phosphorylation dependent (Mathieu et al., 1996). We therefore used the protein kinase inhibitor staurosporine to test whether this is also true for the effect of chitosan in *C. rubrum* cells. When the cells are stimulated with chitosan in the presence of staurosporine, the inducing effect of chitosan on mRNA levels of PAL and CIN1 is inhibited and the cytoplasmic pH is not altered. No change in either pH occurs when the cells are treated with chitosan in the presence of the biosynthesis inhibitor cycloheximide which correlates with an inhibition of the inducing effect on mRNA levels. Fusicoccin is an activator of the plasmamembrane ATPase. Thus, treatment with fusicoccin results in the extrusion of protons from the cytoplasm, K^+ uptake and hyperpolarization of the transmembrane electric potential (Marrè, 1977). Therefore, fusiccocin was used as a tool to reverse the conditions induced by chitosan. As it was expected, treatment with

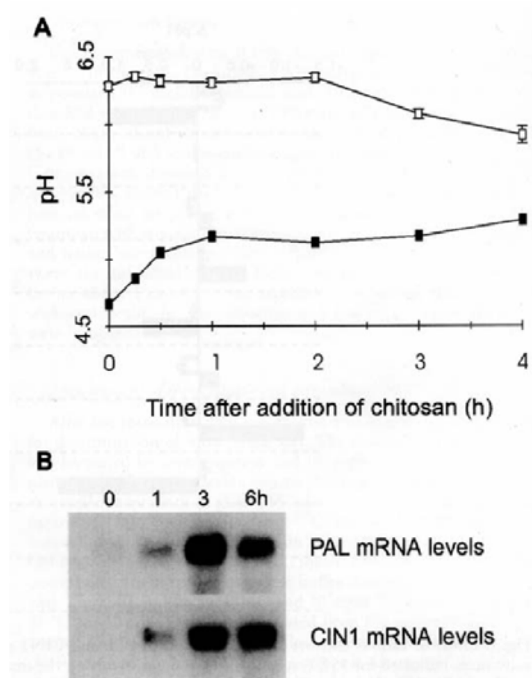

Fig. 2: Time course of the effect of chitosan on mRNA levels of PAL and CIN1 and intracellular and extracellular pH value. Cells were treated with 0.1% chitosan and samples were removed at the time-points indicated. Total RNA was subjected to Northern-blot analysis with PAL and CIN1 cDNAs as homologous probes. **A:** Time-course of the effect on intracellular pH (open symbols) and extracellular pH (filled symbols). **B:** Time course of PAL and CIN1 mRNA regulation.

fusicoccin led to an intracellular alkalisation and extracellular acidification. Furthermore, fusicoccin also induces PAL and CIN1, demonstrating that the activation of these genes is regulated independently of the direction of changes in cytoplasmic proton concentrations.

To further substantiate the results that protons are not involved in the transduction of the chitosan generated signal, the time-course of PAL and CIN1 induction was compared with the effect on intracellular and extracellular pH values. As Fig. 2A shows, changes in intracellular pH do not occur before 2h after

addition of chitosan. As it is the case with all other stimuli used, the change in intracellular pH is persistent throughout the time of the experiment. With the method used in this study, the time course of intracellular pH changes can be resolved down to 15 minute intervals, but no changes in intracellular pH during the first two hours after addition of chitosan were detected. Fig. 2B shows the time course of PAL and CIN1 induction as revealed by northern-blot analysis. The corresponding mRNAs are already elevated after 1 hour indicating that both genes are induced within the first hour after addition of chitosan. Fig. 2 also shows the effect of chitosan on the extracellular pH: the extracellular pH is rapidly shifted to a more alkaline value and remains constant throughout the time of the experiment. The comparison of the time courses of intracellular and extracellular pH changes clearly shows that these two parameters are independently regulated.

Discussion

Changes of the intracellular and extracellular pH as an early elicitor response of suspension-cultured plant cells have been reported from a number of different plant species (Felix et al., 1993; Mathieu et al., 1996; Fukuda, 1996; Kuchitsu et al., 1997). Although it has been suggested that cytosolic protons may function as second messengers in elicitor induced mRNA regulation in tobacco cells (Lapous et al., 1998), the role of these pH-shifts in pathogen response reactions is still not clearly defined. The present study demonstrates that induction of mRNAs for two defence related enzymes, PAL and extracellular invertase, is independent of changes in the intracellular and extracellular pH of autotrophic suspension culture cells of *C. rubrum* based on following reasons:

(1) Addition of the fungal elicitor chitosan results in induction of mRNAs for CIN1 and PAL which correlates with intracellular acidification and extracellular alkalisation and corresponding pH shifts induced by elicitors that have been reported from other species (Felix al., 1993; Mathieu et al., 1996). However, various other elicitors or stimuli that result in the activation of PAL and CIN1 mRNA production do not result in a uniform pattern in terms of intracellular and extracellular proton concentration as would be expected for a common intracellular signal. Xylanase and pectolyase had little or no effect on either pH, and polygalacturonic acid led to an acidification of the growth medium without altering the intracellular pH value. Fusicoccin can be used as a tool to artificially alkalise the cytoplasm, also induces PAL and CIN1 mRNA production although the pH shifts are reversed with respect to the effect of chitosan. This demonstrates that the analysed defence response can be induced under conditions where protons are actively exported into the culture medium. Similar pH shifts can be induced by the addition of benzoic acid. The finding that changes of intracellular and extracellular pH induced by the various elicitors are inconsistent suggests that alterations in intracellular and extracellular pH values are not related to the signalling

pathway leading to the induction of mRNAs for defence related enzymes. The results that elicitor responses of soybean (Horn et al., 1992) and *Catharanthus roseus* (Lundberg et al., 1997) suspension culture cells were not accompanied by cytoplasmatic pH changes further support our conclusion.

(2) The finding that the pH shifts induced by the elicitors used in the present study are persistent is also not compatible with a signal transduction function of changes in proton concentration which would be expected to be of transient nature. It has been reported that tobacco cells respond to oligogalacturonides and crude elicitor preparations from *Phytophtora megasperma* with a rapid cytosolic acidification accompanied by extracellular alkalisation (Mathieu et al., 1996). The time course of the intracellular pH-changes induced by these two elicitors, however, are different. In response to oligogalacturonides, pH_c-changes are transient, whereas a persistent change is found for the crude elicitor preparation. Although, due to the limit of time resolution of the method applied, fast transient changes below 15 min may not be excluded, the transient cytoplasmatic pH changes observed in Tobacco cells (Mathieu et al., 1996) could have been resolved under our experimental conditions. Persistent intracelluar pH-changes after treatment with an elicitor have also been reported for parsley cells (Kneusel et al., 1989) and for *Morinda citrifolia*, *Petunia hybrida* and *Linum flavum* cells (Hagendoorn et al., 1994).

(3) The finding that PAL and CIN1 mRNA induction precedes the chitosan-induced pH_c-transition by at least 1 hour also excludes protons as second messengers in the underlying signal transduction pathway.

A correlation between the extent of extracellular alkalisation and the induction of chitinase in response to a crude elicitor preparation from *Phytophtora infestans* has been described for tobacco cells (Fukuda, 1996; Rouet-Mayer et al., 1997). Similar results were reported for parsley cells (Nürnberger et al., 1994). In *C. rubrum*, the addition of chitosan also correlated with an increase in extracellular pH, whereas differential persistent effects were observed in response to other stimuli tested. Controls showed that the addition of the chitosan preparation to medium supernatant or fresh growth medium resulted in a rapid alkalisation of the medium (data not shown), indicating that this response may be related to a chemical effect of the chitosan preparation used.

The comparison of the time courses of shifts in extracellular and intracellular pH shows that these changes are independent from each other, which indicates that proton fluxes in whatever direction cannot be involved in the transduction of an elicitor generated signal. An independent regulation of elicitor induced extracellular and intracellular pH changes is further supported by studies on elicitation of benzophenanthridine alkaloid-biosynthesis in cultured cells of *Escholtzia californica* (Roos et al., 1998).

It has been reported previously (Mathieu et al., 1996) that intracellular acidification in tobacco cells that were treated

with oligogalacturonides depends on protein phosphorylation. The experiments with chitosan in the presence of staurosporine corroborate these findings: when protein phosphorylation is inhibited by staurosporine, modification of the intracellular pH is not taking place and transcriptional activation of PAL, as well as CIN1 is unresponsive. Furthermore, the induction of PAL and CIN1 mRNA production is, as is any intracellular pH shift, inhibited by cycloheximide. This suggests that *de novo* protein synthesis is required both for the generation of the intracellular acidification and for the induction of PAL and CIN1 (Ehness et al., 1997) in response to chitosan.

Our results show that long term alterations in intracellular and extracellular pH that occur after treatment with an elicitor do not function as a second messenger for the activation of PAL and CIN1 transcription since individual elicitors have differential effects, changes in intracellular proton concentration are persistent and do not precede the transcriptional activation of both PAL and CIN1. Therefore the suggestion of Hagendoorn et al. (1994) discussing cytosolic protons as part of a metabolic shift becomes more favourable: in our system, cytosolic protons are likely to be a result of the activity of secondary metabolism, rather than a prerequisite for its activation. The conclusion that cytoplasmic acidification is not serving as a second messenger is further supported by Kneusel et al. (1989). Since elicitor effects on cytoplasmic and extracellular pH are frequently observed, elucidation of the underlying mechanisms will be important for understanding the biological function in plant defence responses.

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The hexokinase inhibitor glucosamine exerts a concentration dependent dual effect on protein kinase activity *in vitro*

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The Hexokinase Inhibitor Glucosamine Exerts a Concentration Dependant Dual Effect on Protein Kinase Activity *in vitro*

Hexokinase inhibitors are used as tools to address the function of hexokinase in sugar dependent signal transduction pathways of plants. We have characterised glucosamine, N-acetyl-glucosamine and mannoheptulose for their ability to inhibit hexokinase and found pronounced differences in the inhibition of glucose-phosphorylating activity. As it is known that phosphorylation reactions are involved in sugar signalling, we have also evaluated the effect of the three hexokinase inhibitors on the activity of a glucose activated MAP kinase purified from tomato suspension culture cells. Glucosamine was shown not only to be a weak hexokinase inhibitor but specifically affected protein kinase activity. Depending on the glucosamine concentration the *in vitro* activity of the protein kinase was stimulated or inhibited. In contrast, the hexokinase inhibitors mannoheptulose and N-acetylglucosamine did not show this effect.

Introduction

A number of studies support an important role of metabolic regulation by sugars in higher plants. Photosynthesis is affected by feedback inhibition, several classes of genes were shown to be regulated by carbohydrates and glucose has been suggested to function as mitotic stimulus (Koch, 1996; Sheen, 1999). In contrast, only little is known about the mechanisms of sugar sensing and transduction of the sugar-generated signal. A regulatory function of hexokinase (HK) in sugar responsive signal transduction pathways is supported by several studies. However, whether HK is acting as a sugar sensor or is involved in intracellular transduction of the sugar signal is currently under controverse discussion (see Roitsch, 1999; Halford et al., 1999 and Sheen et al., 1999 for reviews). Indirect evidence for the involvement of HK in signal transduction is based on the use of non-metabolizable glucose analogs. In some experimental systems only 2-desoxyglucose, a glucose analogue that may be phosphorylated by HK, is able to mimic the effect of glucose whereas analogs that are no substrates for HK are inactive (Feletti and Gonzalez, 1998; Jang and Sheen 1994; Perata et al., 1997; Umemura et al., 1998). More direct evidence for a role of HK in sugar signalling was obtained from a study where it has been shown that modulation of HK activity in transgenic *Arabidopsis* plants affects sugar dependent gene expression and growth responses (Jang et al., 1997). Overexpression and antisense repression resulted in plants that were sugar hyposensitive and hypersensitive, respectively. Further evidence for the involvement of HK in the transduction of a sugar-generated signal was derived from studies involving competitive HK inhibitors, such as mannoheptulose, glucosamine and N-acetylglucosamine (Salas et al., 1965; Wilson and Perie, 1999). The findings that sugar effects such as induction or repression of genes (Umemura et al., 1998; Jang and Sheen, 1994) or inhibition of germination (Pergo et al., 1999) were abolished in the presence of HK inhibitors support a role of HK in the underlying signal transduction pathway.

Several studies indicate a critical role of protein phosphorylation in sugar dependant signal transduction pathways. Sugars were shown to stimulate the autophosphorylation of three calcium dependent protein kinases of tobacco (Ohto and Nakamura, 1995). In cultured rice cells, the expression of the sugar-repressible α-amylase gene (Umemura et al., 1998) is greatly enhanced by treatment with phosphatase inhibitors (Lue and Lee, 1994). In addition, it has been shown that glucose elicits a rapid and transient activation of a mitogen activated protein (MAP) kinase in photoautotrophic cultures of *C. rubrum* (Ehness et al., 1997) and tomato (this study).

The fact that only the HK inhibitor glucosamine but not mannoheptulose did prevent α-amylase gene repression by sugars in rice embryos (Umemura et al., 1998) whereas only the latter HK inhibitor was able to abolish the inhibitory effect of sugars on photosynthetic gene expression in maize

protoplasts (Jang and Sheen, 1994) and germination of *Arabidopsis* (Pergo et al., 1999) question the specificity of these inhibitors for the highly conserved glycolytic enzyme HK. Since HK inhibitors appeared to be valuable tools to study the involvement of HK in sugar signal transduction pathways we tested the possible effect of N-acetylglucosamine, glucosamine and mannoheptulose on protein phosphorylation, which was also shown to be involved in sugar signalling. The results of the present study demonstrate that glucosamine is not only a weak hexokinase inhibitor but may also affect protein kinase activity. Depending on the glucosamine concentration the in vitro activity of a glucose activated protein kinase from tomato was stimulated or inhibited.

Materials and Methods

Plant material

Photoautotrophic suspension cultures of *Lycopersicon peruvianum* were maintained as described in (Stöcker et al., 1993). Cells were harvested by centrifugation, immediately frozen in liquid nitrogen and stored at –70°C until further use.

Preparation of crude protein extracts

Crude protein extracts for the purification of the glucose activated MAP kinase and for protein kinase assays were prepared by thawing cells ground in the presence of liquid nitrogen in 1/5 volume of buffer containing 50 mM HEPES-KOH pH 7.5, 1 mM EGTA, 1 mM EDTA, 2 mM DTT, 20 mM β-glycerophosphate, 20% glycerole, 10 μM endothall, 10 mM NaF, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, 2 mM PMSF, 100μM benzamidine, 1μg/ml each of leupeptin and antipain and 5μg/ml pepstatin. After centrifugation at 13000g for 10min., the supernatants were stored at –70°C and used as crude protein extracts. Extracts for HK assays were prepared by slowly thawing ground material in 170 mM Tricine/NaOH pH 7.5, 10 mM DTT. After centrifugation, the extracts were stored at 4°C until further use. Protein contents were determined according to Bradford (1976).

Assay of HK activity in vitro

The assay for HK activity was carried out in a final volume of 1mL in the presence of 170 mM Tricine/NaOH pH 7.5, 1 mM ATP, 0.1 mM NADP, 0.3U of glucose 6-phosphate-dehydrogenase (from yeast, grade II, Boehringer, Mannheim, Germany) and 100μg of crude extract. The reaction was started by addition of 0.5mM glucose and the reduction of NADP was monitored by the absorption kinetics at 340nM.

Assays of protein kinase activity

The *in vitro* assay for protein kinase activity was carried out in a final volume of 15μl containing 0.5mg/mL of substrate (either MBP, histone IIIS or casein), 1 mM DTT, 1 mM EGTA, 25 μM ATP, 2μCi γ^{32}P ATP (Hartmann Analytik, Braunschweig, Germany), 7.5 mM $MgCl_2$, 25 mM Tris HCl pH 7.5 and 1.2μg of the enzyme preparation. The reaction was carried out for 20min at room temperature and then terminated by the addition of 4μl of SDS polyacrylamide gel loading buffer. The reactions were then separated on 15% SDS polyacrylamide gels, the gels were dried and

then subjected to autoradiography or quantification using a phosphorimager (Packard Phosphor Storage Systems, Meriden, CT, USA). In-gel protein kinase assays, using MBP as substrate polymerised in the gel matrix, were performed as described before (Ehness et al., 1997).

Results and Discussion

In an increasing number of studies HK inhibitors such as mannoheptulose, glucosamine, and N-acetylglucosamine are used as a tool to address the role of HK in sugar dependant signal transduction pathways in higher plants (Jang and Sheen, 1994; Umemura et al., 1998; Pergo et al., 1999; Perata et al., 1997; Chiou and Bush, 1998; Dai et al., 1999). Since several studies suggest that sugar dependent pathways involve protein kinases (Ohto and Nakamura, 1995; Lue and Lee, 1994; Ehness et al., 1997) the possible effect of HK inhibitors on a glucose activated protein kinase has been tested to control the specificity of HK inhibitors.

It has been shown that glucose rapidly and transiently activates a protein kinase that phosphorylates myelin basic protein (MBP), a typical substrate of MAP kinases, in photoautotrophic cultures of *C. rubrum* (Ehness et al., 1997). The finding that the metabolic stimulus glucose results in the activation of a MAP kinase could be extended to tomato cells by the present study. The in-gel protein kinase assay shown in Fig. 1A demonstrates that incubation of suspension culture cells of *Lycopersicon peruvianum* in the presence of 50 mM glucose for 5 min

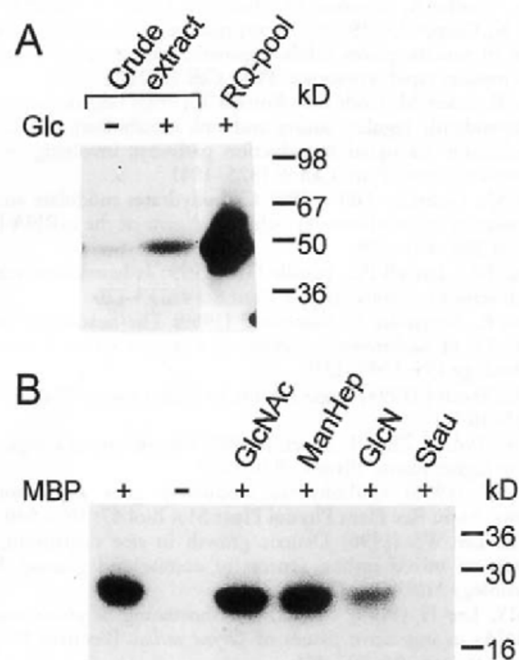

Fig. 1. Protein kinase activity assays. (A) Purification of a glucose activated MAP kinase from tomato suspension culture cells. MAP kinase activity was determined by an in-gel assay with the substrate MBP polymerised in the gel matrix. Crude extract were prepared from control cells and from cells incubated for 5 min in the presence of 50mM glucose. The glucose activated MAP kinase was purified as described in materials and methods and the highly enriched enzyme preparation eluting from a Resource Q anion exchange column was used throughout the present study. For the in-gel assays, 30µg of crude extracts and 8µg of the Resource Q eluate were loaded. (B) Effect of HK inhibitors on the activity of the purified glucose activated MAP kinase. MAP kinase activity was determined by an *in vitro* assay and phorsphorylated MBP was separated on a SDS-polyacrylamide gel and visualised by autoradiography. The activity of the glucose activated MAP kinase was tested in the absence and presence of MBP. Where indicated the HK inhibitors N-acetylglucosamine (GlcNac), mannoheptulose (ManHep), and glucosamine (GlcN) were included in the test at a concentration of 100mM and the protein kinase inhibitor staurosporine was included at a concentration of 1 µM.

results in the activation of a MBP phosphorylating activity. This glucose activated MAP kinase was partially purified. The purification scheme involves $(NH_4)_2SO_4$-

precipitation, ultracentrifugation and final separation on a Resource Q anion exchange column (to be published elsewhere). The purification yielded a highly enriched MBP phosphorylating activity as shown in figure 1A. The active fractions eluting from the Resource Q column were pooled and used for the present study. The strong preference for MBP as a phosphate acceptor over casein and histone IIIS, which are both phosphorylated at less than 10% efficiency compared to MBP (data not shown) supports that the pooled fractions contain only a single kinase activity, i.e. the glucose activated MAP kinase, since no MBP phosphorylation was present in the cells before stimulation with glucose.

The three HK inhibitors mannoheptulose, glucosamine, and N-acetyl-glucosamine were used to address their effect *in vitro* on the HK activity present in crude extracts prepared from autotrophic suspension culture cells of *L. peruvianum*. Figure 2 demonstrates that the three HK inhibitors are able to reduce HK activity although only at high concentrations and to a different extent. Glucosamine was the least effective HK inhibitor resulting in 21% and 39% inhibition of the HK activity present in the crude extract when used at 10mM and 100mM, respectively. 10mM N-acetylglucosamine resulted in 73% inhibition and completely abolished HK activity when used at 100mM and thus was the most potent inhibitor of tomato HK activity. Mannoheptulose resulted in 77% inhibition at 10mM which was further increased to only 83% inhibition when used at a tenfold higher concentration. These inhibitors inhibit HK activity competitively (Salas et al., 1965; Wilson and Perie, 1999) and 100mM of the inhibitors represent a 200 fold excess compared to the glucose concentration present in the assay. The finding that only N-acetylglucosamine is able to abolish HK activity indicates a low affinity of glucosamine and mannoheptulose toward tomato HKs.

A purified preparation of the glucose activated MAP kinase from tomato described above was used to study the effect of the three HK inhibitors mannoheptulose, N-acetylglucosamine and glucosamine on protein kinase activity *in vitro*. Figure 1B shows the effect of the three HK inhibitors on the phosphorylation of MBP by the protein kinase preparation. The purified glucose activated protein kinase shows a high *in vitro* activity. A signal is absent in the control incubation without the substrate which demonstrates the specificity of the test and the absence of any autokinase activities. The kinase activity is completely abolished by 1µM of the protein kinase inhibitor staurosporine. Neither mannoheptulose nor N-acetyl-glucosamine do effect protein kinase activity at concentrations required to substantially reduce HK-activity *in vitro* (100mM). In contrast, 100mM glucosamine strongly inhibits phosphate transfer from ATP to MBP. The inhibition is about 60% compared to the control incubation without inhibitor. The fact that 100mM mannoheptulose and 100mM N-acetylglucosamine do not affect protein kinase activity shows that the reduction of kinase activity by glucosamine is not an unspecific effect due to the high sugar concentration *per se*. Therefore the inhibition represents a specific effect of glucosamine on the

phosphorylating activity of the glucose activated protein kinase.

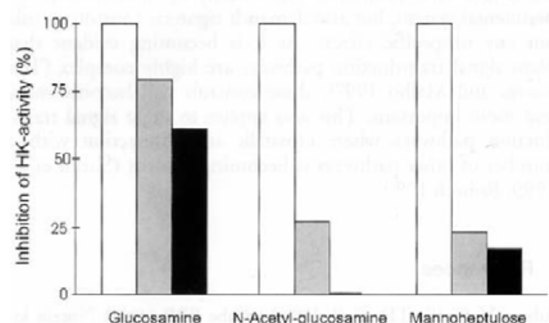

Fig. 2. Effect of HK inhibitors on HK activity *in vitro*. 100µg of a crude extract prepared from tomato suspension culture was assayed for HK activity in the absence (open bars) or the presence of 10mM (grey bars) and 100mM (black bars) of the HK inhibitors indicated in the figure. The HK activity of the extract in the absence of inhibitors was set to 100%.

To further characterise the inhibition of protein kinase activity by glucosamine, dose response curves of the three HK inhibitors were determined (figure 3). The *in vitro* test was carried out under standard conditions in the presence of 10µM to 100mM of HK inhibitors and the phosphorylation of MBP was quantified using a phosphorimager. N-acetylglucosamine and mannoheptulose do not show a significant effect on the glucose activated MAP kinase activity over the range of concentrations tested. Glucosamine exerts a dual effect on phosphate transfer to MBP. At low concentrations, above 0.1mM, protein kinase activity is stimulated and a maximum stimulation of 100% is observed at 1mM. In contrast, high concentrations result in inhibition of protein kinase activity as shown above, with a maximum inhibition of 55% at a concentration of 100mM glucosamine. The present study demonstrates that the use of hexokinase inhibitors to address a possible role of HK in sugar signal transduction pathways is complicated by their rather low efficiency in inhibiting HK and possible unspecific effects on protein kinase activity. This applies in particular to glucosamine, which was shown to result in only 39% inhibition of HK activity even at 100mM. In addition, this HK inhibitor has a pronounced, concentration dependent differential effect on protein kinase activity.

The fact that glucosamine is a weak HK-inhibitor may be explained by the presence of several HK isoforms with differential sensitivity toward glucosamine, which has been recently reported for rice embryos (Yamaguchi et al., 1999). It is striking, that the HK isoenzyme assumed to be involved in sugar signalling of rice embryos was shown to be in particular sensitive to glucosamine (Yamaguchi et al., 1999). A regulatory role of HK in sugar signalling in yeast is well established (Randez-Gil et al., 1998) and it has been shown that specific HK isoenzymes of yeast (Herrero et al., 1989) and rat brain (Adams et al., 1991) possess protein kinase activity. The findings that glucosamine appears to be a specific inhibitor of HK isoenzymes involved in signal transduction (Yamaguchi et al., 1999) and the inhibition of a glucose-activated MAP kinase as shown in the present study indicate that the target of glucosamine inhibition may be a potential protein kinase function of specific plant HKs involved in sugar signalling in addition to the inhibition of the sugar phosphorylating activity.

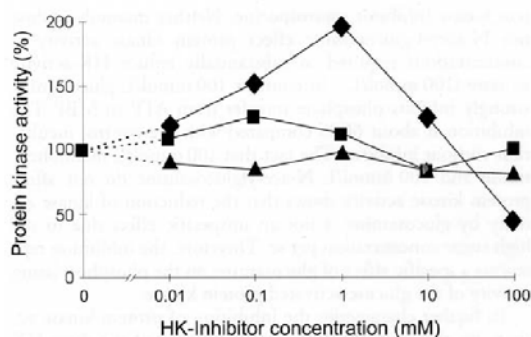

Fig. 3. Effect of different concentrations of HK inhibitors on the activity of the glucose activated protein kinase purified from tomato suspension culture cells. *In vitro* protein kinase assays were carried out in the presence of different concentrations of glucosamine (♦), N-acetylglucosamine (■), and mannoheptulose (▲). Phosphorylated MBP was separated on a SDS-polyacrylamide gel and phosphorylation was quantified by a phosphoimager. The rates of phosphorylation were plotted against the inhibitor concentration present in the assay whereas the protein kinase activity in the absence of HK inhibitors was set to 100%.

This study indicates that the use of HK inhibitors to study sugar signalling in plants not only requires to determine the degree of inhibition of HK activity in an individual experimental system, but also demands rigorous controls to rule out any unspecific effects. As it is becoming evident that signal transduction pathways are highly complex and that they comprise complicated networks with crosstalk between different pathways (Trewawas and Malho, 1997), these controls will become more and more important. This also applies to sugar signal transduction pathways where crosstalk and interaction with a number of other pathways is becoming evident (Sheen et al., 1999; Roitsch, 1999).

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Metabolizable and Non-Metabolizable Sugars Activate Different Signal Transduction Pathways in Tomato

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Metabolizable and non-metabolizable Sugars Activate Different Signal Transduction Pathways in Tomato

To gain insight in the regulatory mechanisms of sugar signalling in plants the effect of derivatives of the transport sugar sucrose, the sucrose isomers palatinose and turanose and the sucrose analogue fluorosucrose were tested. Photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum* L.) were used to study their effect on the regulation of marker genes of source and sink metabolism, photosynthesis and the activation of mitogen activated protein kinases (MAPKs). Sucrose and glucose resulted in reverse regulation of source and sink metabolism. Whereas the mRNA level of extracellular invertase (Lin6) was induced, the transcript level of small subunit of ribulosebisphosphate carboxylase (RbcS) was repressed. In contrast, turanose, palatinose, and fluorosucrose only rapidly induced Lin6 mRNA level whereas the transcript level of RbcS was not affected. The differential effect of the metabolizable and non-metabolizable sugars on RbcS mRNA regulation was reflected by the fact that only sucrose and glucose inhibited photosynthesis and chlorophyll fluorescence. The activation of different signal transduction pathways by sugars was further supported by the analysis of the activation of MAPKs. MAPK activity was found to be strongly activated by turanose, palatinose and fluorosucrose but not by sucrose and glucose. To analyze the role of sugars in relation to pathogen perception, an elicitor preparation of *Fusarium oxysporum lycopersici* was used. The strong activation of MAPKs and the fast and transient induction of Lin6 expresssion by the fungal elicitor resembles the effect of turanose, palatinose, and fluorosucrose and indicates that non-metabolizable sugars are sensed as stress related stimuli.

INTRODUCTION

In recent years sugars have been recognized as important signal molecules that affect a variety of physiological responses and in particular regulate genes involved in photosynthesis, sink metabolism and defence response (Koch 1996; Smeekens 1998; Roitsch 1999; Sheen et al., 1999). Whereas the effect of sugars on gene regulation is well established, the nature of the sugar signal, and the molecular mechanisms involved in sugar perception and intracellular signal transmission are largely unknown. Sucrose is the major form of translocated carbon in higher plants and was shown to regulate a number of carbohydrate responsive genes. Whereas in many cases the effects of sucrose could be mimicked by hexoses, such as glucose and fructose, a few studies demonstrated the existence of sucrose specific regulatory pathways (Rook et al., 1998; Chiou and Bush 1998). In principle, a sugar signal could be generated by extracellular recognition via a soluble or membrane bound receptor molecule or by intracellular sensing at different stages of sugar metabolism. For hexoses a dual role of hexokinase in sugar-sensing and glycolysis has been proposed (Jang et al., 1997; Jang & Sheen 1997) which, however, is a matter of a controversial debate (Halford et al., 1999). Additional membrane based sensing systems have been implied both for hexoses and sucrose. Primary lines of evidence are the finding that non-phosphorylatable glucose analogs can mimic the effect of glucose on the regulation of specific genes (Godt et al., 1995; Roitsch et al., 1995) and transgenic studies with specific subcellular targeting of a yeast invertase (Herbers et al., 1996). Hexose transporters were shown to function as membrane sugar sensors in yeast (Özcan et al., 1998) and a dual function of plant sugar carriers in transport and sensing of sugars has been proposed (Lalonde et al., 1999). Additional sensing mechanisms involving metabolism of sugars such as glycolysis have also been suggested (Koch et al., 2000). In summary, results obtained with various experimental approaches and systems indicate the existence of different sensory mechanism and parallel sugar signalling pathways in higher plants (Jang et al., 1997; Lalonde et al., 1999; Smeekens and Rook 1997).

To gain insight into disaccharide specific signal transduction pathways derivates of the transport sugar sucrose were used in the present study. Turanose (3-O-α-D-glucopyranosyl-fructose) and palatinose (6-O-α-D-glucopyranosyl-fructose) are structural isomers of sucrose composed of glucose and fructose with a different glycosidic linkage. They are not synthesized in higher plants and can not be cleaved or transported by plant enzymes. Using these non-metabolizable sucrose isomers evidence for extra-cellular sugar sensing has been obtained in barley and potato tubers (Loreti et al., 2000; Ferni et al. 2001).

A sucrose analogue that is not cleaved by invertase is 1´ deoxy-1´flurosucrose (1-deoxy-1-fluorofructofuranosyl-α-D-glucopyranosid, fluorosucrose). Since this sugar analogue is not commercially available

it was synthesized by an optimized protocol. We here report a highly effective synthesis of fluorosucrose with a recombinant sucrose synthase 1 from *Solanum tuberosum* to address the possible signalling function of this sucrose analogue.

Acitvation of mitogen activated protein kinases (MAPK) was shown to be involved in the stress induced signal transduction pathways (Zhang et al. 1998; Zhang and Klessig, 1998). Thus we also studied the activation of MAPK by different sucrose analogs. Pathogen infection is known to affect source/sink relations and different lines of experimental evidence suggest a role of sugars in plant defense responses (Herbers et al., 1996; Ehness et al., 1997).

Photoautotrophic cultures proved to be useful to study various aspects of sugar regulation (Roitsch et al., 1995; Krapp and Stitt 1994; Godt and Roitsch 1997). Sugar responses may be analyzed without the necessity of a sugar depletion period required for heterotrophic cultures and signal transduction events may be related to both photosynthetic gene expression or photosynthetic activity as well as source/sink regulations. Using photoautotrophic suspension culture cells of *Chenopodium rubrum* it has been shown that source/sink relations and defense mechanisms are co-ordinately regulated both by sugars and stress related stimuli (Ehness et al., 1997).

A photoautotrophic culture of the model plant species tomato (*Lycopersicon peruvianum* L.; Beimen et al., 1992) was used in the present study to test the effect of sucrose, glucose and the non metabolizable sucrose derivatives turanose, palatinose, and fluorosucrose on different cellular responses. The results were compared with the effect of an elicitor preparation of the tomato pathogen *Fusarium oxysporum lycopersici.* Extracellular invertase is the key enzyme for phloem unloading via an apoplastic pathway and was used as a representative marker gene for sink metabolism (Sturm 1999; Roitsch et al., 2000). The analysis of the regulation of the mRNA for the small subunit of the Calvin cycle enzyme ribulose bisphosphate carboxylase (RbcS) was complemented by the measurement of the rate of photosynthetic oxygen evolution and chlorophyll fluorescence. The present study demonstrates that metabolizable and non metabolizable sugars activate different signal transduction pathways. They were shown to differentially affect photosynthesis as well as MAP kinase activation. In contrast to sucrose and glucose the sucrose derivatives had no effect on RbcS expression and photosynthesis but resulted in strong MAP kinase activation. The facts that the non metabolizable sugars, like the fungal elicitor, activate MAPKs and results in a fast induction of Lin6 expression indicates that they are sensed as stress related stimuli rather that extracellular carbohydrate signals.

RESULTS

Inverse Regulation of mRNAs for Extracellular Invertase Lin6 and RbcS by Sucrose and Glucose

The time course of the regulation of mRNAs for the sink specific extracellular invertase

Lin6 and the photosynthetic marker gene RbcS by metabolizable sugars was analyzed by the addition of 50 mM glucose or sucrose to autotrophically growing tomato cell cultures. Samples were taken prior to the addition of the sugars and after 1 hr, 4 hr, 9 hr, 24 hr, and 48 hr and mRNA levels were determined by RNA gel blot analysis.

The low level of mRNA for extracellular invertase Lin6 was already elevated after 1 hr in response to both glucose and sucrose, further increased up to 24 hr and then declined (Fig. 1A). In contrast, the mRNA level of the photosynthetic protein was inversely regulated. The high steady state of RbcS mRNA was repressed already after 4 hr by both sugars and further declined up to 48 hr. Addition of 50 mM Mannitol to the cultures as an osmotic control did not resulted either in the induction of LIN6 or repression of RbcS transcript level (data not shown).

Differential Uptake of Hexoses, Sucrose and the Sucrose Isomers Turanose and Palatinose by Tomato Suspension Culture Cells

It has been shown previously that the sucrose isomers turanose and palatinose are neither recognized nor transported by Suc transporters of soybean cotyledons and broad bean leaves (M´Batchi and Delrot 1988; Li et al. 1994). To rule out a possible uptake of these two sucrose derivatives by the tomato suspension cultures used for the experiments, 50 mM of these sugar were added to the suspension culture cells and the concentration of the two sucrose isomers in the culture supernatant were determined during a 48 hr

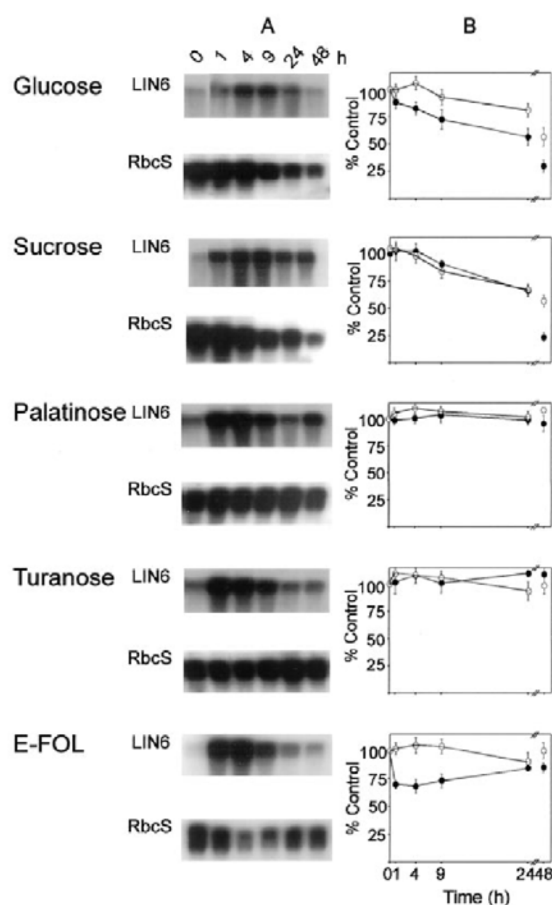

Fig. 1. Differential effect of metabolizable sugars, sucrose isomers and an elicitor preparation of *Fusarium oxysporum lycopersici* (E-FOL) on mRNA regulation and photosynthetic parameters.

(A) Regulation of mRNAs for extracellular invertase Lin6 and RbcS. 30 µg of total RNA was separated on formaldehyde agarose gels, blotted onto nitrocellulose and probed with random primer labeled cDNA fragments. Equal loading of RNA was confirmed by ethidium bromide staining of the rRNA (data not shown). The data presented are representative of five independent sets of experiments.

(B) Regulation of the rate of oxygen evolution (•) and effective photochemical yield Y (o). The data represent the mean values of 5 independent experiments.

incubation period. Figure 2A demonstrates that the extracellular concentration of palatinose and turanose did not change even after a prolonged incubation time of 48 hr compared to the initial concentration determined. No glucose could be detected

during the course of the experiment. These result demonstrate that the two sucrose isomers are neither cleaved nor taken up by the tomato suspension culture cells. In comparison, control incubations with 50 mM glucose or sucrose demonstrate a fast decrease of the concentrations of these sugars in the culture supernatant. Fig 2A shows that the glucose concentration starts to decrease after 1 hr and further declines to 11 mM after 48 hr. The concentration of sucrose gradually declines to 0.3 mM at 48 hr (Fig. 2B). Determination of the sucrose cleavage products revealed a differential accumulation of glucose and fructose. Glucose can be detected only after 24 hours and the peak concentration of 12.7 mM at 24 hr decreases to 9.4 mM at 48 hr. In contrast, fructose starts to accumulate after 1 hr which further increases up to 29 mM at 48 hr.

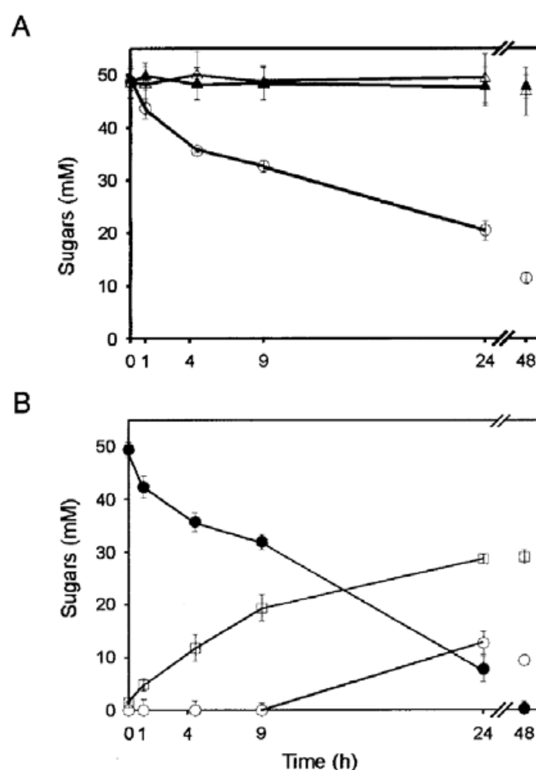

Fig. 2. Time course of changes in external sugar concentrations.

Suspension cultures cells were treated with 50 mM of the indicated sugars and the remaining concentration in the culture supernatant was determined at the given time points.

(A) Suspension cultures were treated with glucose (o), palatinose (▲) or turanose (Δ).

(B) Suspension cultures were treated with sucrose and the concentration of sucrose (•), glucose (o) and fructose (□) in the same supernatant were determined. The data represent the mean values of six independent measurements.

Sucrose Isomers Differentially Affects the mRNAs for Extracellular Invertase Lin6 and RbcS

The time course of the regulation of mRNAs for the sink specific extracellular invertase Lin6 and the photosynthetic marker gene RbcS by metabolizable sugars was analyzed by the addition of 50 mM turanose and palatinose to autotrophically growing tomato cell cultures. Samples were taken prior to the addition of the sugars and after 1 hr, 4 hr, 9 hr, 24 hr, and 48 hr and mRNA levels were determined by RNA gel blot analysis.

A fast and strong induction of the Lin6 gene could be observed in response to both sucrose isomers. The low level of mRNA for Lin6 was highly induced already after 1 hr and the elevated level declined after 24 hr (Fig. 1A). In contrast, neither turanose nor palatinose had an effect on the high RbcS mRNA level throughout the 48 hr experiment. Thus addition of the two non-metabolizable sucrose isomers turanose and palatinose results in a differential effect on the source and sink specific marker enzymes tested.

The Differential Effect of Glucose, Sucrose and Sucrose Isomers on RbcS mRNA is Reflected by the Rate of Photosynthetic Oxygen Evolution and Chlorophyll Fluorescence

In further experiments it has been addressed whether the differential effect of the metabolizable and non-metabolizable sugars on the mRNA level of the photosynthetic gene RbcS is reflected by physiological parameters.

The rates of oxygen evolution was measured with the help of a liquid phase oxygen electrode. Glucose treatment results in an immediate decrease of the rate of oxygen evolution which further declines up to 48 hr (Fig. 1B). Sucrose treatment also resulted in a pronounced reduction of the rate of oxygen evolution although with a different time course. An initial lag phase of 4 hr hours was followed by a constant decline to result in a final reduction to values comparable to the glucose treated cultures. In contrast, the non-metabolizable sucrose analogs palatinose and turanose did not reduce the rate of oxygen evolution throughout the experiment.

To analyze whether the photosynthetic apparatus is also differentially affected by the metabolizable sugars and the sucrose analogs, chlorophyll fluorescence measurements were carried out with a PAM 2000 portable fluorometer as described in materials and methods. The Fv:Fm values, reflecting the maximal photochemical quantum efficiency of PSII reaction centers of dark adapted samples, remained unchanged by all the treatments (data not shown). Photochemical yield Y, an indicator of effective photochemical quantum efficiency of illuminated sample, was differentially affected by the metabolizable and non-metabolizable sugars. Glucose and sucrose resulted in a constant decline of the photochemical yield Y with comparable low values after 48 hr (Fig 1B). In contrast, the photochemical yield remained unchanged in turanose and palatinose treated cultures.

Fast, transient, and Inverse Regulation of mRNAs for Extracellular Invertase Lin6 and RbcS by an Elicitor Preparation of *Fusarium oxysporum lycopersici*

Using photoautotrophic cultures of *C. rubrum* it has been shown that during a short incubation time of 6 hr, the mRNA for an extracellular invertase and RbcS are coordinately regulated both by glucose and the fungal elicitor chitosan (Ehness et al., 1997). These findings were re-evaluated and extended in the present study by comparing the effect of sugars with a fungal elicitor on the autotrophic tomato suspension culture over a 48 hr period. *Fusarium oxysporum lycopersici* is a wilt inducing pathogenic fungus specific for tomato (Armstrong and Armstrong 1981). An elicitor preparation of this fungus (E-FOL), shown to elicit secondary metabolite production in the photoautotrophic tomato suspension culture line used for the experiments (Beimen et al., 1992), was used to address the regulation of Lin6 and RbcS in response to this stress related stimulus.

Treatment of the tomato suspension culture with 150 µg mL^{-1} E-FOL resulted in fast and transient effects on the levels of mRNAs for Lin6 and RbcS (Fig. 1A). After 1

and 4 hr the Lin6 mRNA was highly induced then declined to a low level again. The transient effect on RbcS mRNA showed a similar time course. The high level of mRNA for RbcS was most strongly repressed at 4 hr and then increased again to the normal level.

E-FOL treatment resulted in a fast and pronounced decrease of the rate of oxygen evolution after 1 hr which recovered after 24 hr, although values were still reduced compared to the control cultures (Fig. 1B). In contrast to the oxygen production, photochemical yield Y was not affected in the cultures treated with E-FOL (Fig. 1 B).

MAPK Activity is Induced Only by Non-Metabolizable Sugars and an Elicitor Preparation of *Fusarium oxysporum lycopersici* but not by Metabolizable Sugars

MAPKs play a key role in signal transduction cascades of animals and yeast. They are rapidly and transiently activated and characterized by phosphorylating the model substrate myelin basic protein (MBP) in an in-gel assay. There is accumulating evidence for the importance of MAPKs also in the transduction of various, in particular stress related stimuli in higher plants. Therefore we have compared the effect of sucrose, glucose and the sucrose derivatives with the effect of the fungal elicitor E-FOL on MAPK activation. Tomato suspension culture cells were treated with the different stimuli for five min and crude extracts were analyzed for MAPK activity by in-gel kinase assays with the model substrate MBP assay. The dose response shown in Fig. 3 demonstrates that concentrations of up to

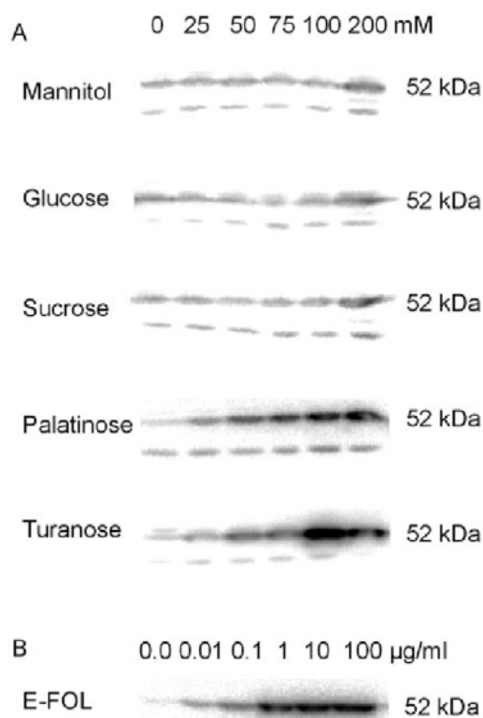

Fig. 3. Differential effect of metabolizable sugars, sucrose isomers and an elicitor preparation of *Fusarium oxysporum lycopersici* (E-FOL) on activation of MAPK. Cells were harvested exactly after 5 min after the addition of stimuli. A. Study of activation of MAPK by different sugars. Mannitol was taken as osmotic control for the different concentrations of sugars used. B. Activation of MAPK by different amount of E-FOL. The data presented in A and B are representative of five independent sets of experiments.

100 mM of either glucose or sucrose had no effect on MAP kinase. Only a concentration of 200 mM of the two metabolizable sugars results in a weak MAPK activity. Control incubations demonstrate that mannitol also result in weak MAPK activation at a concentration of 200 mM. This finding indicates that the weak MAPK activation by 200 mM glucose and sucrose represent an osmotic effect rather than a specific effect of the two sugars applied. In contrast to the two metabolizable sugars, both palatinose and

turanose resulted in strong MAPK kinases activation. The dose response shown in Fig. 3 demonstrates that concentration of 25 mM both sucrose isomers were sufficient to result in MAPK activation which further increases at concentration of up to 100 mM (Fig3a). The elicitor preparation E-FOL also strongly activated MAPK even at the lowest concentration of 0.1 µg ml^{-1} tested (Fig 3b). The observed differential effect of metabolizable and non metabolizable sugars on MAP kinase activation further supports the activation of different signal transduction pathways.

Synthesis of the Sucrose Analogue Fluorosucrose

In further experiments the question has been addressed whether the differential effect of the sucrose isomers and sucrose and glucose is related to the fact that turanose and palatinose are not transported. Fluorosucrose was used as a sucrose analogue that is not subject to invertase hydrolysis, only slowly metabolized (Hitz et al., 1985) but efficiently transported into plant cells (Thom and Maretzki, 1992. We found 80-85% of fluorosucrose were taken up by the cell cultures within 6 hours of incubation..

Since fluorosucrose is not commercially available it was synthesized by an optimized procedure. Card and Hitz (1984) previously synthesized fluorosucrose by using a sucrose synthase from barley seeds with an overall yield of 59% (507 mg). In the present paper we could improve the synthesis with reference to the enzyme productivity by repetitive use of recombinant sucrose synthase and alkaline phosphatase (Fig. 4). The synthesis yield after three batches was 100% with reference to the

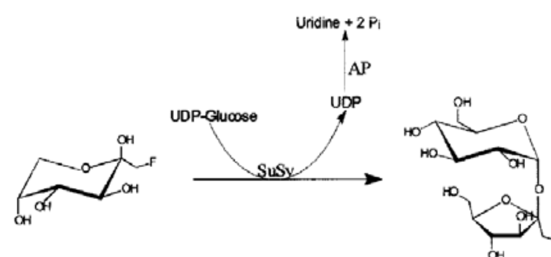

Fig. 4. Enzymatic synthesis of 1´-deoxy-1´-fluorosucrose with a recombinant sucrose synthase (SuSy) from *Solanum tuberosum* and alkaline phosphatase (AP)

acceptor substrate. After purification an overall yield of 85% (860.5 mg) for fluorosucrose was obtained. The analysis by NMR confirmed the structural integrity of the product as described previously (Card and Hitz 1984).

The Sucrose Analogue Fluorosucrose Differentially Affects he Regulation of mRNAs for Extracellular Invertase Lin6 and RbcS and Activates MAPKs

Due to the limited amount of fluorosucrose available, the regulation of the mRNAs for the marker enzymes Lin6 and RbcS was analyzed only at one time point, 6 hr after the addition of the sucrose analogue. Fig. 5A shows that 20 mM fluorosucrose strongly induces Lin6 mRNA whereas the level of the RbcS mRNA was not affected. Thus fluorosucrose, like the sucrose isomers, specifically affects only the expression of the sink specfic extracellular invertase in contrast to the metabolizable sugars that also repress RbcS mRNA level.

To further substantiate the similar effect of fluorosucrose and the sucrose isomers

MAPK activation was tested. The in-gel assay shown in Fig. 5B demonstrates that addition of 20 mM fluorosucrose for 5 min also results in strong MAPK activation whereas sucrose was inactive. Thus also the ability of fluorosucrose

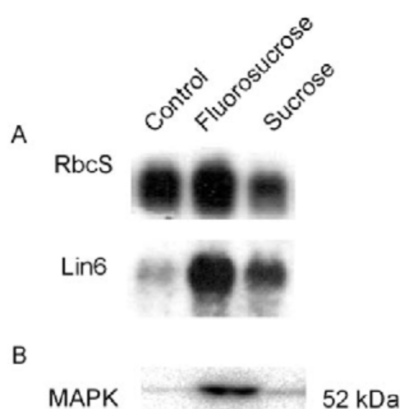

Fig. 5. A. Regulation of mRNAs of extracellular invertase Lin6 and RbcS by fluorosucrose and sucrose. Suspension culture cells were treated with 20 mM of fluorosucrose and 50 mM sucrose for 6 hr. 30 µg of total RNA was separated on formaldehyde agarose gels, blotted onto nitrocellulose and probed with random primer labeled cDNA fragments of extracellular invertase Lin6 or RbcS. Equal loading of RNA was confirmed by ethidium bromide staining of the rRNA (data not shown). B. Activation of MAPK by fluorosucrose and sucrose. The cell cultures were incubated with 20 mM of fluorosucrose and 50 mM of sucrose and harvested exactly after 5 min for the MAPK assay. The data presented in A and B are representative of three independent sets of experiments.

to activate MAPK activity resembles the effcet of turanose and palationse shown above.

Control experiments were carried out to rule out intracellular cleavage of fluorosucrose by sucrose synthase. Crude extracts of fluorosucrose treated cells were analyzed by ^{19}F-NMR. The ^{19}F-NMR spectra revealed that fluorosucrose was taken up by the tomato suspension culture cells but that this sucrose analogue has not undergone any change in structure under the experimental conditions used (data not shown).

DISCUSSION

Although sugar mediated signal transduction pathways have been recognized to be important to regulate a variety of physiological responses, the analysis in particular of the effect of the transport sugar sucrose is complicated by the fact that it is readily cleaved by extracellular invertase. To circumvent this problem the non-metabolizable sucrose derivatives turanose and palatinose have been used to address disaccharide specific signalling (Loreti et al., 2000; Ferni et al., 2001). The present study demonstrates that metabolizable sugars and non-metabolizable sucrose derivatives activate distinctly different signal transduction pathways in photoautotrophic tomato suspension culture cells. The data indicate that different disaccharide specific pathways exist and that non-metabolizable sucrose derivatives are sensed as stress related stimuli.

Photoautotrophic suspension culture cells of the model plant species tomato and sucrose derivatives were used to get further insight in the mechanisms that mediate sugar recognition and signal transduction. Turanose and palatinose are isomers of sucrose that differ in their glycosydic linkage between glucose and fructose. These two sucrose isomers were shown neither to be cleaved nor taken up by the tomato suspension culture cells used. These data support previous findings that turanose and palatinose are not recognized or transported by sucrose transporter (M'Batchi et al. 1984; M'Batchi and

Derot, 1988; Li et al. 1994). Recently also Fernie et al. (2001) showed very poor absorption of palatinose by slices of potato tubers. The analysis was further complemented by the use of fluorosucrose, a sucrose derivative that is not subject to invertase hydrolysis but efficiently transported into plant cells (Thom and Maretzki, 1992). It was and synthesized by optimizing the protocol of Kragl et al. (1993) using a recombinant sucrose synthase. The results obtained with metabolizable sugars (glucose and sucrose) and non-metabolizable sugars (turanose, palatinose and flurosucrose) were compared with the effect of an elicitor preparation of the tomato pathogenic fungus *Fusarium oxysporum lycopersici* (E-FOL) as a specific and physiological stress related stimulus.

Metabolizable Sugars and Non-metabolizable Sucrose Derivatives Result in Differential Gene Regulation

In the present study the regulation of mRNAs for extracellular invertase Lin6 and RbcS, chosen as representative marker enzymes for sink and source metabolism, have been analyzed over a 48 hr incubation time. The metabolizable sugars glucose and sucrose induce the expression of Lin6 while RbcS was repressed. This finding confirms results obtained with various experimental systems involving both monocotyledonous and dicotyledonous species showing that metabolizable sugars in general seem to repress photosynthetic genes whereas sink specific enzymes are induced (Ehness et al., 1997; Sheen et al., 1999; Roitsch 1999; Pego et al., 2000). Whereas turanose and palatinose resulted in a strong, fast and transient induction of extracellular invertase Lin6, the level of RbcS mRNA was not affected. Likewise the sucrose analogue flurosucrose, tested only with an incubation time of 6 hr due to the limited amount available, also showed a fast induction of Lin6 transcript level while RbcS transcript level was not affected. Palatinose was shown to stimulate sucrose degradation also in discs of growing potato tubers (Fernie et al., 2001). The differential effect of the metabolizable sugars and the non-metabolizable sucrose isomers on RbcS expression indicate that they activate distinctly different signal transduction pathways. The fungal elicitor E-FOL resulted in fast and transient repression of RbcS and induction of Lin6 mRNA. Induction of Lin6 pathway seems to be activated by all the stimuli tested although at different time courses. The sucrose derivatives and stress stimuli resulted in faster activation than the metabolizable sugars. In addition, the effect of the sucrose isomers was transient like the elicitor, although it was not consumed like sucrose.

The differential effect of the metabolizable sugars and the sucrose isomers on RbcS expression is substantiated by the analysis of two physiological photosynthetic parameters. The correlation between the regulation of RbcS mRNA, the rate of oxygen evolution and the photochemical yield Y supports the use of RbcS mRNA as an appropriate marker for photosynthesis. The regulation of RbcS mRNA by E-FOL is also

reflected by a transient decrease of the rate of oxygen evolution whereas photochemical yield Y is not affected.

Metabolizable Sugars and Non-metabolizable Sucrose Derivatives Results in Differential MAP Kinase Activation

The differential effect of sugar analogs and metabolizable sugars were further substantiated by the study of activation of MAPK, which is an important enzyme in a number of signal transduction cascades. MAPKs have been reported to be activated by several stresses in plants such as elicitors (Zhang et al. 1998), wounding (Stratmann and Ryan, 1997), cold and drought stress (Jonak,et al. 1996), salinity (Munnik et al. 1999) and endogenous signal (Zhang and Klessig, 1997). In the present studies from the suspension cell cultures of tomato MAPK was found to activated not only by the fungal elicitor, E-FOL but also by the sugar analogs, turanose, palatinose and fluorosucrose. In contrast, sucrose and glucose did not result in MAPK activation at the corresponding concentrations. These results suggests that the signal perception and transduction by non-metabolizable sucrose analogs and metabolizable follows different pathways.

Implications for the Analysis of Sugar Signal Transduction Pathways

The finding that the metabolizable sugars and the different non-metabolizable sucrose derivatives tested, turanose, palationose and fluorosucrose, activate distinctly different signal transduction pathways further supports the complexity and importance of carbohydrate mediated signal transduction in higher plants.

The differential effect of the non-metabolizable sucrose isomers and sucrose on a very fast signal transduction event, the activation of MAPK, indicates the existence of distinctly different disaccharide specific pathways. Within the very short incubation time of five minutes cleavage of sucrose by extracellular invertase can be neglected. Thus the observed difference between sucrose and the non-metabolizable sucrose may not be due to conversion of sucrose into the hexose monomers. Using the sucrose isomers turanose and palatinose Loreti et al. (2000) also have demonstrated that both glucose and disaccharide-sensing mechanisms modulate the expression of -amylase mRNA in barley embryos. Since effects on gene regulation have been analyzed, incubation times of at least several hrs were required. The resulting sucrose/glucose interconversion ruled out the comparison between the effect of sucrose and the sucrose isomers.

The finding that neither turanose nor palatinose is transported supports an extracellular recognition of these carbohydrate signals, which has been suggested before (Loretti et al., 2000; Fernie et al., 2001). Since the transportable fluorosucrose elicited the same responses as turanose and palatinose the corresponding effects are independent of the lack of a transport system. Both with respect to the time course of Lin6 mRNA induction and MAP kinase the effect of the non-metabolizable sucrose derivatives resemble the effect of the fungal elicitor E-FOL. These observations indicate that these

sucrose derivatives that are not naturally occurring in plants are sensed as stress signal rather than a metabolic signal. The physiological significance of this assumption is supported by the fact that phytopathogens such as specific strains of *Erwinia* are able to transform sucrose into palatinose (Huang et al. 1998). By this mechanism sucrose is retrieved from the plants and converted to a form unavailable for the plant metabolism. Thus the presence of unusual sucrose derivative may be signals for the presence of a pathogen. Therefore these sugar analogs may not be appropriate tools to address extracelluar and disaccharide specific sensing mechanism in plants per se.

Non-metabolizable sucrose derivative were shown to activate different signal transduction pathways than metabolizable sugars thus demonstrating the complexity of carbohydrate mediated regulatory mechanisms. Distinct sugar sensing mechanisms and parallel signal transduction pathways may be a central part of a complex regulatory network of higher plants to integrate metabolism with development and defense responses.

MATERIALS AND METHODS

Growth of Suspension Culture Cells

Photoautotrophic suspension cell culture cells of *Lycopersicon peruvianum* L. were established by Beimen et al. (Beimen et al., 1992) These cell cultures are being subcultured every two weeks in MS-medium and are incubated shaking under continuous light conditions with an atmosphere containing 2% CO_2.

Preparation of an Elicitor from *Fusarium oxysporum lycopersici*

The pathogenic fungus *Fusarium oxysporum lycopersici* Schlecht. Fr.f.sp *lycopersici* (Sacc.) was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). The fungus was cultured in a medium containing 50 g L^{-1} glucose, 8 g L^{-1} casamino acids, 0.5 g L^{-1} yeast extract, 0.2 g L^{-1} $MgSO_4$ and $FeSO_4$ each, 20 mg L^{-1} $CaCl_2$, 1.5 mg L^{-1} $MnSO_4$ and Na_2MoO_4 each in 25 mM potassium phosphate at pH 7.5. After four days of shaking at 28°C the culture was autoclaved, dialyzed against water and lyophilized. For the induction of stress response, 150 mg L^{-1} of the dried hyphae were added to a tomato cell suspension culture.

Extraction of mRNA and RNA Gel Blot Analysis

For the isolation of RNA, cells were harvested by centrifugation, snap frozen in liquid nitrogen and ground in the presence of liquid nitrogen. Total RNA was isolated according to the methods of Chomczynski and Sacchi (1987). Northern blot analysis was carried out as described previously (Godt and Roitsch 1997).

Determination of Sugars

The concentrations of glucose were determined by a commercially available test system (GOD test, Roche, Mannheim, Germany). For the determination of sucrose concentrations, the sucrose present in the samples were hydrolyzed by 100 U of yeast

invertase (Grade VII, Sigma) incubated at 30 ^{o}C for 1 hr. Glucose concentration was determined before and after hydrolysis by invertase and the difference between these values was taken as the actual amount of sucrose in the sample. The supernatant of sucrose treated cells were used to estimate the build up of hexoses, glucose and fructose. Glucose was estimated by the GOD test while fructose was estimated as described by Bernt and Bergmeyer (1970). Turanose and palatinose concentrations were determined according to Dubois et al. (1956).

In-Gel Kinase Assay For MAPK

The enzyme was extracted from the ground tissue in an extraction buffer consisting of 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 glycerophosphate, 0.1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM bezamidine and 0.1µg ml^{-1} antipain. The crude extract were centrifuged at 4 ^{o}C at 20,000rpm for 10 min and an aliquot of supernatant equivalent to 40 µg of protein analysed by bradford assay (Bradford, 1956) was used for in gel kinase assay. 10% polyacralymide gel embedded with 0.3 mg ml^{-1} of mylein basic protein (MBP UPstate Biotechnology) as substrate was used for the kinase assay. For control MBP was substituted with histone or casein. After electrophoresis proteins were renatured and assayed for kinase assay as described by Zhang and Klessig , 1997. Activity were visualized by autoradiography and phosphor imager (Cyclone Phosphor Storage system, madison.

Measurement of Rate of Oxygen Evolution

Rate of oxygen evolution of the cell cultures were measured using a liquid phase oxygen electrode (Frank Bros Ltd., Cambridge, UK) in the presence of saturating light provided by a halogen lamp projector. The cells in the sample cuvette were first allowed to respire for one minute in the dark and then exposed to light for the measurement of oxygen evolution. Equal volumes of cells were used each time and immediately after the measurements, the cells were taken out to determine the fresh weight. The rate of oxygen evolution was calculated on the basis of fresh weight and represented as relative units.

Chlorophyll Fluorescence Measurements

Modulated chlorophyll fluorescence of the tomato cell suspension culture were measured using a PAM 2000 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). Maximum PSII quantum yield of a dark adapted sample- Fv:Fm and effective PSII quantum yield of illuminated sample- Y; (for nomenclature see van Kooten and Snel 1990) were measured on cells dark adapted for 15 minutes as described by Schrieber et al. (1986). The preprogrammed protocol (Standard Run 3) was used for the determination of Fv:Fm and 'Y' in a special cuvette designed for the purpose. The steady state value obtained at the end of Run 3 were reported as the values of 'Y'.

Synthesis of 1-deoxy-1-fluorofructose

1-Deoxy-1-fluoro-D-fructose was obtained by the reaction described by Card

and Hitz (1984). In brief, the readily available 2,3:4,5-di-o-isopropylidene-D-fructopyranose was converted into the trifylate by the procedure described by Binkley et al (1980). Trifylate was fluorinated by TASF (tris(dimethylamino)sulfur (trimethylsilyl)difluoride, Aldrich Chemicals) in refluxing tetrahydrofuran. After removal of the isopropylidene protection groups, 1-deoxy-1-fluoro-D-fructose was obtained as a syrup in 75% yield. The synthesis of 1'-deoxy-1'-fluorosucrose was carried out by the repetitive-batch-technique (Kragl et al., 1993). The reaction mixture (100 ml) containing 0.96 mmol 1-deoxy-1-fluorofructose (176 mg) and 1 mmol UDP-α-D-glucose (Sigma, Deisenhofen) in 200 mM HEPES buffer, pH 8,0, was gently stirred at 30 ^{o}C after the addition of 40 U recombinant SuSy 1 from *Solanum tuberosum* (Zervosen et al., 1998; Zervosen and Elling 1999), and 200 U alkaline phosphatase (Roche Diagnostics, Mannheim). The course of reaction was controlled by HPLC analysis of the product with an Aminex HPX-87C column (300 x 7,8 mm, BioRad, Munich) by elution with distilled water at 85 °C. After 48 hr the enzymes were recovered by ultrafiltration and used in a second and third batch, respectively, by the addition of new substrates.

The yield of the combined product solutions was 2.9 mmol (100%) for 1´-deoxy-1´-fluorosucrose. For isolation the product solution was adjusted to pH 8.6 and loaded onto an anion exchanger column ($HCOO^-$-form) filled with AG 1-X8 resin (100-200 mesh, 122 ml bed volume BioRad, Munich), which was equilibrated with distilled water. Elution with distilled water (linear flow-rate: 56.5 cm h^{-1}) gave a product pool, which was concentrated by *in vacuo* evaporation to a final volume of 5 ml. The disaccharide was further purified by chromatography on a AG 50W-X8 resin column (200-400 mesh, Ca^{2+}-form, 1532 ml bed volume, BioRad, Munich). Elution with distilled water (linear flow rate: 3 cm h^{-1}) gave the fractions containing the disaccharide, which were pooled and lyophilized. The dry product was dissolved in 10 ml absolute methanol and crystallized at 25°C. 1´-deoxy-1´-fluorosucrose was obtained in an overall yield of 85% corresponding to 2.5 mmol (860.5 mg) with a HPLC purity of 89%. NMR spectroscopy (11.7T) of 1'-deoxy-1'-fluorosucrose revealed the typical couplings between ^{19}F and ^{1}H or ^{13}C: ^{1}H-NMR(D_2O): $H_{1'}$ δ: 4.39ppm, m, $J_{1H\text{-}19F}$: 46.6Hz, $J_{1H\text{-}1H}$: 10.4Hz; ^{19}F-NMR(D_2O): δ_{CFCl3}: -229.4ppm, m, $J_{19F\text{-}1H}$: 46.6Hz.; ^{13}C-NMR(D2O): $C_{1'}$ δ:80.7ppm, d, $J_{13C\text{-}19F}$: 174.2Hz; $C_{2'}$ δ:101.8ppm, d, $J_{13C\text{-}19F}$: 19.6Hz.

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Biochemical evidence for the Activation of Distinct Subsetd of Mitogen-Activated Protein Kinases by Voltage and Denfense-Related Stimuli

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BIOCHEMICAL EVIDENCE FOR THE ACTIVATION OF DISTINCT SUBSETS OF MAP KINASES BY VOLTAGE AND DEFENSE RELATED STIMULI

Activation of MAP kinases is a common reaction of plant cells in defense related signal transduction pathways. To gain insight into the mechanisms that determine specificity in response to a particular stimulus, a biochemical approach has been employed. Photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum* L.) were used as experimental system to characterize MAP kinase activation by different stress related stimuli. An elicitor preparation of the tomato specific pathogen *Fusarium oxysporum lycopersici* was shown to result in the simultaneous induction of four kinase activities that could be separated by ion exchange chromatography. The simultaneous activation of multiple MAP kinases was further substantiated by distinct pharmacological and immunological properties: a differential sensitivity towards various protein kinase inhibitors and a differential cross reaction with isoform specific MAP kinase antibodies. In contrast to the two fungal elicitors chitosan and the *Fusarium* preparation, the plant derived stimuli polygalacturonic acid and salicylic acid were shown to activate distinctly different subsets of MAP kinases. Application of a voltage pulse was introduced as a transient stress related stimulus that does not persist in the culture. Voltage application activates a distinct set of MAP kinases, resembling those activated by salicylic acid treatment, and generates a refractory state for the salicylic acid response. The inhibitory effect of nifedipine indicates that current application may directly affect voltage gated calcium channels thus providing a tool to study various calcium dependent pathways.

Introduction

During their whole life plants need to cope with a variety of attacking pathogens. They developed appropriate defense responses which protect them against impairment by most of them. These defense responses against several different pathogens have been extensively studied, however, the components that determine the specificity of the defense gene activation remain to be elucidated. After a plant cell is challenged by an elicitor, one or more signal transduction pathways are invoked by a ligand-receptor interaction (Nürnberger, 1999) that lead to the activation of a set of defense related genes appropriate for the defense against the attacking pathogen (Jabs et al., 1997).

The involvement of MAP kinases in biotic and abiotic stress-mediated defense gene activation has been extensively studied and MAP kinases that respond to elicitors (Zhang et al., 1998), wounding (Stratmann and Ryan, 1997), cold and drought stress (Jonak et al., 1996), salinity (Munnik et al., 1999) and endogenous signals (Zhang and Klessig, 1997) have been described. The activities were assigned to MAP kinase genes by the observation of co-ordinated transcriptional upregulation and the use of specific antibodies. In-depth analysis of MAP kinase activation in tobacco revealed that the MAP kinase SIPK is activated by salicylic acid (Zhang and Klessig, 1997), fungal elicitors (Zhang et al., 1998), wounding (Zhang and Klessig, 1998) and tobacco mosaic virus infection (Zhang and Klessig, 1998). Therefore activity in response to several stimuli was ascribed to only one MAP kinase. This raises the question, which factors determine the specific pattern of gene activation induced by defense related stimuli. A specific response may arise from the activation of more than one MAP kinase pathway in response to a particular stimulus. Indeed it has been demonstrated by isoelectric focussing that after wounding 4 MAP kinases were activated with 3 of them having the same molecular weight (Usami et al., 1995). Recently Cardinale et al. (2000) showed that a yeast elicitor resulted in the activation of four different MAP kinases as revealed by immunoprecipitation with peptide specific antibodies.

An involvement of calcium signalling upstream of MAP kinase pathways has been proposed based on the observation that MAP kinase activation was prevented by the addition of calcium channel blockers (Lebrun-Garcia et al., 1998). Calcium concentration is kept very low in the cytosol, enabling fast responses by the opening of calcium channels. Thus calcium is capable of fast transduction of stress and pathogen signals. Calcium increases were reported in response to several stress related stimuli like touch, wind and cold (Knight et al., 1991). There is also an increasing number of publications on the importance of calcium in defense signalling in response to pathogen related signals (Kawano et al., 1998), pathogens (Xu and Heath, 1998), race specific peptides (Gelli et al., 1997), peptide receptor interaction (Blume et al., 2000) or oligosaccharides (Mithoefer et al., 1999).

In the present study we investigated the response of photoautotrophic suspension culture cells of tomato to fungal elicitors, endogenous stress related signals and application of voltage. A combination of biochemical methods, the use of various protein kinase inhibitors and immunocomplex kinase assays substantiates the activation of multiple MAP kinases by one particular stimulus. Comparison of the effects of different pathogen related stimuli revealed that the activation of specific subsets of kinases results in different physiological responses. The activation of MAP kinases was shown to require calcium influx across the plasma membrane. Voltage application was introduced as a transient stimulus that does not persist in the culture medium. Based on the sensitivity towards the calcium channel inhibitor nifedipine we propose that voltage application acts directly on voltage gated calcium channels providing a tool to study the role of calcium signatures in signal transduction pathways.

Results

An elicitor preparation of the wilt inducing fungus Fusarium oxysporum lycopersici *leads to MAP kinase activation*

To investigate the defense response of tomato against one of its naturally occurring pathogens, an elicitor preparation of the wilt inducing fungus *Fusarium oxysporum lycopersici,* referred to as E-FOL, was used to challenge photoautotrophically growing

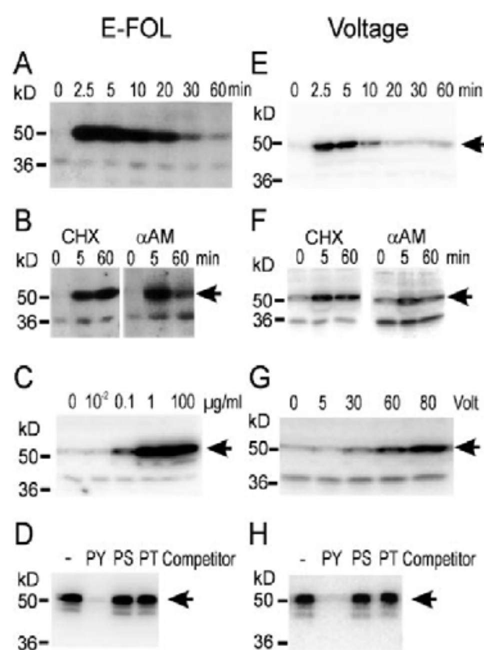

Figure 1. MAP kinase activation by treatment with E-FOL or voltage. Suspension cultures were treated with 100 µg/ml E-FOL (A, B) or 60 volts (E, F) at timepoint 0. Samples were taken at the indicated timepoints and analyzed by in-gel kinase assay with MBP as substrate. **(B), (F)** Cells were pre-treated with cycloheximide (CHX) or α-amanitin (αAM) 15 min before stimulation. **(C), (G)** Cells treated with the indicated concentrations of E-FOL (C) respectively volts (G) were collected 5 minutes after stimulation and analyzed by in-gel kinase assay. **(D), (H)** Immunoprecipitation with phosphotyrosine specific antibody 4G10. Crude extract of E-FOL (D) or voltage (H) treated cells was used for immunoprecipitation without competitor (-) or in the presence of 1 mM phosphotyrosine (PY), phosphoserine (PS) or phosphothreonine (PT). The precipitate was analyzed by in-gel kinase assay.

suspension culture cells of tomato (*Lycopersicon peruvianum* L.).

To assay for possible MAP kinase activation upon treatment with E-FOL, in-gel kinase assays with the model substrate Myelin Basic Protein (MBP) were performed and revealed the fast and transient activation of a kinase of 52kD molecular mass (Fig. 1A). Since previous reports on stress activated MAP kinases demonstrated that the activation of those kinases occurs posttranslationally

(Zhang et al., 1998), we pre-treated the cells with the RNA-polymerase II inhibitor α-amanitin or the protein biosynthesis inhibitor cycloheximide. The results shown in Fig. 1B demonstrate that the activation of the 52kD kinase only depends on posttranslational modifications since it is not blocked by either inhibitor. Inactivation in contrast is dependent on transcription and translation of a negative regulator as is revealed by the sustained activity after treatment with both inhibitors.

To further characterize the activation of the 52kD MBP phosphorylating kinase activated by treatment with E-FOL, we conducted dose response experiments (Fig. 1C) that demonstrate the induction of maximal kinase activity by addition of 1 µg/ml E-FOL preparation.

A known feature of MAP kinases is their activation by phosphorylation on the conserved sequence motif TXY, both at tyrosine and threonine (Canagarajah et al., 1997). To further support that the 52kD MBP-phosphorylating kinase activated by E-FOL treatment belongs to the MAP kinase family, immunoprecipitation with the phosphotyrosine specific antibody 4G10 and subsequent analysis with an in-gel kinase assay were performed. As shown in Fig. 1D, the E-FOL activated protein kinase is recognised by the 4G10 antibody. To ensure the specificity of the antibody, competition of the immunocomplex formation by the phosphoaminoacids phosphotyrosine, phosphoserine and phosphothreonine was tested. The results demonstrate specific recognition of phosphotyrosine and thus support tyrosine phosphorylation of the activated MAP kinase.

Application of a voltage pulse leads to MAP kinase activation

To challenge the cells by a stress related stimulus that does not persist in the culture we introduced a novel type of stress stimulus: a constant voltage was applied to the suspension culture for a limited time. Two concentric rings of platinum wires were submerged as electrodes into a 50 ml culture of tomato cells with a distance of 2.5 cm between the electrodes. With a direct current power supply 60 volts were applied for 10 seconds. As revealed by the in-gel kinase assay shown in Fig. 1E, this treatment resulted in a fast and transient kinase activation. Analysis of the dose response reveals that the lowest inducing dc voltage was 30 volts (Fig. 1G). Comparison of the effects of E-FOL and voltage treatment reveals a lower activity and a faster deactivation after voltage treatment as compared to E-FOL. This may reflect the duration of perception of the signal: while the voltage treatment lasts only 10 seconds, the elicitor treatment is a persistent signal and therefore stimulates the cells longer.

Further characterization of the activation of the MBP phosphorylating protein kinase reveals that both α-amanitin and cycloheximide do not interfere with the activation but inhibit the fast inactivation (Fig. 1F). Thus activation is independent of transcription and protein translation whereas inactivation requires synthesis of a regulatory protein. Tyrosine phosphorylation was demonstrated by immunoprecipitation by the phosphotyrosine specific antibody 4G10 (Fig. 1H). These data substantiate that voltage, as shown before for

E-FOL, results in the activation of a protein kinase belonging to the MAP kinase family.

Calcium influx is necessary for MAP kinase activation

Calcium has been shown to be involved in the initiation of plant defense responses and was a prerequisite for MAP kinase activation in response to hypoosmotic shock and elicitor treatment (Takahashi et al., 1997). To test whether calcium is involved in the signal transduction leading to MAP kinase activation after treatment with E-FOL and voltage, we used the calcium channel inhibitor gadolinium. Fig. 2A and 2B show that after 4 minutes of preincubation with 1 mM gadolinium the response to E-FOL and voltage is reduced. The kinase activation is also reduced if the suspension media is depleted of calcium by addition of 8 mM EGTA 2 minutes prior to stimulation. To demonstrate the specificity of the EGTA treatment we added 8 mM $CaCl_2$ together with 8 mM EGTA. In combination with calcium, EGTA did not interfere with MAP kinase activation (Figs. 2A and 2B). This demonstrates that the influx of calcium is responsible to invoke MAP kinase activation after treatment with E-FOL or voltage.

Voltage treatment acts on voltage dependent calcium channels

To further substantiate the results of the general calcium channel blockers we used the calcium channel inhibitor nifedipine which specifically blocks voltage gated calcium channels of the L-type in animal cells (Catterall and Striessnig, 1992) and has been used successfully to block voltage gated

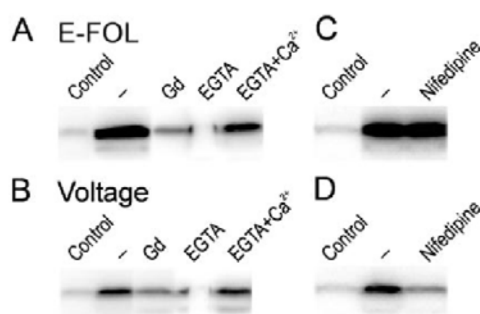

Figure 2. Calcium is necessary for MAP kinase activation. Untreated cells (Control) or cells treated with E-FOL **(A)** or voltage **(B)** were analyzed by in-gel kinase assay. Cells were pre-treated 4 minute before stimulation with the calcium channel blockers gadolinium(III) chloride (Gd) or nifedipine. EGTA or EGTA and calcium chloride (EGTA+Ca^{2+}) was added 2 minutes before stimulation to test for the involvement of extracellular calcium.

channels in plants (Gelli and Blumwald, 1997). Addition of a final concentration of 20 µM nifedipine reduced MAP kinase activation in response to voltage treatment to about 60 % (Fig. 2D). In contrast the E-FOL induced MAP kinase activation is not inhibited by nifedipine (Fig. 2C) demonstrating the specificity of the inhibitor since calcium-mediated signal transduction is not blocked in general. This result indicates that voltage treatment acts specifically through voltage gated calcium permeable channels and the applied voltage possibly directly opens these channels.

Characterization of effects of several pathogen related stimuli

To further characterize the response to E-FOL and voltage treatment we tested the production of H_2O_2 as a common physiological response to pathogen infection. E-FOL treatment induced a fast increase of H_2O_2 concentration up to 5 µM in the media while voltage treatment did not elicit H_2O_2 production.

To characterize the specificity in MBP-kinase activation in response to different signals we tested three further stress signals, chitosan, polygalacturonic acid (PGA) and salicylic acid. Chitosan is a known elicitor of plant defense responses and is part of fungal cell walls (Hadwiger and Beckman, 1980). Polygalacturonic acid (PGA) is a degradation product of plant cell walls and thus a host-derived elicitor (Ryan, 1987). Salicylic acid is an important signalling molecule involved in intra- and inter-cellular defense signalling (Reymond and Farmer, 1998).

In addition to E-FOL only the other fungal elicitor chitosan, but none of the plant derived signals PGA and salicylic acid, led to H_2O_2-production (Fig. 3). The tomato suspension culture cells differentially responded towards pathogen related signals that are endogenously produced by the plant in response to infection and stimuli derived from pathogens such as E-FOL or chitosan. Only upon contact with the latter signal H_2O_2 was produced.

Fig. 3 shows by in-gel kinase assay that all of the tested stimuli induce MAP kinase activity. Using histone or casein instead of MBP as substrate resulted in strongly reduced activity as is expected for MAP kinases (data not shown). E-FOL, chitosan and PGA resulted in a higher MAP kinase activity compared to the other stimuli. This different magnitude of MAP kinase activation correlates to the effect on the regulation of defense related genes (Fig. 3). As markers for defense gene activation we probed for phenylalanine-ammonium-lyase

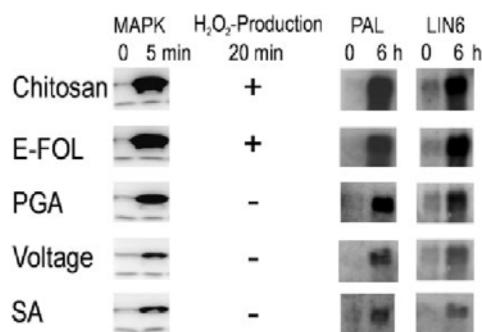

Figure 3. Downstream effects of defense related signals. Cells treated with chitosan, E-FOL, PGA, voltage or salicylic acid (SA) were assayed for MAP kinase activation, H_2O_2-production and gene induction. 5 minute samples were subjected to in-gel kinase assay. In chitosan and E-FOL treated cultures H_2O_2-concentration had increased to 5 µM above untreated cultures within 20 minutes. Gene regulation was determined by northern blot analysis with probes for *Pal* and *Lin6*.

(*Pal*) expression, which is the key enzyme of the phenylpropanoid pathway involved in the production of various defense related products including salicylic acid (Coquoz et al., 1998). As a second marker gene we probed for Lin6, an extracellular invertase, which is upregulated upon stress to increase sink strength and supply the cell with additional sugar as energy source during defense reactions (Ehness and Roitsch, 1997). Northern blot analysis revealed that all stimuli lead to induction of the defense genes *Pal* and *Lin6*, while the differential level of mRNA induction correlates with the level of MAP kinase activity.

Several MAP kinases are activated by one specific stimulus

The differences in H_2O_2-production and the levels of kinase activity and mRNA regulation indicated that differences in MAP kinase activation may reflect the different stimuli. We characterized the MBP-kinase activities in

response to various signals by biochemical separation.

Anion-exchange chromatography was used to separate the protein extracts of control cells and of cells treated for 5 min with E-FOL. As shown in Fig. 4A by an in-gel kinase assay we were able to separate four peaks with MBP-phosphorylating activity after E-FOL treatment. The untreated cells had no comparable activity (data not shown). In-solution kinase assay as used for experiment of Fig. 4C and 5A revealed a similar activity profile. This separation indicates that there is not only one but at least four kinases activated by treatment with E-FOL. There is no detectable elution of MBP-phosphorylating kinase activity at higher salt concentrations. Comparison with in-gel kinase assays with casein and histone as substrate revealed that MBP is the preferred substrate (data not shown), which is in accordance with the published substrate specificity for MAP kinases. To further support the identity as MAP kinases the peak fractions were immunoprecipitated with the phosphotyrosine specific antibody 4G10. As shown by in-gel kinase assay in Fig. 4B each peak contains tyrosine phosphorylated protein kinases that are able to phosphorylate MBP, strongly supporting that the separated kinases belong to the MAP kinase family.

The different peak MAP kinase activities display differential inhibitor sensitivity

To ensure that the different activity peaks eluting from the Resource Q column are due to different MAP kinases, we applied a set of inhibitors to determine their effects on the separated MPB phosphorylating protein kinases. For these experiments, we used the purine analogs olomoucine, bohemine and roscovitine. These inhibitors are used as potent inhibitors of cyclin-dependent kinases (Cdk) (De Azevedo et al., 1997) and were shown in one study to also inhibit an alfalfa MAP kinase (Binarová et al., 1998). Furthermore we included the kinase inhibitor staurosporine that was shown to discriminate between different MAP kinases (Romeis et al., 1999).

We tested the effect of 100µM olomoucine, roscovitine, bohemine or 1µM staurosporine on the four MAP kinase fractions in an in-solution kinase assay. Fig. 5A displays that the four different inhibitors act differentially on the four different fractions. Each peak is characterized by a unique sensitivity profile with respect to the four different inhibitors. The differential effect of the purine analogs as well as staurosporine on the MBP phosphorylating activity in the four fractions supports that the four separated peaks reflect different MAP kinases with distinct properties. Since there was only low activation in peak 1 we can not exclude that the distinct inhibitor profile of peak 1 is due to other MBP phosphorylating kinases which coelute.

Immunoprecipitation with specific MAP kinase antibodies

The activation of at least four kinases by E-FOL, which is evident from the biochemical separation and the inhibitor studies described above, raises the question whether homologs of known MAP kinases are among those. Since for tomato no MAP kinase

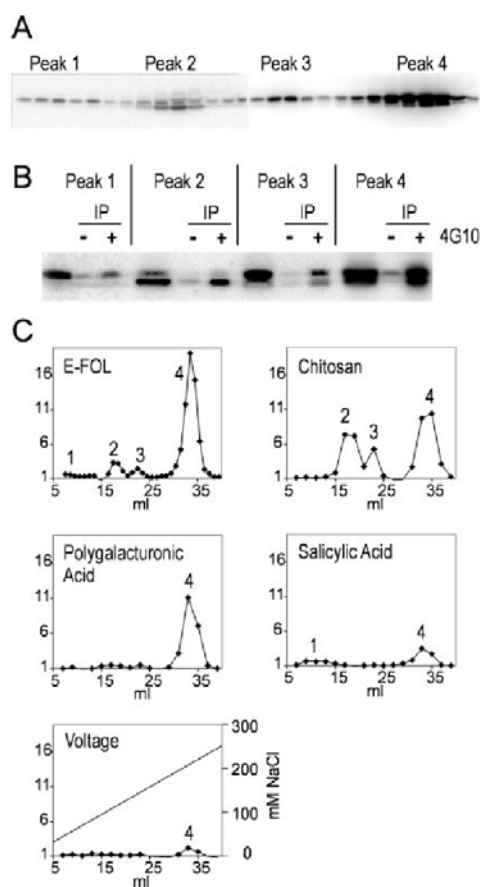

Figure 4. Separation of cell extracts by anion exchange chromatography reveals four kinase activity peaks. **(A)** Chromatography fractions 10 to 37 of E-FOL treated cells were analyzed by in-gel kinase assay. **(B)** Peak fractions were immunoprecipitated with P-Tyr specific antibody 4G10 and analyzed by in-gel kinase assay. Peak fraction and same fraction immunoprecipitated without antibody (-) and with 4G10 (+) are depicted. **(C)** Extracts from cells treated with the indicated stimuli were separated and analyzed by in-solution kinase assay. Activity was quantified by phosphor imager and diagrammed as x-fold activity of untreated cells. The salt-gradient is exemplary depicted in the voltage diagram.

antibodies are available, we used previously characterized antibodies directed against MAP kinases from alfalfa. Isoform specific antibodies directed against SIMK, MMK2, MMK3 and SAMK were shown to recognize specific MAP kinases without crossreacting against other MAP kinase homologs and are thus used to identify distinctly different MAP kinases involved in various physiological events (Cardinale et al., 2000). Since synthetic peptides of the poorly conserved C-termini were used for generation of the four alfalfa MAP kinase antibodies, we searched EST databases for putative MAP kinase homologs in tomato which in particular are characterized by a high degree of homology at the C-terminus with the alfalfa MAP kinases. The search revealed tomato MAP kinase homologs to SIMK (AW933300), MMK2 (TC52211) and SAMK (TC78176), which share a conserved C-terminal motive of 6, 5, and 6 amino acids out of the corresponding 7, 11, and 6 amino acid peptide sequences used for antibody generation respectively (Cardinale et al., 2000, TC numbers: TIGR LeGI database). This indicates that these alfalfa antibodies may be a suitable tool to also precipitate MAP kinases of tomato. No tomato MAP kinase with high C-terminal homology to the C-terminal peptide sequence of MMK3 was found in the database.

Fig. 5B shows that the MBP phosphorylating kinase activity of peak 2 was precipitated by SIMK and MMK2-antibody. No activity was precipitated from peak 1 and 3. The activity of peak 4 was solely precipitated by the SAMK-antibody suggesting the presence of a tomato MAP kinase with homology to SAMK. The MAP kinases present in peak 1 and 3, that are distinguished by their inhibitor sensitivity, apparently represent tomato MAP kinases with homology too low to be precipitated by the applied alfalfa antibodies. The differential cross reactions of the MAP kinase fractions with the four alfalfa MAP kinase antibodies further supports that a set of at least four MAP

kinases are simultaneously activated by E-FOL and separated by ion exchange chromatography.

Distinct subsets of MAP kinases are activated by different stimuli

After establishing an experimental system to separate and distinguish different MAP kinases activated after treatment with an individual stimulus, the fungal elicitor E-FOL, we investigated the effect of different stress-related stimuli on MAP kinase activation. We used the in-solution kinase test to compare the activation profiles after treatment with the different stimuli.

From the profiles depicted in Fig. 4C it is obvious that there is activation of different subsets of kinases depending on the stimulus used: Treatment with E-FOL, PGA and chitosan resulted in strong activity in peak 4. But while also peak 2 and 3 had a high activity after E-FOL and chitosan treatment, PGA treated cells displayed only little activity in these fractions. Both salicylic acid and voltage treatment resulted in a comparable low MAP kinase activity in peak 4. Thus treatment of the cell cultures with different pathogen related stimuli leads to unique profiles of MAP kinase activation. The voltage treatment results in a profile resembling salicylic acid treatment.

Refractory experiments support the activation of an identical MAP kinase pathway by salicylic acid and voltage

The result that both voltage treatment and salicylic acid induced activation of the same MAP kinase subsets and did not elicit H_2O_2-production indicated similarities between these two stimuli. To further substantiate this, we made use of the fact that a MAP kinase

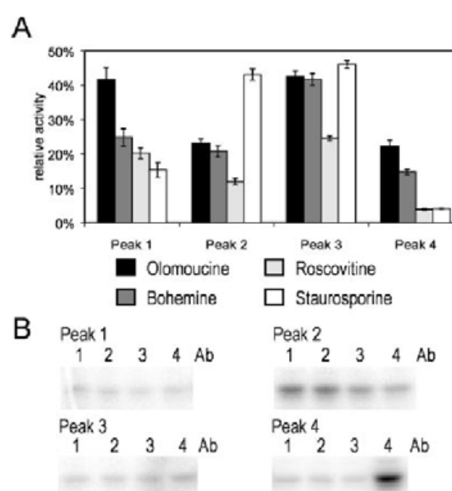

Figure 5. Separated peaks of E-FOL treated cells analyzed by inhibitor sensitivity and immunoprecipitation. **(A)** Peak fractions were analyzed by in-solution kinase assay in the presence of the inhibitors olomoucine, bohemine, roscovitine or staurosporine and without inhibitor. The activity was quantified and is depicted as percentage of the activity without addition of inhibitor. Standard deviation of 3 experiments is depicted. **(B)** Peak fractions were used for immunoprecipitation with the antibodies against the alfalfa MAP kinases SIMK (1), MMK2 (2), MMK3 (3) or SAMK (4) and subsequent in-solution kinase assay.

pathway, once activated, remains in a refractory state for some time (Bögre et al., 1997). A common feature of MAP kinase pathways is a fast deactivation even if the stimulus is not removed. As shown in Figs. 1B and 1F the activation of MAP kinases by E-FOL and voltage can be preserved by inhibiting transcription or translation. This demonstrates that gene activation and protein synthesis is required for deactivation of the MAP kinase. This may be caused by induction of a specific phosphatase which inactivates the MAP kinase (Gupta et al., 1998). For a second treatment or other stimuli acting through the same pathway this results in a refractory time during which a stimulus does not invoke a response because

dephosphorylation is competing phosphorylation. On whole plants a refractory time has been shown for repeated wounding of leaves (Bögre et al., 1997). In cell cultures the difficulty arises that the stimuli usually applied to elicit defense responses can not be removed from the system without applying additional stress. The newly established voltage stimulus is in particular suited for testing of a refractory state since it is applied only for a defined short time and thus not persistently present in the medium unlike fungal elicitors.

In an initial experiment addressing the refractory phase of the voltage induced MAP kinase activation, we found that the refractory time period lasts at least 40 minutes after voltage treatment since during this time period a second stimulation induces only weak MAP kinase activation. After 3 hours the responsiveness of the signal transduction pathway is completely recovered (Fig. 6A).

We now used this experimental setup to investigate the connection of voltage induced signalling with the pathways activated by E-FOL and salicylic acid. Reviewing the results described above, the voltage treatment mimics salicylic acid-treatment most: Both stimuli lead to a comparable MAP kinase activation in in-gel kinase assays, the activity mainly elutes in peak 4 of anion-exchange chromatography and both stimuli do not result in H_2O_2-production. In accordance to these results voltage treated cells were refractory for salicylic acid treatment (Fig. 6B). In contrast, E-FOL application after voltage treatment resulted in a normal activation of MAP kinases. Thus voltage treatment results in the activation of the same specific MAP kinase pathway as salicylic acid treatment presumably by imitating the calcium signature of salicylic acid signalling.

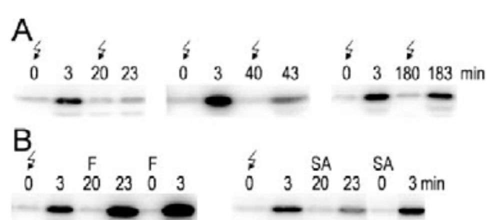

Figure 6. Voltage generates a refractory state for MAP kinase activation. Samples were analyzed by in-gel kinase assay. **(A)** Cells were treated with voltage (⚡) at 0 minutes. After 20, 40 or 180 minutes the culture was treated a second time. **(B)** Cells were treated 20 minutes after voltage treatment with E-FOL (F) or salicylic acid (SA). To compare with non refractory cells a second culture was directly treated with E-FOL (F) or salicylic acid (SA).

Discussion

A pivotal role of MAP kinases in the initiation of defense response of higher plants has been demonstrated in several cases. In the present study we focus on the analysis of how signals are integrated and specified by the activation of MAP kinases during the initiation of a defense response. MAP kinases integrate signals transduced by various mechanisms e.g. calcium, receptor tyrosine kinases or G-proteins (Widmann et al., 1999) and an increasing number of publications demonstrate the activation of MAP kinases by diverse stimuli in plants (Ligterink, 2000). However, the data elaborated so far yielded a limited number of MAP kinases that are positioned upstream of a far bigger number of physiological responses. This discrepancy might be due to other signal transduction

pathways and so far unknown MAP kinases crossreacting with the known MAP kinase pathways. Recently analysis of alfalfa cells by immunokinase assays revealed the activation of four different MAP kinases upon treatment with yeast elicitor (Cardinale et al., 2000). Immunoprecipitation limits the results in general to the proteins against which antibodies exist. To overcome this limitation we used a biochemical approach: photoautotrophic tomato suspension culture cells were treated with an elicitor preparation of the tomato pathogen *Fusarium oxysporum lycopersicon* (E-FOL). The treatment resulted in a strong, posttranslational activation of a MAP kinase as revealed by in-gel kinase assay with myelin basic protein as substrate and immunoprecipitation with a phosphotyrosine specific antibody. By partial purification on an anion-exchange column, we were able to separate the activity into four distinct protein kinase activities. The in-gel kinase assay of the separated fractions revealed in two of the four peaks two MBP phosphorylating kinases of different molecular weights. The additional bands might be due to degradation products or they might represent additional kinases. Therefore up to six proteins are activated in response to one stimulus. The finding that the separated kinases are tyrosine phosphorylated supports their identity as MAP kinases.

The simultaneous activation of several kinases was substantiated by two further sets of experimental evidence, to rule out that complex formation with other proteins or different phosphorylation states account for the separation. In a previous study bohemine, olomoucine and roscovitine, inhibitors of cyclin-dependent kinases, have been shown to also inhibit the MMK1 MAP kinase from alfalfa (Binarová et al., 1998). Along with these, the general protein kinase inhibitor staurosporine was applied. A comparison of the sensitivity profiles of the separated protein kinases towards the four inhibitors reveals characteristic differences, thus demonstrating the presence of proteins with distinct biochemical properties, and thus most likely different MAP kinases. Further we used antibodies directed against the alfalfa MAP kinases SIMK, MMK2, MMK3 and SAMK (Munnik et al., 1999) to immunoprecipitate the separated protein kinases. The MAP kinase activity of peak 2 and 4 are precipitated by different antibodies, while no activity can be detected after precipitation of peak 1 and 3 (Fig. 5B). Therefore the separation of the MAP kinase activities by ion exchange chromatography, the differential inhibitor sensitivity and the differential cross reaction with the four alfalfa MAP kinase antibodies support that at least four different MAP kinases are activated by E-FOL stimulation.

After establishing a purification procedure suitable to distinguish four different MAP kinases activated after elicitation with E-FOL, we compared this profile with that induced by several other stress related stimuli. Given the diversity of the stimuli applied in this study, we anticipated that they elicit distinguishable responses. A test for different physiological responses is provided by the measurement of H_2O_2-production after elicitation of the cells. These experiments demonstrated, that only the fungal elicitors E-FOL and chitosan elicited

H_2O_2-production whereas the endogenous effectors PGA and salicylic acid as well as voltage treatment did not. However, the mRNA for the defense related genes *Pal* and *Lin6* are induced by all of these stimuli. Further analysis of the elution profiles of those MAP kinases activated by PGA, voltage and salicylic acid demonstrated that these only induce subsets of the kinases activated by E-FOL or chitosan treatment. The use of overlapping sets of MAP kinases seems to be an economical way of plants to cope with the big numbers of different stimuli they are exposed to, while maintaining the capability to elicit specific responses with a limited number of proteins.

We used the described analysis of MAP kinase profiles to compare the downstream effects invoked by voltage with the effects of the physiological stimuli characterized before. This comparison elucidated a striking similarity of the effects invoked by voltage and salicylic acid treatment. To further substantiate that both stimuli actually induce the same pathway we tested the refractory properties of the MAP kinases activated. The activity of MAP kinases is kept transient by a concomitantly induced phosphatase which inactivates the MAP kinase module. Thus, a second stimulation of the same pathway during the time of presence of the phosphatase results only in a weak activation of the MAP kinase: the refractory state. In accordance to this voltage induced a refractory time period during which a second voltage treatment resulted in a highly reduced activation. While this treatment did not interfere with signalling of E-FOL, the salicylic acid induced activity was also markedly reduced. This strongly indicates that voltage indeed results in a natural response activating a MAP kinase pathway that is normally activated after reception of salicylic acid.

Calcium has been reported to be involved in signal transduction invoked by a variety of stimuli. There has been a great progress in calcium signalling research by the establishment of methods to record calcium concentrations in living cells by calcium dyes and the aequorin system (Knight et al., 1991). Enabled by these techniques spatial differences in calcium concentration have been reported as well as temporal patterns of several peaks of defined amplitudes and repeated spikes (Rudd and Franklin-Tong, 1999). Yet the significance of these observations for specific signalling remains to be elucidated. As we have shown in this study, calcium is a prerequisite for full activation of MAP kinases of tomato in response to stimulation with E-FOL or voltage. Voltage induced signalling is in contrast to the E-FOL response sensitive to nifedipine, a known inhibitor of voltage dependent calcium channels. This indicates that the applied voltage might act directly on voltage gated calcium channels. We conclude from the refractory experiments with voltage and salicylic acid that both treatments resulted in the activation of the same signalling pathway. Presumably because the chosen voltage treatment resulted in a calcium influx and signature that mimics the salicylic acid induced calcium signature. Application of voltage pulses to elevate intracellular calcium would open a field of potential applications to study various calcium-dependent pathways.

Increasing the cytosolic calcium concentration in a controlled way by voltage treatment would provide a tool to specifically generate calcium signatures. Using this unique experimental approach it would be possible to address the physiological relevance of a distinct calcium signature for the induction of a specific physiological response.

Materials and methods

Growth of suspension culture cells

Photoautotrophic suspension culture cells of *Lycopersicon peruvianum* L. were established by Beimen et al. (1992) and are being subcultured every two weeks in MS-medium and incubated shaking under continuous light conditions with an atmosphere containing 2% CO_2. Cells were used for the experiments during the second week after subculturing. To ensure same starting conditions for all cultures of an experiment, cultures were mixed and divided again 2 hours before treatment.

Stimuli and inhibitors were applied at the following concentrations unless indicated differently: E-FOL, 1 µg dry hyphae/ml culture; chitosan, 100 µg/ml; polygalacturonic acid (PGA), 100 µg/ml; salicylic acid, 250 µM; cycloheximide, 5 µg/ml; α-amanitin, 5 µM; gadolinium(III) chloride, 1mM; nifedipine, 20µM; EGTA, 8mM, calcium chloride 8mM.

1% (w/v) PGA (Sigma) was dissolved in 0,1 N NaOH and dialysed for 18 h in water (Ohto et al., 1992). 1% (w/v) chitosan (Roth) was dissolved in 1 M acetic acid and dialysed for 18 h in water.

Voltage treatment

To generate an electrical field in culture vessels two platinum wires were submerged as electrodes in a cell culture of 50 ml. The electrodes were designed as a large and a small circle creating a distance in-between of 25 mm. Voltage was applied for 10 seconds by two Gene Power Supply GPS200/400 (Pharmacia). A current of 0.7 ampere was observed at 60 volt.

Preparation of an elicitor from Fusarium oxysporum lycopersici

The pathogenic fungus *Fusarium oxysporum* Schlecht.: Fr.f.sp. *lycopersici* (Sacc.) was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). The fungus was cultured in a medium containing 50 g/l glucose, 8 g/l casamino acids, 0.5 g/l yeast extract, 0.2 g/l $MgSO_4$ and $FeSO_4$ each, 20 mg/l $CaCl_2$, 1.5 mg/l $MnSO_4$ and Na_2MoO_4 each, in 25 mM potassium phosphate at pH 7.5. After four days of shaking at 29°C the culture was autoclaved, washed 3 times with water, and lyophilized.

Extraction of mRNA and RNA gel blot analysis

For the isolation of RNA, cells were harvested by centrifugation, snap frozen in liquid nitrogen and ground in the presence of liquid nitrogen. Total RNA was isolated according to the method described in Chomczynski and Sacchi (1987). Northern blot analysis was carried out as described previously (Godt and Roitsch, 1997).

H_2O_2-determination

For H_2O_2-determination 100µl of culture supernatant was mixed with 800 µl of 70 µM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in 50 mM $KHPO_4$ pH 7.9. Light emission was started by addition of 100 µl 12,5 mM $K_3Fe(CN)_6$ in water and

quantified with a FB12 luminometer (Berthold, Germany).

Preparation of crude extracts

Cells were harvested by centrifugation, snap frozen in liquid nitrogen and ground in the presence of liquid nitrogen. Extracts from ground cells were prepared in the following buffer: 100 mM Hepes/KOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 10% (w/v) glycerol, 7.5% (w/v) polyvinylpolypyrrolidone, 1 µg/ml antipain, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM benzamidine.

In-gel kinase assay

For the determination of kinase activity in polyacrylamide gels, crude extracts were centrifuged for 10 min at 20,000g. An aliquot of the supernatant containing 40 µg total protein as determined by bradford assay (Bradford, 1976) was loaded on a 10% polyacrylamide gel embedded with 0.3 mg/ml of myelin basic protein (MBP, Upstate Biotechnology) in the separating gels as substrate for the kinase. As control for substrate specificity MBP was substituted by the same amount of histone or caseine. After electrophoresis proteins were renatured and assayed for kinase activity as described by Zhang and Klessig (1997). Activities were visualised by autoradiography and by a phosphor imager (Cyclone, Phosphor Storage Systems, Madison).

In-solution kinase assay

For kinase determination in solution 5 µl of sample was mixed with reaction buffer to give a final volume of 15 µl containing 25 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5 mg/ml MBP, 25 µM ATP and 1 µCi ^{32}P-γ-ATP. Incubation at room temperature was stopped after 20 min by addition of 10 µl 5xSDS sample buffer. The unincorporated ^{32}P-γ-ATP was separated from the MBP by SDS-gel electrophoresis. Activities were visualized and quantified using a phosphor imager (Cyclone, Phosphor Storage Systems, Madison).

Inhibitor studies

The inhibitors bohemine (6-benzylamino-2-(3-hydroxypropylamino)-9-isopropylpurine), olomoucine (6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine) and A.2.3.9R (6-(3-hydroxybenzylamino)-2-(R)-(1-(hydroxymethyl)propylamino)-9-isopropylpurine) were synthesised (Havlicek et al., 1997) and prepared as 100 mM stock solutions in pure dimethyl sulfoxide. A.2.3.9R is derivative of roscovitine with an additional hydroxy group on the N6 benzyl ring. It is referred to as roscovitine throughout this publication. A 100 mM solution in dimethyl sulfoxide was diluted in water to a final concentration of 100 µM in the in-solution kinase assay. Staurosporine (Boehringer Mannheim) was used at a final concentration of 1 µM.

Immunoprecipitation with MMK antibodies

The antibodies specific for SIMK (M23), MMK2 (H140), MMK3 (H141) and SAMK (M24) were a generous gift from H. Hirt (University of Vienna, Vienna, Austria). 1 µl serum, 0.1% (v/v) Nonidet P-40 and 75 mM NaCl were added to 60 µl of the chromatography fraction to be analyzed. After 2 hours of shaking at 4°C 20 µl protein A-

sepharose (50% suspension, Calbiochem) was added. After another 3 hours we proceeded with washings and kinase assay exactly as described by Munnik et al. (1999) to ensure the same specificity.

Immunoprecipitation with phosphotyrosine specific antibody

For the immunoprecipitation 200 µg of total protein in a crude extract was brought to 150 mM NaCl and 1% (v/v) Nonidet P40. After addition of 1 µg of the phosphotyrosine specific monoclonal antibody 4G10 (UBI, Lake Placid, USA) the assay was shaken at 4°C for two hours and, after the subsequent addition of 15 µl of protein A-sepharose (50% suspension, Calbiochem), for another 4 hours. Competing phosphoaminoacids phosphoserine, phosphothreonine and phosphotyrosine were included in the precipitation mixture in a concentration of 1 mM. The protein A-sepharose with the bound antigen was pelleted at 20,000 g for 2 minutes and washed with 500 µl of 100 mM Hepes/KOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 10% (w/v) glycerol, 1 µg/ml antipain, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM benzamidine, 150 mM NaCl and 1% (v/v) Nonidet P40. The pellet was resuspended in 40 µl of SDS-loading buffer and incubated at 37°C for 15 minutes and at 65°C for another 15 minutes. After centrifugation the supernatant was analyzed by in-gel kinase assay with MBP as substrate.

Chromatographic separation

Ground cells of a 50 ml culture were used to prepare the crude extract with 20 ml of buffer A (25 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 5% (w/v) glycerol, 20 mM β-glycerophosphate, 10 mM NaF) containing 0.1 mM PMSF, 1 mM benzamidine and 1 mM Na_3VO_4. After 40 minutes of centrifugation at 120,000 g supernatant containing 20 mg total protein was loaded on the strong anion-exchange column Resource Q (6 ml, Pharmacia). The column was washed with 30 ml of buffer A and then eluted with a 80 ml linear gradient of 0 to 500 mM NaCl in buffer A. Fractions were analyzed by in-solution kinase assay or in-gel kinase assay and quantified by phosphor imager. Relative activities depicted in Fig. 4C were calculated by dividing the measured activity by the basal activity of unstimulated cells processed in the same way.

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A Heat-activated MAP Kinase in Tomato: a possible regulator of the heat stress response

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A HEAT ACTIVATED MAP KINASE IN TOMATO: A POSSIBLE REGULATOR OF THE HEAT STRESS RESPONSE

Adaptation to elevated temperatures is of major importance for the survival of plants. The role of kinases in heat stress response was studied in tomato by *in gel* and *in solution* kinase assays using myelin basic protein as substrate. The application of a heat stress in a naturally occurring temperature range resulted in a fast and transient activation of a 50kD MAP kinase both in a photoautotrophic cell suspension culture and in leaves of mature plants. The heat activation of the MAP kinase was shown to be calcium dependent. The specific phosphorylation of tomato heat stress transcription factor HsfA3 by a partially purified preparation of the heat activated MAP kinase supports a physiological role of the identified kinase activity in transducing the heat stress signal.

1. Introduction

Changes in environmental temperature are a highly parameter to which cellular functions of plants have constantly to adapt. As sessile life forms, plants have had to develop mechanisms that can sense and respond to temperature changes in a very fast and flexible way to ensure cellular functions over a surprisingly large range of temperatures. The response to heat stress is usually transient in nature and helps to maintain cellular function over a rather short time of heat exposure, like during the daily hours of higher temperatures but also may lead to an increased overall thermotolerance [1]. The heat stress response is characterised by a large attenuation of transcriptional and translational activity with the exception of heat shock proteins (HSPs) which accumulate in a dose dependent manner and seem to account for a major part of thermotolerance [2,3]. The regulation of HSP activity occurs mainly on the transcriptional level, via heat shock elements (HSEs) located in the promotors of the heat shock genes. The HSEs in turn provide binding sites for the heat stress factors, transcription factors that become activated upon heat stress and drive HSP expression, with HsfA1, which is a thermo inducible protein itself as a central regulator [4]. However, only little is known about the molecular events leading to Hsf activation and Hsp expression to induce thermotolerance. It has been shown for higher eucaryotes, that upon heat stress, Hsfs undergo trimerisation and thereby acquire high binding affinity to HSEs and transactivation potential [5]. In addition, phosphorylation events have been implicated in the regulation of Hsfs and it has been shown, that Arabidopsis Hsf1 is a substrate of CDC2 [6]. Mitogen activated protein (MAP) kinases are involved in biotic and abiotic stress mediated defence reactions [7-11] and were found to phosphorylate Hsfs in yeasts and mammals [12-14]. This study is focussed on the characterisation of the very early responses of tomato plants and cell cultures to heat stress. Here we present data on the specific activation of a 50kD MAP kinase by heat stress and provide indication on the possible involvement of this MAP kinase in the regulation of the heat stress response by phosphorylating a heat stress transcription factor.

2. Materials and Methods

2.1 Cell culture conditions and plant growth

Photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum)* were established by [15] and were subcultured every 2 weeks in Murashige and Skoog medium under constant illumination and in a 2% CO_2 atmosphere. Cells were used for experiments as described in [16]. Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were grown in the greenhouse under a 16h light, 8h dark regime. Plants were used for the experiments after 9 weeks. When experiments involved a change in the environment for the plants, they were allowed to adapt for 16h.

2.2 Heat stress treatments

Heat stress was applied to the suspension cultures by the addition of prewarmed, preconditioned culture medium to the cells. For a single experiment, the appropriate amount of cell cultures were mixed and redistributed into the culture flasks. Remaining cell culture was cleared and the cell free medium was used as preconditioned medium for the experiments. Unless otherwise indicated, experiments were performed by adding 21ml of 80°C preconditioned medium to 40ml of cell culture, which resulted in a temperature increase of 12°C. The cells were then incubated at 26°C. To challenge plants, they were either exposed to direct sunlight, shifted from a 26°C to a 37°C growth chamber or by dipping leaves into 37°C water.

2.3 MAP kinase assays and inhibitor treatments

In gel kinase assays, *in solution* assays, immunoprecipitations and Ca^{2+}-Channel inhibitor treatments were performed as described in [16].

2.4 Partial purification of the heat activated MAP kinase

To enrich the heat activated MAP kinase for in solution kinase assays, crude cell extracts were applied to a ResourceQ anion exchange column and processed as described in [16]. One single peak of kinase activity eluted from the column at concentrations of 150 to 200mM NaCl.

2.5 HsfA1 and HsfA3 overexpression and purification

A C-terminal His-tag fusion to HsfA1 was created by insertion of a synthetic oligonucleotide linker (5` tatgctcgtgcctcgcggcagccatcaccatcaccatcactaa gcttggtac 3´) into pRTHsf8LS [17] cut with NdeI and Asp718I. The coding sequence of the fusion protein was released with EcoRI and ApaI and religated into pJC20 to create pJCA1HC which was used for overexpression. HsfA1 was overexpressed in *E.coli* BL21 by addition of 0.4mM IPTG followed by 3h incubation at 30°C. The protein was purified using the HisTrap system (Pharmacia, Freiburg, Germany) by sonyfying the cells in 25mM HEPES/KOH pH 7.6, 500mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 10% glycerol, 100µM PMSF. Columns were washed with 20mM Na-Phosphate buffer containing 10mM imidazole and eluted with the same buffer containing 250mM imidazole. To overexpress HsfA3, the DNA binding domain of the factor was deleted by PCR using the oligonucleotides HsfA3/MBP5` (gaattcatctctttatatgatatgaacc) and HsfA3/MBP3` (gcagcctgaagcttttcattaacggatcc) with pJCHN-HsfA3 [18] as a template. The resulting fragment was subcloned into pGEM-T (Promega, Madison, WI, U.S.A.) and released with EcoRI and PstI to be ligated into pMALc (NEB, Schwalbach, Germany) to create an N-terminal in-frame fusion with the maltose binding protein. Induction of protein synthesis was carried out by addition of 0.5mM IPTG and incubation for 16h at 26°C. The fusion protein was purified on amylose resin (NEB, Schwalbach, Germany) according to the manufacturer's instructions. For *in*

solution kinase assays, 3µg of each purified protein were added per reaction.

2.6 Antibody production

To generate antibodies directed against different MAP kinases a cDNA fragment spanning the eleven domains that are conserved among MAP kinases [19] was cloned from MAP kinase MMK 4 of *Medicago* [7]. Using primers OMK3 (TCCTGGATCCATGCCGATTGGTCGTGGTGC) and OMK4 (TTTTAAGCTTTAAGCATACTCAGGATTG) a 1000 bp cDNA fragment with a BamHI site at the 5´-end was amplified by reverse transcriptase PCR and cloned into the SmaI site of pUC18. The insert of the resulting plasmid pMK3-4 was released as BamHI fragment and cloned into pMalc (NEB, Schwalbach, Germany) to generate an N-terminal in frame fusion with the maltose binding protein. Induction of protein synthesis and purification of the fusion protein was carried out as described above. Polyclonal antibodies directed against the purified fusion protein were generated in New Zealand White Rabbits as described before [20].

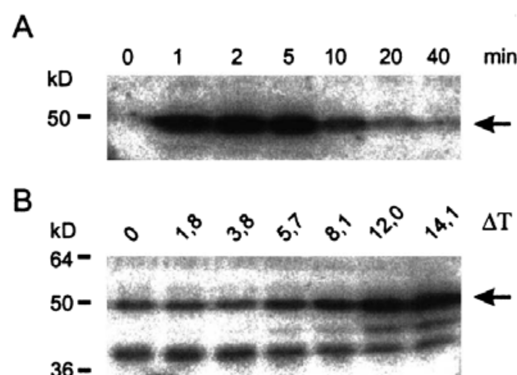

Fig. 1. Heat induced MAP kinase activation. **A**. Time course of activation of kinase after adding 21ml of 80°C preconditioned media to a 40 ml tomato cell culture resulting in a temperature increase of 12°C. **B.** Dose response of increase in temperature by adding 21ml of medium at different temperatures. Samples were taken 1 min after the temparuture shift. Kinase activity was analysed by an *in gel* kinase assay with MBP as substrate.

3. Results and discussion

1. A 50kD MAP kinase is activated in response to heat stress in tomato cell suspension cultures

In gel kinase assays using myelin basic protein (MBP) as substrates were carried out to determine the possible involvement of kinases in early heat stress signalling. To investigate fast responses to elevated temperatures in higher plants, we used photoautotrophic suspension culture cells of tomato (*L. peruvianum*) that were heat stressed by a fast increase in the temperature of the growth medium. To induce a heat stress in these cell cultures, we added heated, preconditioned medium to the cells. This treatment was found to be superior to e.g. shifting the cultures to the warmer environment of a different growth chamber since the resulting temperature changes were much more reliable in terms of extend and timing of the increase/decrease of the temperature gradient. For a first characterisation, the cells were challenged with a temperature increase from 26 to 38°C and cell extracts were tested at different time points for the activation of MBP-phosphorylating protein kinases. As shown in Fig. 1A, a protein kinase with an apparent molecular weight of 50kD is transiently

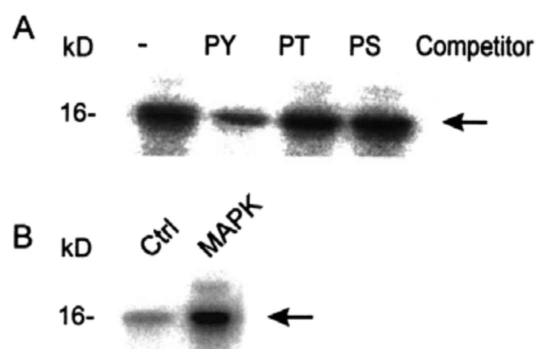

Fig. 2. Immunoprecipitation with phosphotyrosine and MAP kinase specific antibodies. A crude extract of heat stressed cells was subjected to immunoprecipitation and subsequent *in solution* kinase assay with MBP as substrate. **A.** Phosphotyrosine specific antibody 4G10 and competition with 1 mM phosphotyrosine (PY), phosphothreonine (PT) or phosphoserine (PS). **B.** Preimmuneserum (Ctrl) and serum with antibodies against conserved MAP kinase domains (MAPK).

activated only 1 minute after the treatment and is found to be deactivated approximately 10 minutes after the heat stress. This fast and transient activation is a typical feature of early response elements and was found to often occur in the activation patterns of stress-related MAP kinases [21-24]. To further investigate the specificity of the heat stress treatment, we conducted a dose response experiment (Fig. 1B) which reveals, that the protein kinase is not activated by temperature shifts of less than 5.7°C and that the activation is maximal at a temperature difference of 12°C. At activating temperatures, an additional but weaker phosphorylation signal becomes evident. However, since this signal is not present in the experiment shown in Fig. 1A and appears in parallel with the 50kD signal, it is considered as a degradation product of the activated protein kinase.

Apart from the fast and transient activation, another typical feature of MAP kinases is their phosphorylation on tyrosine residues upon activation [25]. To test whether this feature is also present in the heat activated protein kinase, we performed immunoprecipitations using the phosphotyrosine specific monoclonal antibody 4G10. As it is shown in Fig. 2A, this antibody effectively precipitates the heat activated protein kinase. The specificity of this reaction is demonstrated by the use of P-tyr, P-thr and P-ser as competitors of whom only P-tyr was able to hinder binding of the antibody. To gain further support to place the heat activated protein kinase into the group of MAP kinases, we also performed an immunoprecipitation using a polyclonal serum directed against the conserved MAP kinase domains. The antibody was raised against mmk4 MAP kinase excluding its N-terminus, and should thus be able to recognise tomato MAP kinases since the kinase domains of mmk4 and those encoded by tomato MAPK ESTs are highly homologous and show several identical stretches. As depicted in Fig. 2B, this polyclonal serum effectively precipitates the heat activated protein kinase, whereas the control reaction using the preimmune serum does not display any kinase activity in the precipitate. These various lines of experimental evidence support that the heat activated protein kinase belongs to the family of MAP kinases.

MAP kinases have also been reported to be involved in the regulation of the heat stress response of yeast and mammals [14,26]. The involvement of a MAP kinase in the heat response of plants thus demonstrates that the heat response is a conserved mechanism

probably following analogous pathways in all eucaryotes.

2. The activation of the heat activated MAP kinase is dependent on calcium influx

Calcium signalling is involved in several stress related signal transduction processes. Specifically, for the induction of a heat stress response a strong dependence on extracellular calcium has been demonstrated in sugar beet cells [27]. In another report, the dependence of heat stress factor activation in rabbit heart cells on calcium was demonstrated by the use of the calcium channel inhibitor gadolinium [28]. To elucidate the underlying signal transduction pathway of heat MAP kinase activation in plant cells, we tested the involvement of calcium in heat stress signalling by the use of calcium channel inhibitors. As demonstrated by the *in gel* kinase assay shown in Fig. 3, treatment of the cells with gadolinium and lanthanium effectively inhibited MAP kinase activation by the heat stress. Likewise, depletion of calcium ions from the culture medium by EGTA also hindered MAP kinase activation by the heat stress, which was only retained by the concomitant addition of equal molar amounts of calcium. Therefore, the heat activated MAP kinase acts downstream of a calcium mediated signal transduction process.

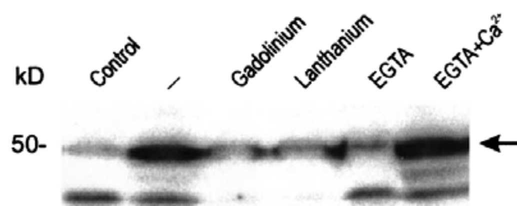

Fig. 3. Heat induced MAP kinase activation depends on calcium signalling. Gadolinium (1mM), Lanthanium (1mM), EGTA (8mM) or EGTA (8mM) and calcium (8mM) were added 4 min before heat stress treatment (temperature increase of 14°C). Samples taken 1min after stimulation were analysed by an *in gel* kinase assay with MBP as substrate.

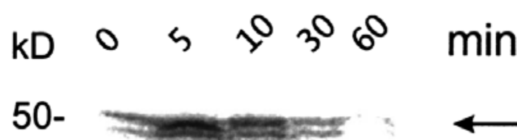

Fig. 4. Activation of heat activated MAP Kinase *in planta*. Tomato plants were moved to bright sunlight and the leaves were analysed for activation of MAP Kinase at the indicated time points by an *in gel* kinase assay with MBP as substrate.

3. Activation of the Heat Activated MAP kinase in planta

To relate our observations in the cell culture system to the physiological significance *in planta*, we determined the temperature increase on leaf surfaces that were suddenly exposed to bright sunlight. Interestingly, and in accordance with the dose response pattern observed in the cell cultures, we found this temperature increase to be approximately 11°C. *In gel* kinase assays were performed with leaves of plants that were shifted to bright sunlight. The experiments were carried out in the greenhouse to exclude the effect of UV light exposure. The results from a representative sunlight experiment are shown in Fig. 4. By analysis with *in gel* kinase assays using MBP as substrate, a 50kD protein kinase is found to be activated shortly after exposure to sunlight. As it is the case in cell cultures, the activation of the MAP kinase is fast and transient, showing activation after 5

minutes and a return to steady-state activity after 30 minutes. Similar results for heat activation of MAP kinase activity *in planta*, although with different activation kinetics, were obtained with other heating methods such as dipping detached leaves into warm water or moving plants to another climate chamber (data not shown). In order to exclude light as an activator of the MAP kinase [29,30] plants were shifted between two climate chambers without exposing them to daylight. This experiment also lead to the activation of the heat activated MAP kinase (data not shown). In contrast to our findings, a heat inactivated MAP kinase has been described in heterotrophic tomato cell cultures [31]. However, our study used autotrophic cell cultures and mature leaves, which both can be considered as source organs. Interestingly, the inactivation of the MAP kinase was observed in a system rather resembling a sink organ, thus pointing towards different modes of regulation of MAP kinase(s) in response to heat stress in source and sink organs.

4. The heat activated MAP kinase phosphorylates HsfA3

In the yeast *Saccharomyces cerevisiae*, the heat stress response is part of the cell integrity pathway, in which gene activation in response to heat is mediated by a MAP kinase module [14] which ultimately leads to the production of heat stress proteins (Hsps), that seem to account for a major part of induced thermotolerance [32]. One major component of this pathway is the heat stress factor 1 (Hsf-1), a transcription factor that is involved in the upregulation of Hsps, which was demonstrated by temperature sensitive mutants lacking a functional Hsf1 [33]. In plants, several homologs to Hsf1 from yeast have been described [18,34]. Interestingly, some of these Hsfs, along with another homolog from Drosophila can functionally complement Hsf function in yeast [35,36] which shows that the signal transduction pathway leading to the activation of a heat stress response is highly conserved among eucaryotes. All four known heat stress factors in tomato contain several copies of consensus MAP kinase phosphorylation sites ((PX)[(S/T)P]) [18,34], with HsfA1 (9 times (S/T)P, 1 time ((PX)[(S/T)P]) and HsfA3 (8 times (S/T)P, 1 time ((PX)[(S/T)P]) having the highest number of sites. The factors were thus expressed in *E. coli* as maltose binding protein (A3) or His-Tag (A1) fusions and the purified proteins were used in *in solution* kinase assays to test their potence as MAP kinase substrates. As it is shown in Fig. 5, when purified HsfA3 is incubated in the presence of a partially purified heat activated MAP kinase, a strong phosphorylation of the recombinant protein is observed at the predicted molecular mass of 70 kD. No phosphorylated protein is observed at this position when the substrate is omitted from the reaction or is replaced by the maltose binding protein. Myelin basic protein was included as a positive control. Interestingly, HsfA1 is not phosphorylated in this assay, indicating a selective recognition of HsfA3, since both proteins display MAP kinase phosphorylation sites. The specific phosphorylation of HsfA3 thus supports the idea of an involvement of a MAP kinase(s) in the regulation of the heat stress response of

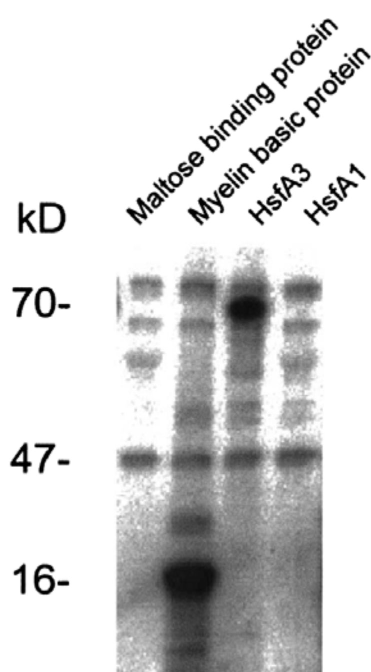

Fig. 5. Purified, recombinant heat stress factors HsfA3 and HsfA1 were used as substrates for the partially purified heat activated MAP kinase in an *in solution* kinase assay. As controls, purified maltose binding protein alone and myelin basic protein (MBP) were also used as substrate in separate reactions.

higher plants. This implies that not only the molecular mechanisms of transcriptional regulation but also upstream components of the heat stress induced signalling are conserved in eucaryotes. MAP kinases were also found in mammals to be involved in heat stress signalling [12,13], although the system appears to be regulated by more complex mechanisms than it is the case for yeast [37,38]. However, in yeast as well as mammals, MAP kinases have been shown to directly phosphorylate Hsf1 *in vivo* [39]. Although MAP kinases belonging to several subfamilies have been identified in many plant species and were shown to be involved in various signal transduction pathways [6-10], so far no physiological substrate has been identified in plants. The finding that the heat activated MAP kinase from tomato specifically accepts HsfA3 as a substrate, but not HsfA1 gives a strong indication for the first physiological MAP kinase-substrate described for plants. Recently, it has been published that tomato HsfA1 is a central regulator of the heat stress response that seems to affect HsfA2 and HsfA3 activity [4]. The heat activated MAP kinase could thus act as a “safety” switch to also initiate the heat stress response. Further investigation will aim to show the direct interaction of the heat activated MAP kinase with HsfA3 *in vivo* to elucidate the exact regulatory role of the heat activated MAP kinase in the heat stress response.

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Phosphorylation of LpWRKY1: Transient activation of two protein kinases during the elicitation of defence response in tomato

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Phosphorylation of LpWRKY1: Transient activation of two protein kinases during the elicitation of defence response in tomato

The initiation of defence responses in plants is accompanied by fundamental changes in gene expression: the expression of pathogenesis-related genes is co-ordinately regulated with metabolic changes such as downregulation of photosynthesis and induction of sink metabolism. To identify candidate regulators of this co-ordinated regulatory mechanism, the role of WRKY transcription factors in the initiation of defence response was analysed in tomato. A WRKY- type transcription factor (LpWRKY1) from tomato was cloned by a reverse Northern approach. The corresponding mRNA is rapidly and transiently induced after challenging the cells with an elicitor-preparation derived from the wilt inducing fungus *Fusarium oxysporum lycopersici* (E-FOL) and the fungal elicitor chitosan, whereas the endogenous signals systemin and salicylic acid are inactive. Inhibition of protein biosynthesis by cycloheximide results in sustained induction of mRNA for LpWRKY1. In contrast, the transient induction of the gene encoding LpWRKY1 in response to elicitation by E-FOL is inhibited by the protein-kinase inhibitor staurosporine and may be mimicked by the phosphatase inhibitors endothall and cantharidine indicating the involvement of protein phosphorylation in the regulation of WRKY-type transcription factors. Direct proof of this postranslational modification of LpWRKY1 was obtained by demonstrating in vivo phosphorylation and by in-gel kinase assays using recombinant LpWRKY1 as substrate. A 44kDa and a 67kDa protein kinase were shown to be transiently activated to phosphorylate LpWRKY1 protein in response to elicitation with E-FOL.

Introduction

If a plant is challenged by a pathogen, several waves of gene activation or repression occur. Depending on the pathogen, appropriate defence-response genes are activated (1) so that the plant restricts the growth and spread of fungi or bacteria. The expression of defence-related genes is invoked by a ligand-receptor interaction (2) which leads to the expression of immediate early genes. The products of these genes are usually believed to be regulatory proteins that control subsequent cellular reactions. Recently a class of transcription factors that is involved in defence responses and that shows the characteristics of immediate early genes has been discovered in plants (3-5). After a strictly conserved amino acid motif, these proteins are called WRKY transcription factors, which comprise a superfamily of 75 members in *Arabidopsis*. The WRKY domain contains the WRKYGQK motif and a putative zinc finger domain of the C_2-H_2 type at its C-terminal end (6). The WRKY domain occurs either once or twice in the known WRKY proteins, which separates them into class 1 WRKY proteins (two WRKY domains) and class 2 proteins (one WRKY domain). A third group of WRKY proteins carries one WRKY domain with a C_2-HC zinc finger (5). All WRKY proteins characterised so far bind to the cis-acting element (T)(T)TGAC(T/C) known as the W-box (3,6-8) which is found in the promoter regions of WRKY (9) and defence-related genes (10,11). The fact that the promoters of the genes encoding sporamine and β-amylase from sweet potato (8) are characterised by binding sites for WRKY transcription factors indicates that the regulation of diverse processes such as hormone and metabolic regulation as well as trichome development involve WRKY transcription factors. Adding up to this complexity are more than 500 ESTs encoding putative WRKY proteins present in the databases, which points towards a central regulatory role of these plant specific transcription factors.

Elicitation of photoautotrophic suspension culture cells of *Chenopodium rubrum* with either the stress related stimulus chitosan or the metabolic stimulus D-glucose results in the downregulation of photosynthesis, whereas sink metabolism is induced (12). Simultaneously defence responsive genes like phenylalanineammonia-lyase are upregulated. We have shown, that the signal transduction pathways leading to this co-ordinated regulation share at least in part common components, and that these pathways involve the rapid and transient activation of MAP-kinases (12). To further dissect the signal transduction pathway leading to a co-ordinated regulation of the defence response and source/sink relations, we investigated the mechanism that initiates this concerted gene regulation in photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum*). To get a detailed insight into the regulation of the defence response we studied the possible role of immediate-early genes as the final targets of this signal transduction pathway, that display candidate early regulators of those genes subject to regulation

by pathogen infection. An elicitor preparation of the wilt inducing fungus *Fusarium oxysporum lycopersici* (E-FOL), a naturally occurring pathogen of tomato, was shown to result in various elicitor-induced reactions. Rapid induction of changes in phenylpropanoid metabolism (13) are accompanied by effects on source/sink relations (14) supporting the co-ordinated regulatory mechanism demonstrated in *C. rubrum*.

In the present study, a WRKY transcription factor was cloned from tomato by a reverse-northern approach that is rapidly induced by E-FOL and the fungal elicitor chitosan in autotrophic suspension culture cells. We demonstrate, that the expression of this transcription factor is dependent on protein phosphorylation and that the WRKY protein is phosphorylated by two transiently activated kinases. Based on the findings presented in this study, we propose an extension of the current model of gene regulation through WRKY transcription proposed by Eulgem et al. (9).

Materials and Methods

Growth of cell suspension culture cells—Photoautotrophic suspension culture cells of *L. peruvianum* L. were established by (13) and are being sub cultured every two weeks in MS-medium and incubated shaking under continuous light conditions with an atmosphere containing 2% CO_2. Cells were used for the experiments during the second week after sub culturing. Stimuli and inhibitors were applied at the following concentrations: FOL, 150µg dry hyphae/ml culture; chitosan, 0.1%; SA, 250µM; systemin, 250nM; cycloheximide, 5µg/ml; staurosporine, 2µM; endothall, 50µM; cantharidine, 50µM.

Preparation of an elicitor from F. oxysporum lycopersici—The pathogenic fungus *F. oxysporum* Schlecht.: Fr.f.sp. *lycopersici* (Sacc.) was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). The fungus was cultured in a medium containing 50 g/L glucose, 8 g/L Casamino acids, 0.5 g/L yeast extract, 0.2 g/L $MgSO_4$ and $FeSO_4$ each, 20 mg/L $CaCl_2$, 1.5 mg/L $MnSO_4$ and Na_2MoO_4 each, in 25mM potassium phosphate at pH 7.5. After four days of shaking at 28°C the culture was autoclaved, washed with water, and lyophyllised. For the induction of stress response, 100 mg/L of the dried hyphae were added to a tomato cell suspension culture.

Construction of the WRKY cDNA library and reverse-northern screening—A cDNA library was constructed from tomato cells that were previously treated with E-FOL for 30 min. Total RNA was used for reverse transcription using random hexamere primers and M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturers instructions. Subsequently, PCR was performed with the degenerate oligonucleotides Omh-7 (5`-GAY GGW TAY AAY TGG MGD AAR TAY GGW CAR AA-3´) and Omh-8 (5´-RTG RTY RTG YTK WCC YTC RTA WGT WGT –3´) which are directed against the conserved WRKY domains. The products of this PCR were then cloned into pUC57 using the T/A cloning kit (MBI, St. Leon-Rot, Germany). For reverse-northern,

DNA was isolated from these clones and PCR was performed in 96 well plates to amplify the inserts of the individual clones, using Omh-7 and Omh-8 as primers. The PCR reactions were evaluated by agarose gel electrophoresis and then diluted with 1.5M NaCl, 0.5M NaOH. One half of this dilution was then spotted on one nitrocellulose filter prewetted in the same solution using a 96 well vacuum manifold. After UV-crosslinking, the membrane was blocked in a modified church buffer (0.24M Na_2HPO_4 pH 7.2, 7% SDS, 1mM EDTA). For the detection of putatively regulated clones, total RNA from control and FOL treated cells was reverse-transcribed in the presence of ^{32}PdCTP. This reaction mixture was then treated with 1.3mM NaOH at 95°C and was then added to the prehybridisation solution. After overnight incubation, the membranes were washed with 2XSSC/ 0.1%SDS and 0.2XSSC/ 0.1%SDS and then evaluated using a phosphorimager. Those clones showing a positive signal compared to the corresponding control membrane were then re-evaluated by northern-blot analysis.

Cloning of LpWRKY1, heterologous expression and plasmid constructions—After the identification of an E-FOL inducible clone, the corresponding cDNA fragment was cloned into the pMALc vector to create an in frame maltose binding protein fusion. Heterologous expression and purification by affinity chromatography was performed according to the manufacturers instructions (New England Biolabs, Schwalbach, Germany). To obtain the full lengh sequence, available EST sequences were used to define the 3`end sequence. The 5`end was cloned by two successive genome walks. The full-length sequence was obtained by RT-PCR using the primers W13flf (atgatgaactcatcaacatcacc) and W13flr (aagtatatcaatgtcacttgtgg). The product was cloned into the SmaI site of pUC19 which yielded the plasmid pW13-5`. The missing 3` end was then isolated from the EST cLEC17K24 using the MluI and XbaI sites. This fragment was then fused to the 5`end in pW13-5´ by using the endogenous MluI site in LpWRKY1 and XbaI. Since we failed to express the full length sequence of LpWRKY1 in E.coli and in yeast, we sub cloned the two WRKY domains using the EcoRI and PstI sites into pMalc to express this polypeptide to be used in gelshift assays. To create a 6XHis tagged version of LpWRKY1, the near full length cds was released from pUC19 with KpnI and SacI and cloned into pYES2/CT (Invitrogen). From this plasmid, the construct was released with KpnI and MmeI, which collects the His-tag and a stop codon from pYES2/CT and was cloned into the KpnI and Ecl136II sites of pPS1, which contains a 35S promoter and terminator. This construct was used for the transformation of BY2 cells.

Extraction of mRNA and RNA Gel Blot Analysis—For the isolation of RNA, cells were harvested by centrifugation, snap frozen in liquid nitrogen and ground in the presence of liquid nitrogen. Total RNA was isolated according to the method described in (15). Northern blot analysis was carried out as described previously (16).

In-gel kinase assay—For the determination of kinase activity in polyacrylamide gels, extracts from ground

cells were prepared in the following buffer: 100mM Hepes/KOH pH 7.5, 5mM EDTA, 5mM EGTA, 10mM DTT, 10mM Na_3VO_4, 10mM NaF, 50mM β-glycerophosphate, 10% glycerol, 7.5% polyvinylpyrolidone. The extracts were loaded on 10% polyacrylamide gels (2 ml bed volume) embedded with 600µg of maltose-binding protein/LpWRKY1 fusion or 600µg of maltose binding protein. For controls without any substrate, the according volume was substituted for by water and processed as described in (17).

Transformation of BY2 cells—to transform BY2 cells, cells were co-cultivated with agrobacteria carrying the LpWRKY1::6XHis expression plasmid for three days in B5 Medium (4.3g/L MS medium, 2g/L MES, 100mg/L Inosit, 30g/L sucrose, 200mg/L KH_2PO_4, 1mg/ml Thiamin, 1mg/ml 2,4D, pH5.8), washed in B5 medium and then plated on B5/3% gelrite in 2% agarose containing 500µg/ml carbenicilin and 100µg/ml kanamycine. Plates were incubated at 28°C for several weeks until calli formed. Individual calli were then solubilised in B5 medium and subcultured every week. The cells were tested for the transgenes by RT-PCR.

In vivo phosphorylation of LpWRKY1—BY2 control cells and BY2 cells expressing LpWRKY1::6XHis were grown in the presence of 200µCi ^{32}P phosphoric acid and 50µM endothall in B5 medium. Cells were harvested at different time points by filtration, snap frozen in liquid nitrogen und ground. The material was then thawed in 20mM phosphate pH 7.5, 500mM NaCl, 10mM Imidazole, 10mM Na_3VO_4, 10mM NaF, cleared and loaded onto Probond Resin (Invitrogen). After three washes with 10 times the resin volume of lysis buffer, bound protein was eluted in the same buffer containing 300mM Imidazole. Eluted protein was then subjected to SDS-PAGE followed by phosphorimager analysis.

Gelshift assay—gelshift assays were performed using 2fmol of a 200bp fragment from the LpWRKY1 promoter containing the W-boxes per reaction. The DNA probe was end labelled with ^{32}P using T7 polynucleotide kinase. LpWRKY1 was purified from E.coli BL21 cells by affinity chromatography on amylose resin (New England Biolabs, Schwalbach, Germany) and was used at a concentration of 1µg per reaction. As noncompetitor, a 300bp fragment from the LpWRKY1 cds was used. Standard reactions contained 10% glycerole, 25mM Tris/Cl pH 7.5, 10mM $MgCl_2$, 0.2mM $CaCl_2$, 1mM DTT and 0.6µg polydIdC. Reactions were incubated at room temperature for 20 minutes and were then run on 7.5% acrylamide gels in 0.5X TBE.

Results and Discussion

Cloning of the LpWRKY1 cDNA by reverse northern—To gain insight into the regulation of the defence response of tomato in response to infection by the wilt inducing fungus F. oxysproum lycopersici, we investigated the role of the recently discovered WRKY transcription factors (for a recent review refer to (5). To identify possible candidate genes that are induced after treatment with an elicitor preparation of F. oxysporum (E-FOL), we designed degenerate oligonucleotides directed against conserved

Fig.1 Schematic diagram of LpWRKY1. In A, a sketch of the WRKY gene is shown with the position of the start and stop codons and of the two WRKY domains is given. Restriction sites used to generate plasmids used in this study are indicated. In B, the sequence 5\` of the start codon is shown, W boxes are indicated by arrows. The underlined portion of the sequence is the fragment used for gelshift assys. The start codon is boxed.

domains of a class 1 WRKY protein consensus sequence. Using these oligonucleotides we constructed a cDNA library from E-FOL treated photoautotrophic suspension culture cells of tomato (L. peruvianum), which was then screened by using a reverse-northern approach (see methods section for details). Putatively positive clones were then re-evaluated by northern blot analysis. This procedure yielded four clones that proved to be induced after treatment with E-FOL. These four clones were subjected to DNA sequencing and were found to be identical, showing about 95% homology to the NtWRKY1 protein (Acc. No AB022693) on the amino-acid level. Sequence comparison revealed that the cDNA fragment we obtained encodes a polypeptide that contains two WRKY domains that comply with the WRKY consensus sequence (3), showing the WRKYGQK motif and a putative zinc finger domain of the C-X_4-C-X_{22}-H-X_1-H type downstream of that motif. We designated the cloned cDNA fragment as LpWRKY1.

Transient induction of the mRNA for LpWRKY1 by fungal elicitors—To investigate the regulation of the LpWRKY1 gene, we conducted time courses on the induction of mRNA production in response to different stimuli. The northern blot analysis shown in figure 2A demonstrates that the LpWRKY1 gene is rapidly induced after treatment with E-FOL and chitosan, but not after treatment with salicylic acid or systemin. The concurrent activation of a defence response was, as a positive control, evaluated by probing the expression of the phenylalanineammonia-lyase gene (PAL). Since systemin and salicylic acid lead to the expression of PAL, but not to induction of LpWRKY1, it appears that the induction of this gene is independent of the endogenous, secondary signals systemin and

salicylic acid and thus appears to be specifically induced by elicitors not invoking production of those signals. The induction of LpWRKY1 by E-FOL and chitosan occurs in a fast and transient manner. mRNA production starts as early as 10 minutes after the application of either elicitor, with the maximum transcript abundance at 40 minutes. After 120 minutes, no mRNA encoding LpWRKY1 can be detected anymore. A fast and transient induction of immediate early transcription factors has been described before (3,18,19) and is considered as a common feature of these genes. The induction kinetics of LpWRKY1 is considerably faster than the one for the elicitor-inducible WRKY proteins 1 and 3 from parsley (3) and resembles more closely the induction kinetics of the tobacco wounding-induced WIZZ protein (18).

Since FOL is a naturally occurring tomato pathogen and since it is conceivable that E-FOL invokes similar reactions as chitosan does (Sinha and Roitsch, unpublished results), we focused the following studies on the elicitation of defence response by E-FOL. Figure 2B shows an analysis of how the protein biosynthesis-inhibitor cycloheximide affects expression of LpWRKY1 in response to treatment with E-FOL. As it can be expected for an immediate early gene, induction of mRNA production is not dependent on protein biosynthesis, however, this has not been demonstrated for WRKY-type transcription factors before. As it has already been observed for immediate-early genes (19,20), treatment with cycloheximide alone also leads to the induction of LpWRKY1. The induction kinetics is identical to that observed in the presence of

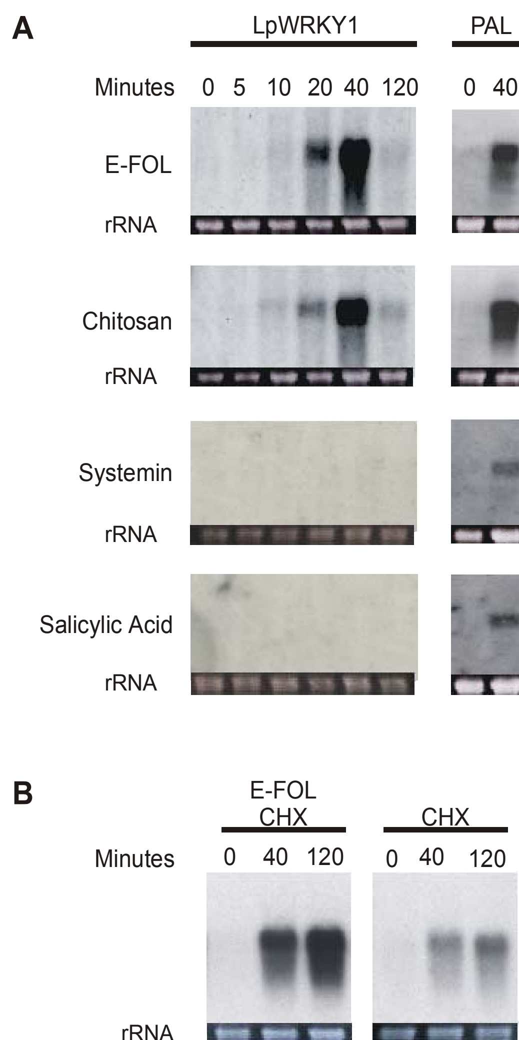

Fig. 2. Expression analysis of *Lycoper-sicon peruvianum* (Lp) WRKY1. (A) time courses of mRNA induction in response to an elicitor preparation from *Fusarium oxysporum lycopersici* (E-FOL), chitosan, systemin and salicylic acid. To verify the induction of a defence response, induction of PAL was also probed as a positive control. (B) Effect of cycloheximide (CHX) on LpWRKY1 induction. For both LpWRKY1 and PAL homologous probes were used. 20µg of total RNA was loaded and equal loading was verified by staining the rRNA bands with acridineorange.

E-FOL, however, the transcripts are far less abundant. Fukuda (21) demonstrated that the

binding of a nuclear factor to a W-box element is abolished by treatment of tobacco cells with cycloheximide together with an elicitor. Compared with our results, this demonstrates that LpWRKY1 is either induced by other factors or, according to the current model of WRKY gene regulation (9), the constitutively present amount of LpWRKY1 is not sufficient for secondary gene activation. Interestingly, by treatment with cycloheximide, the induction of the gene is conserved so that after 120 minutes transcripts are still present. Thus, cycloheximide appears either to inhibit the synthesis of a negative regulator of LpWRKY1 transcription or might simply affect the turnover of LpWRKY1 mRNA.

Effect of protein-kinase and protein-phosphatase inhibitors on LpWRKY1 mRNA induction—To shed further light on the regulation of the LpWRKY1 gene, we tested the effect of staurosporine, a general protein-kinase inhibitor, on the induction of LpWRKY1 by treatment with E-FOL. We chose a protein-kinase inhibitor as a starting point since it has been shown previously that elicitor-mediated stress responses include the activation of protein kinases (12). Secondly, phosphorylation is expected to be a common mechanism for the regulation of transcription factors (22) although this has only been shown for a few plant transcription factors (19,23-26). Finally, Yang et al. (11) have shown that the DNA binding activity of the tobacco WRKY protein TDBA12 can be abolished by phosphatase treatment, indicating the involvement of a protein kinase in the regulation of WRKY-type DNA binding proteins. As it is shown in figure 3A, induction of the LpWRKY1 gene can be abolished by concomitant treatment with staurosporine. Thus, it is likely that one or more phosphorylation events occur during the signal transduction pathway leading to the expression of LpWRKY1. Furthermore, this result is in accordance with the previously published report that staurosporine abolishes *in vitro* binding of nuclear factors from tobacco to W-box containing oligonucleotides (21). However, despite we demonstrate that LpWRKY1 is not induced in the presence of staurosporine, a posttranslational modification of WRKY-type transcription factors should not be excluded.

Since *de novo* protein synthesis is not needed for LpWRKY1 induction (figure 2B), all components of the signal transduction pathway should be constitutively present at a low level. Thus, a negative regulator has to be present that prevents inadvertent activation of the signal transduction pathway leading to LpWRKY1 gene induction. As staurosporine abolishes the activation of LpWRKY1 transcription and thus points towards the involvement of a protein kinase, such a negative regulator might be a phosphatase. We therefore studied the effect of the phosphatase inhibitors endothall and cantharidine on LpWRKY1 induction. As depicted in figure 3B, treatment of tomato cells with both endothall and cantharidine in absence of the elicitor E-FOL results in the induction of the LpWRKY1 gene. Interestingly, this induction is delayed in comparison to that occurring after treatment with E-FOL. This finding supports our hypothesis of a phosphatase as a negative regulator of a

protein kinase that invokes LpWRKY1 induction. The induction of a defence response in response to treatment with a

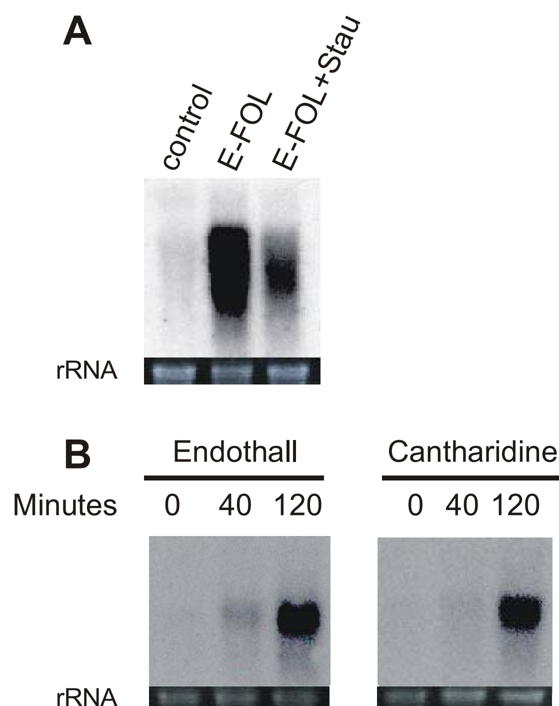

Fig. 3. Effect of protein-kinase and protein-phosphatase inhibitors on *Lycopersicon peruvianum* (Lp) WRKY1 induction. (A) Effect of staurosporine (Stau) on the induction of LpWRKY1 by an elicitor preparation from *Fusarium oxysporum lycopersici* (E-FOL). (B) Effect of endothall and cantharidine on the induction of LpWRKY1 in absence of an elicitor. 20µg of total RNA was loaded and equal loading was verified by staining the rRNA bands with acridineorange.

phosphatase inhibitor has also been shown for photoautotrophic *C. rubrum* cells (12) and the involvement of a phosphatase in the regulation of stress activated signal transduction pathways has directly been shown for Alfalfa (27). The delay in gene induction in comparison to treatment with E-FOL might thus be explained by the inhibition of the phosphatase: Since the basal activity of an inactive protein kinase, i.e. not phosphorylated, complexed with an inhibitor or at the wrong subcellular localisation is low compared to the activated state, but not absent, LpWRKY1 is induced if the phosphatase is inhibited, but not as efficiently as in the presence of an elicitor.

LpWRKY1 recognises W-boxes within its own promoter—Another prominent feature of WRKY proteins is their ability to recognise W-boxes located in their own promoter sequence (9,28). To test whether this is also true for LpWRKY1, we performed a 5´directed genome walk to clone the corresponding promoter sequence. Sequence analysis revealed three W-boxes located 5´of the translation start of LpWRKY1 (figure 1) that closely resemble W boxes described in other promoters of WRKY genes (9). Since we failed to express the full-length sequence of LpWRKY1 in *E.coli* and in yeast, we subcloned the two WRKY domains to be expressed as a maltose binding protein for the gelshift assays. As it is shown in figure 4, LpWRKY1 is able to recognise the W-boxes in the promoter fragment used. An excess of unlabelled promoter fragment can effectively compete the binding of the labelled probe, whereas the noncompetitor DNA at the same molar excess is not able to hinder binding to the W-boxes. It can thus be concluded, that LpWRKY1 is able to recognise and bind W-boxes on its own promoter which is in agreement with the model proposed by Eulgem et al. (9).

Phosphorylation of LpWRKY1 in vivo—The data presented in this study indicates a phosphorylation dependent regulation of WRKY gene induction. To further

substantiate our findings, we determined whether LpWRKY1 protein itself is phosphorylated *in vivo*. A 6XHis tagged near

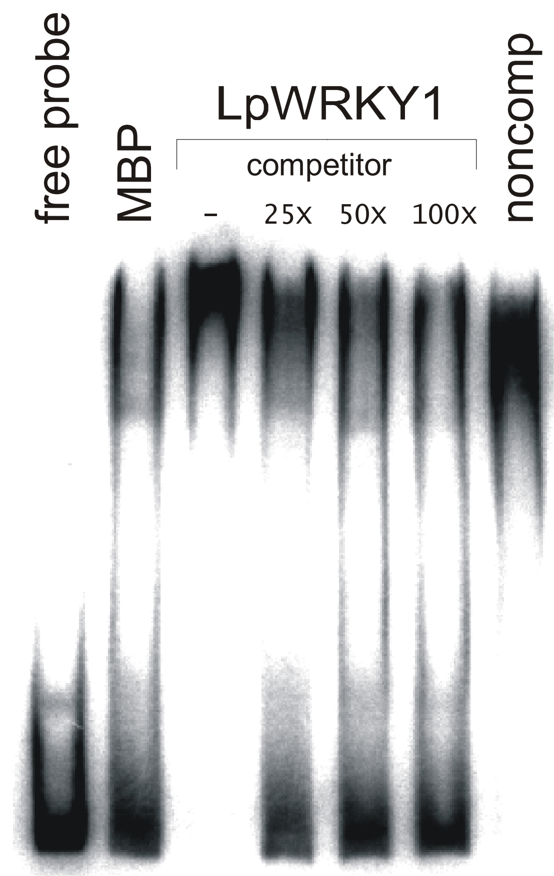

Fig. 4. LpWRKY1 recognises W-boxes within its own promoter. Gelshift assays were performed with the partial LpWRKY1 sequence fused to the Maltose binding protein (MBP). As control, MBP alone was added as well. The competitor was the unlabelled promoter fragment (200bp), the noncompetitor was a 300bp fragment of the LpWRKY1 cds.

full length sequence of LpWRKY1 ranging from the native start codon to the SacI restriction site depicted in figure 1 under control of the CaMV35S promoter was stably transformed into tobacco BY2 suspension cells. Albeit LpWRKY1 is highly homologous to NtWRKY1, compatible regulatory mechanisms in response to elicitors cannot readily be expected. However, preliminary experiments showed that the highly

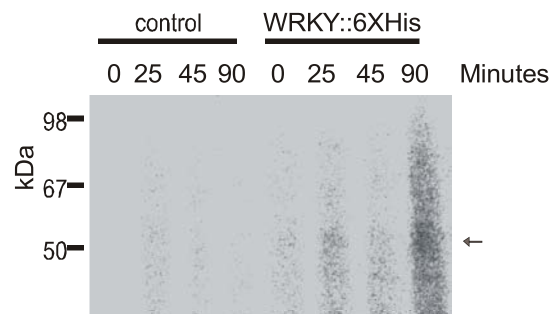

Fig. 5. LpWRKY1 is phosphorylated in vivo. 6XHis tagged LpWRKY1 was expressed in tobacco BY2 cells and the accumulation of 32P was monitored in the presence of the phosphatase inhibitor endothall at the time points indicated. Purified LpWRKY1 was then analysed by SDS-PAGE and autoradiography.

homologous NtWRKY1 is induced in BY2 cells with the same kinetics as LpWRKY1 in tomato cells by the phosphatase inhibitor endothall (data not shown). According to the data presented in this study, endothall might inhibit a constitutively active phosphatase, which counteracts a presumably activating phosphorylation of LpWRKY1. At the same time, endothall should prevent LpWRKY1 from being dephosphorylated, thus altering the equilibrium towards the phosphorylated state. To test this hypothesis and to demonstrate *in* vivo phosphorylation of LpWRKY1, the cells were thus metabolically labelled with ^{32}P orthophosphate in the presence of endothall and LpWRKY1 was purified from crude protein extracts by affinity column chromatography on Ni^{2+}-NTA sepharose at different time points. As it is shown in figure 4, accumulation of ^{32}P is found after 90 minutes of incubation on the

purified material from BY2 cells expressing LpWRKY1::6XHis, but not in eluates from control cells. It can therefore be concluded, that LpWRKY1 undergoes constitutive phosphorylation/dephosphorylation cycles in resting cells.

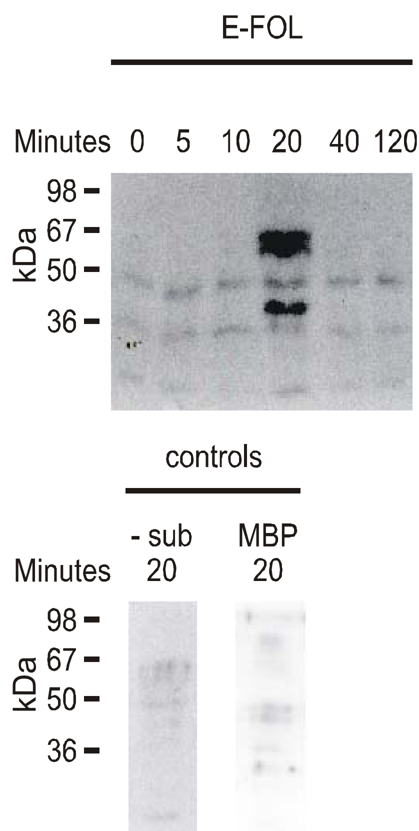

Fig. 6. In-gel kinase assay with a *Lycopersicon peruvianum* WRKY1/maltose binding protein fusion-protein as the substrate. An *in gel* assay using 20µg of crude protein extract from a time-course experiment after treatment with an elicitor preparation from *Fusarium oxysporum lycopersici* (E-FOL) is shown. As controls, the substrate was omitted from the gel (-sub) or substituted for by the maltose binding protein (MBP). The molecular weight standard in kDa is indicated on the left.

Phosphorylation of the LpWRKY1 gene product by transiently activated protein kinases—To further characterise the involvement of protein kinases in the regulation of LpWRKY1, the activation of protein kinases capable of phosphorylating LpWRKY1 was monitored by in gel kinase assays. For this, we expressed the polypeptide encoded by the cloned LpWRKY1 cDNA as a maltose binding protein fusion in *E. coli* and used the purified fusion protein as the substrate in *in gel* kinase assays. Figure 5 shows that LpWRKY1 is phosphorylated by a 44kDa and a 67kDa protein kinase that are transiently activated 20 min after elicitation. To exclude the activity of autokinases with corresponding molecular weights, we omitted the substrate from the gel or substituted it by the maltose binding protein. As these controls show, no signal is found at a molecular weight of 44kDa and a faint signal is visible at a molecular weight of 67kDa. This signal is considerably weaker in intensity and thus might reflect the activity of an autokinase that is able to phosphorylate LpWRKY1. An autokinase, the Pto kinase, that is responsible for the elicitor-dependent activation of the transcription factor Pti4 in tomato has recently been described (19). However, since it is not known whether autophosphorylation of kinases is common during an elicitor dependent activation of a transcription factor, further investigation will have to be conducted on this issue. Interestingly, pretreatment of the cell cultures with cycloheximide before the application of E-FOL abolishes WRKY phosphorylation by the two protein kinases (data not shown). However, whether this is due to inhibited *de novo* synthesis of the protein kinases themselves or their corresponding activators can only be clarified

when more information on the identity of these kinases is available.

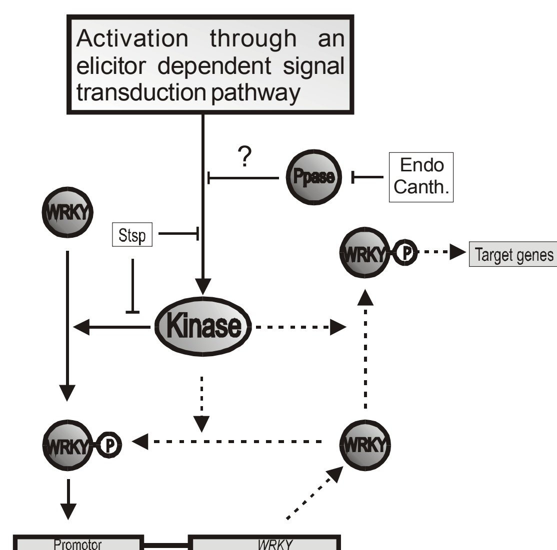

Fig. 7. Proposed model for the regulation of WRKY transcription factors. Lines indicate primary events during the elicitation, scattered lines indicate secondary events. During the elicitation of a defence response, protein kinases become activated to phosphorylate WRKY transcription factors which in turn enhance their own transcription. Later during the event, target genes are activated. Endo.: Endothall, Canth.: Cantharidine, Stau.: Staurosporine, PPase.: Protein-phosphatase.

The current model of WRKY gene activation implies that WRKY proteins are able to generate a feedback loop via their own promoters to modulate their own expression. This feedback loop can either be positive (9) or negative (28). Based on the findings of the present study we propose following extensions of this model by inclusion of a protein kinase that serves as an activator of LpWRKY1. As it is illustrated in figure 5, a protein kinase, which is transiently activated through an elicitor dependent signal transduction pathway phosphorylates LpWRKY1 and might thereby activate the transcription factor. Based on the current model, activated WRKY protein is then able to generate a feedback loop to its own promoter sequence, which in this case results in the upregulation of its own mRNA production. Later during this first event, when activated WRKY protein is sufficiently abundant and has reached a threshold concentration, target genes are activated. The results obtained by treatment with phosphatase inhibitors indicate also the possible involvement of a phosphatase that is responsible for the inactivation of this loop. Thus, the occurrence of all principle key components, i.e. a transcription factor, an activator and a negative regulator, for the transcriptional regulation of WRKY transcription factors, and thus the regulation of downstream events, is demonstrated in this study. The identification of these factors along with their regulation will provide the possibility of a deeper understanding of the regulatory roles of this ubiquitous class of plant specific transcription factors.

Acknowledgements

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Generalised Discussion and Perspectives

Progress in plant signal transduction has been rapid during the last years and the ideas and models to describe information processing in the cell are still growing to higher complexity. It became clear that the view of a `pathway` is insufficient and only describes the situation for very isolated events. Instead, networks are emerging with a multitude of junctions and feedback loops. Initial studies to obtain information on the co-ordinated regulation of source-sink transition and defense response aimed to elucidate the roles of intracellular protons and hexokinase isoenzymes as components of the underlying signal transduction pathways. Changes in extracellular as well as in intracellular pH have been suggested as second messengers leading to pathogen defense (Ojalvo et al., 1987; Lapous et al., 1998). Such pH shifts have been observed in our system after elicitation with different stimuli leading to defense reactions and source-sink transition, however, a systematic study excluded a role for intracellular pH shifts as second messengers. Each stimulus results in an individual pattern of pH changes, which do not precede regulation of defense related or sink specific genes (chapter 1). Nevertheless, the change in pH value either in the cytoplasm or the culture medium is not a secondary effect of the stimulus added but is clearly a cellular reaction. It thus emerged that these physiological changes are a downstream component of the defense response rather than an early component of the signal transduction chain.

Hexokinase isoenzymes have been implicated to play a role in the carbohydrate dependent activation of defense response and source-sink transition. This view is mainly based on the fact that glucose analogs which cannot be phosphorylated by hexokinase fail to induce sink metabolism and defense response. In addition, 2-desoxyglucose which can be phosphorylated by hexokinase but cannot be further metabolised is able to induce cellular reactions (Jang and Sheen, 1994; Jang et al., 1997; Perata et al., 1997; Umemura et al., 1998). Hexokinase inhibitors such as mannoheptulose, glucosamine and N-acetyl glucosamine were

thus widely used to study the involvement of hexokinase in different signal transduction pathways. Since it has been demonstrated that sugar dependent signal transduction is in addition dependent on protein phosphorylation events it was interesting to find that glucosamine not only inhibits hexokinase but as well interferes with the activity of MAP kinases, even with a higher affinity than towards hexokinase (Chapter 2). These findings complicate the use of such hexokinase inhibitors especially in signal transduction cascades that are known to involve protein phosphorylation events. However, it is interesting to mention that specific hexokinase isoenzymes from yeast and rat brain posses protein kinase activity (Herrero et al., 1989; Adams et al., 1991) which possibly points towards a similar function of specific hexokinase isoenzymes in plants. In summary, this study demonstrates that inhibitors can only be applied to complex cellular systems with great caution.

MAP kinase modules clearly serve as integration and divergence points for signals leading to alterations in gene expression or other cellular features. The fact that 20 MAP kinases encoded in the *Arabidopsis* genome face potential activation by up to 60 MAPKKKs through only 10 MAPKKs suggests a high degree of flexibility considering the composition of MAP kinase cascades. It is this flexibility, together with crosstalk on the level of MAPKKs that leads to a specific output. Even on the level of the MAP kinases, interactions allowing the modulation of the output should be taken into consideration. As it was outlined in the introduction, MAPKKs that are able to differentially activate alternative substrates depending on the stimulus serve as points of signal divergence. However, it remains to be elucidated how changes in substrate specificity are regulated in detail. Work presented in this thesis (chapter 4) revealed another prominent feature of MAP kinase cascades. The differential activation of multiple MAP kinase combinations in response to different stimuli described here points towards a signal encoding function also for the endpoints of a MAP kinase cascade. Future research will have to clarify the exact role of concomitant MAP kinase activation. It is conceivable that the activation of multiple MAP kinases leads to an orchestrated regulation of their cellular targets, which in turn specifies the response to the current needs. The question remains whether this specificity is generated by the activation of different MAP kinase subsets or by the

downstream interaction of these MAP kinases or their targets. The most promising approach to clarify this would be multiple MAP kinase knockouts. Plants silenced in one or more MAP kinases would be analysed with respect to altered gene expression patterns upon stimulation with an elicitor that is known to activate the silenced MAP kinases in the wild type. Such an approach would also yield valuable data on the (indirect) targets of MAP kinases. To date, no such systematic approach using MAP kinase knockouts and micro arrays has been carried out but would deliver important information on the downstream effects of defense response.

The purification of MAP kinases (chapters 2, 4 and 5) provides the basis for the nonbiased identification of novel MAP kinases. In the past, MAP kinases were identified in the direction gene to function: antibodies were generated against cloned MAP kinases of which often only little functional data was available and the *in vivo* function of the enzyme was then studied by immonokinase assays. The combination of the purification procedure with mass-spectroscopic techniques will now allow us to reverse this approach. The partial purification of the heat activated MAP kinase (chapter 5) demonstrates that the technique is versatile and not limited to elicitor activated enzymes and might even be applied to other cellular systems.

Chapter 5 also describes the identification of the first possible *in vivo* substrate of MAP kinases. The identification of the heat activated MAP kinase allowed the deduction of possible cellular substrates of which one, HsfA3 was indeed specifically phosphorylated *in vitro*. However, information on the target genes of other MAP kinases and the factors regulating these target genes still is few. Similar strategies for the identification of substrates of other MAP kinases therefore appear to be not very promising. The identification of direct *in vivo* substrates of MAP kinases is one of the most important future tasks in MAP kinase research. The establishment of mass-spectroscopic techniques will in this case also show the way. A practical approach has recently been published which uses two dimensional gel electrophoresis coupled to MALDI-TOF. Although the steady state levels of the substrate protein might not change after the phosphorylation, the pI value of the substrate is substantially altered. Substrate proteins therefore appeared to be up- or downregulated by changing their position according to the new pI value.

In this way, 25 new targets of the mammalian MMK/ERK pathway were identified, including five previously known substrates (Lewis et al., 2000).

A putative target gene for stress activated MAP kinases is the extracellular invertase Lin6 from tomato (Ehness et al., 1997; Link et al., 2002b; Sinha et al., 2002). We were recently able to identify the stress activated MAP kinase which could regulate Lin6 as a WIPK homolog and *in vivo* data will be available by using a knock out approach to establish a direct connection between WIPK activation and Lin6 induction. The enzyme was identified by the comparison of different activity profiles obtained from cells stimulated with different elicitors. It was found that only one specific activity peak was closely connected to invertase regulation. By immunoprecipitation with MAP kinase specific antibodies, this activity was identified as the tomato WIPK homolog (chapter 4, figs. 4 and 5). The classification of the enzyme is a direct outcome from the MAP kinase purification procedure and could greatly facilitate the elucidation of the signal transduction pathways leading to source-sink transitions. Most importantly, WIPK is also a prominent candidate to explain the co-ordinated regulation of defense response and sink metabolism (Ehness and Roitsch, 1997; Sinha et al., 2002) since a known target gene of WIPK is the phenylalanine-ammonia lyase (Yang et al., 2001). If WIPK is responsible for the concomitant regulation of Lin6 and PAL it still remains to be elucidated how the regulation of photosynthesis is connected to this pathway. It will thus be of outstanding interest to investigate photosynthetic performance of plants silenced for the WIPK gene under stress conditions. Yet, a second pathway governs the co-ordinate regulation of Lin6 and PAL. This is demonstrated by studies involving metabolisable sugars like glucose and sucrose which induce the co-ordinated regulation without the activation of the MAP kinase (Sinha et al., 2002). This pathway might be independent from the stress related MAP kinase pathway. However, both pathways are connected through a feedforward loop. Upon elicitor stimulation, the induction of extracellular invertase is considerably faster than the one found after treatment with metabolisable sugars (Ehness et al., 1997; Sinha et al., 2002). Therefore, stress dependent induction of extracellular invertase via the MAP kinase pathway will lead to an increased sucrose import to the stressed tissue, hence a secondary induction of the carbohydrate dependent pathway that as well

leads to the co-ordinate gene regulation observed. In this view, the pathway invoked by metabolisable sugars acts as a bypass to the stress activated MAP kinase pathway.

Photosynthesis appears to be subject to a yet more delicate regulation involving an independent pathway (chapter 3). Depending on the stimulus, RbcS expression can be unaffected, as in the case of nonmetabolisable sucrose analogs, transient as in the case of elicitors and downregulated as after stimulation with metabolisable sugars. This regulatory pattern might however be due to the experimental system. Since photoautotrophic cell cultures were used, sink induction will not lead to an increased carbohydrate status and at least some of the transient regulations of photosynthesis might simply be due to the lacking carbohydrate supply under sink conditions.

In chapter 6, the cloning and characterisation of a novel WRKY transcription factor from tomato is described. The gene was found to encode an immediate early factor that is transiently expressed after treatment with elicitors and that undergoes posttranslational modification by phosphorylation. It has been speculated that WRKY transcription factors are regulated by phosphorylation (Fukuda, 1997; Eulgem et al., 2000), however direct evidence for this modification was missing. The discovery that LpWRKY1 is modified and possibly regulated by phosphorylation might be as well true for other WRKY factors. The binding of WRKY proteins to the W-box can be effectively inhibited by the pretreatment of the cells with staurosporine, a general protein kinase inhibitor. In addition, treatment of the nuclear extracts with a phosphatase abolishes binding as well (Fukuda, 1997). Nevertheless, more research will be required to elucidate the exact role of the phosphorylation of WRKY transcription factors. Phosphorylation might play a role in the regulation of DNA binding activity or transactivation potential, but may be as well involved in the control of dimerisation with interacting partners. Further characterisation of posttranslational modifications of WRKY proteins will require the identification of the phosphorylation site(s), which might as well give insight into the possible identity of the protein kinase(s) involved. Mutation of the phosphorylation site(s) will then allow the investigation of the *in vivo* role of phosphorylation on WRKY proteins. Another interesting, though yet uncharacterised possibility of

WRKY regulation is the interaction with other proteins or transcription factors. Some WRKY proteins contain leucine zipper domains that strongly point towards such interactions (Cormack et al., 2002). In addition, it has been shown that two WRKY factors are downstream targets of the flg22 MAP kinase cascade (Asai et al., 2002). Since WRKYs could not be shown to be direct targets of the MAP kinases involved, it has to be anticipated that a negative regulator is inactivated by the MAP kinases, which in turn allows the WRKY factors to act on their target genes.

The discovery of W-box clusters in the promoter of the extracellular invertase Lin6 makes this gene a putative target for WRKY proteins (R. Proels and T. Roitsch, unpublished). Transactivation studies using protoplast assays to study the regulation of the Lin6 promoter by WRKY proteins might, however, be complicated. WRKY factors are known, that do not show transactivating potential in such assays (Yoda et al., 2002) which might be due to a missing regulatory modification or interacting partners. The most promising WRKY factors described that could be involved in the regulation of extracellular invertase are AtWRKY22 and AtWRKY29 (Asai et al., 2002). Both genes are downstream of the central flg22 MAP kinase cascade that employs the activation of WIPK and SIPK orthologs to control gene activation. WIPK in turn is known to regulate the expression of phenylalanine ammonia lyase (Yang et al., 2001) and is a candidate to also be involved in the regulation of Lin6 (see discussion above). It would thus be interesting to study the involvement of AtWRKY22/29 in the regulation of extracellular invertase. Since the function of these two transcription factors appears to be highly redundant, double knockout mutants would be required to study their impact on the co-ordinate gene regulation of defense response and source-sink metabolism. Another promising candidate to regulate extracellular invertase both under stress and senescence conditions is AtWRKY6 (Robatzek and Somssich, 2001, 2002). The expression of this WRKY factor appears to be sink specific, as it was found in roots and anthers but not in leaves. Moreover, the gene is pathogen inducible and is upregulated in late developmental stages. We have shown previously, that an anther specific invertase is indispensable for pollen grain filling and that silencing of this gene leads to male sterile plants (Goetz et al., 2001). Since the maturation of reproductive organs is closely associated to senescence (Bleecker and Patterson, 1997) it is of

particular interest, that extracellular invertase has also been found to play a pivotal role in the regulation of leaf senescence (E. Balibrea and T. Roitsch, unpublished). However, the identification of the tomato orthologs of AtWRKY22/29 and AtWRKY6 might be complicated by the only limited availability of EST sequences. It would thus be most promising to perform two hybrid screens with the invertase promoter as a bait and cDNA libraries from senescing and pathogen infected tissues as the prey. A major drawback of this approach is the fact that it will only uncover those factors that are capable of transactivation *per se*, which might not be the case for all WRKY or other transcription factors.

The work presented in this thesis developed tools to identify and characterise signal transduction components involved in the co-ordinate regulation of defense response and source-sink transition in tomato. The purification procedure is a versatile method that led to the identification of LpWIPK as a candidate upstream regulator of extracellular invertase (Link et al., 2002b). In addition, the identification of previously unknown MAP kinases will be greatly facilitated. Methods have as well been established to study the involvement of future candidate transcriptional regulators of extracellular invertase in the underlying signal transduction pathway. In combination with different *in vivo* analysis tools, which are currently developed in the lab, this provides the basis to identify and to interlink key regulatory elements on the level of MAP kinases and transcriptional regulation of the co-ordinated defense response and source-sink transition in the near future.

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Publications marked with an asterisk are not part of this thesis

Erklärung nach §4 Abs. 3 der Promotionsordnung

1. Ich habe vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel dafür benutzt.

2. Die vorliegende Dissertation lag bisher in keinem anderen Prüfungsverfahren vor

3. Außer dem Diplom in Biologie habe keine weiteren akademischen Grade erworben oder zu erwerben versucht.

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