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Analysis of pathogen-induced glutathione *S*-transferases in *Arabidopsis thaliana* and a gene related to systemic acquired resistance in cucumber (*Cucumis sativus* L.)

THESE

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par
Damien Lieberherr

de
Chêne-Bougeries (GE)

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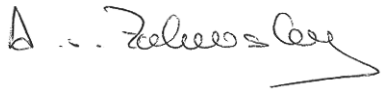
Le Directeur de thèse:

Prof. Dr. Jean-Pierre Métraux

Le Doyen:

Prof. Dr. Alexander von Zelewsky



RESUME

Dans leur environnement naturel, les plantes sont exposées en permanence à une grande variété de microorganismes dont certains sont pathogènes. Ainsi, les plantes ont développé au cours de l'évolution des mécanismes de défense multiples et complexes, qui leur permettent de résister à la plupart des microbes pathogènes. La résistance peut s'exprimer localement au site d'infection et systémiquement selon la nature des agents infectieux. Par exemple, suite à une infection par un agent pathogène provoquant des nécroses, la résistance se développe dans toute la plante y compris dans les tissus éloignés du site initial d'infection. Cette forme de résistance, appelée résistance systémique acquise (SAR), se caractérise par l'accumulation locale et systémique de l'acide salicylique (SA) qui agirait sur une activation concomitante de gènes associés à une défense contre certains microbes. Le SA semblerait jouer le rôle de régulateur endogène. En outre, deux hormones végétales, l'acide jasmonique (JA) et l'éthylène sont indispensables à l'activation d'une autre série de gènes impliqués dans la protection contre d'autres agents pathogènes. A l'heure actuelle, de nombreux chercheurs s'efforcent de répertorier les gènes associés à la défense induite et caractériser les fonctions de leurs produits. A part une résistance déployée directement contre les agents pathogènes, les plantes ont aussi développé des mécanismes qui contribuent à restreindre localement les nécroses, sans pour autant affecter directement la prolifération microbienne. Ces mécanismes de protection restent cependant peu étudiés.

En premier lieu, nous avons analysé l'expression de deux membres de la super-famille des glutathion *S*-transférases (GST), *AtGSTF2* et *AtGSTF6*, chez des plantes d'*Arabidopsis thaliana* inoculées avec une souche avirulente de *Pseudomonas syringae*. Les deux gènes étudiés sont fortement et rapidement induits après inoculation avec un agent pathogène, ceci même avant l'accumulation de gènes de défense tel que *PR-1*. L'expression des deux GSTs est aussi corrélée avec la production d'éthylène et l'accumulation de SA dans la plante infectée; de plus, l'application externe de SA et d'éthylène induit fortement ces deux gènes. Grâce à l'étude de l'expression de *AtGSTF2* et *AtGSTF6* dans des plantes *NahG* et chez les mutants *cpr1*, *npr1* et *etr1*, nous avons mis en évidence que l'expression de ces deux gènes dépend du SA, mais pas de la protéine NPR1. En outre, *AtGSTF2* ne peut être exprimée dans le mutant *etr1*, indicant que l'expression de ce gène est soumis à la présence simultanée de l'éthylène et du SA. Une étude fonctionnelle de la protéine *At*GSTF2 chez la bactérie *E.coli* a permis de mettre en évidence une activité de glutathion peroxidase, capable de détoxifier des hydroperoxides d'acides gras *in vitro*. Cela suggère que *At*GSTF2 pourrait jouer un rôle dans la gestion des dommages cellulaires occasionnés lors de la réaction hypersensible (HR) chez la plante.

Beaucoup d'efforts ont été entrepris pour identifier des gènes induits lors de la SAR. Dans une étude sur le concombre, Marro (1997) a utilisé la technique d'amplification différentielle de l'ARNm, pour isoler de nouveaux gènes exprimés systémiquement après une infection locale. Marro a ainsi pu isoler un certain nombre de candidats groupés sous le terme générique "Cucumber Pathogen-Induced Genes" (CuPi). Nous avons cherché à compléter l'étude de l'expression de CuPi1 par une caractérisation fonctionnelle du produit de ce gène. Le gène, présent en copie unique, est exprimé dans le fruit, induit par des activateurs chimiques de la SAR et par plusieurs agents pathogènes, aussi bien dans les parties infectées que dans les feuilles systémiques des plantes de concombre. Le gène *CuPi1* code pour une protéine de petite taille comprenant 87 acides aminés, qui présente des homologies avec ω-conotoxin, une toxine du venin d'un escargot marin (*Conus geographus*). Certains brefs motifs peptidiques et la plupart des résidus cystéine sont conservés entre les deux séquences. L'expression de CuPi1 s'est révélée toxique dans un système d'expression de type procaryotique, et la proteine a pu finalement être obtenu dans un système d'expression *in vitro*. Cependant, bien que plusieurs systèmes d'expression aient été utilisé pour produire

CUPI1, il n’a pas été possible de l’isoler. D’autes approches, telle que l’analyse de plantes transgéniques exprimant CUPI1 pourraient se révéler prometteuses pour évaluer la fonction de la protéine *in vivo*.

SUMMARY

In response to infection by pathogens, plants deploy various and complex defense mechanisms. Resistance can be expressed locally and systemically, depending on the nature of the inducing stimulus. For instance, after infection by a necrotizing pathogen, resistance develops throughout the plants, in tissues distant from the initial site of infection. This form of resistance, called systemic acquired resistance (SAR), is dependent of the local and systemic accumulation of salicylic acid (SA), which is involved in the concomitant activation of genes associated with defense against certain types of microorganisms. SA seems to play the role of an endogenous regulator. Besides SA, the plant hormones jasmonic acid (JA) and ethylene have been shown to be involved in the activation of separate sets of genes, the products of which mediate resistance against other types of pathogens. Much work is in progress to provide a comprehensive overview of the genes associated with defense and the function of their products. Besides restricting the growth of invading microbes, plants also evolved mechanisms to contain the tissue damage. However, such mechanisms have been poorly studied.

We have first analyzed the expression of two members of the glutathione *S*-transferase (GST) multigene family, *AtGSTF2* and *AtGSTF6* in *Arabidopsis thaliana* inoculated with an avirulent strain of *Pseudomonas syringae*. Both GST genes, were expressed early and to a large extent after the inoculation of the pathogen, before the accumulation of the pathogenesis-related gene *PR-1*. The expression of the pathogen-induced GSTs correlated with the production of ethylene and the accumulation of SA upon pathogen attack, and both genes were strongly induced by application of exogenous SA or ethylene. Moreover expression studies in *NahG* plants, *cpr1*, *npr1* and *etr1* mutants revealed that *AtGSTF2* and *AtGSTF6* were SA-dependent but not under the control of NPR1. Interestingly, *AtGSTF2* expression was also abolished in *etr1*, indicating that *AtGSTF2* gene induction requires the combination of both SA- and ethylene-dependent signaling pathways. Furthermore, *At*GSTF2 expressed in *E.coli* led to active glutathione peroxidase activity, capable to detoxify fatty acid hydroperoxides *in vitro*. Thus, *At*GSTF2 might play a positive role in lowering the cellular damage caused during the process of hypersensitive response (HR).

Large efforts were carried out to identify genes induced during SAR. In his study on cucumber, Marro (1997) has used mRNA differential display to isolate novel genes that are expressed systemically after a local infection. A number of candidates were found and grouped under the generic term of Cucumber Pathogen-Induced Genes (CuPi). We have further attempted to complete the expression study of CuPi1 by a functional characterisation of the protein. The single copy gene*CuPi1* is constitutively expressed in fruit, induced by chemical activators of SAR and by various pathogens. *CuPi1* encodes a small protein of 87 amino acids with homology to ω-conotoxin of a sea snail (*Conus geographus*) venom toxin. Small sequence motifs and many of the cysteine residues were conserved between the two sequences. The expression of CuPi1 was toxic for procaryotic expression systems, and the protein could only be expressed using an *in vitro* expression system. Although, different systems were used to express CUPI1, it was still not possible to purify it and to characterize its function. Other approaches such as the analysis of transgenic *Arabidopsis thaliana* expressing CUPI1 might be promising and could provide an answer to the function of the protein *in vivo*.

Contents

C H A P T E R 1

General introduction

Resistance in plants

In their natural environment, plants are frequently exposed to potentially pathogenic microorganims. Only a small proportion of these pathogens will eventually invade the plant and cause disease. Thus, during evolution plants have developed resistance that allows them to cope with most of potential pathogens. Resistance is the ability of the plant to block or delay the activity of the pathogen and the subsequent infection. During contact with the pathogen, most of the physiological processes controlling plant development and yield production are affected. This has encouraged breeders to select novel crop plants for traits like tolerance or resistance to pathogens. Resistance in plant against pathogenic microorganisms is based on constitutive resistance, and induced defense mechanisms activated once the perception of the pathogen has been established.

Constitutive resistance

Plants possess different preformed natural barriers which prevent pathogens from colonizing the plant. Some structural barriers like wax, cuticle and the cell wall structure strengthen the plant mechanically and prevent the invasion by pathogens penetrating directly through the cell wall (Kerstiens, 1996). Other cell wall components like lignin and suberin offer additional barriers and increase resistance against fungal penetration (Bhaskara-Reddy *et al*., 1999).
Moreover, plants produce a wide range of secondary metabolites which have often antifungal activity. These preformed antimicrobial compounds exist in their biologically active forms or in their inactive forms such as cyanogenic glycosides and glucosinolates. For instance, the inactive glycosides precursors are activated in response to tissue damage or pathogen attack through the catalytic activity of endogenous enzymes released when cell integrity is broken down (Osbourn, 1996).

Induced resistance
Upon recognition of an attacking pathogen, plants induce a number of defense mechanisms and the invader remains localized.

Resistant gene-mediated resistance and hypersensitive response
Disease resistance in plants is often dependent on the ability of the plant to specifically recognize invading pathogens. This mechanism of resistance is controlled at the genetic level and is governed by disease resistance (*R*) genes that confer on the plant the ability to recognize pathogen strains expressing specific avirulence (*avr*) genes (Baker *et al*., 1997). Pathogen *avr* gene products are molecules that directly elicit the rapid induction of defense responses on resistant host plants carrying the corresponding *R* gene. *R* gene-mediated resistance also often appear to involve often the development of necrotic flecks due to host cell death called hypersensitive response (HR) (Hammond-Kosack and Jones, 1996). In addition, defense genes are activated in cells surrounding the lesion, providing direct protection against pathogen invasion, through host cell wall reinforcement, or production of antimicrobial compounds such as phytoalexins as well as defense-related proteins such as pathogenesis-related (PR) proteins.
In *R* gene-mediated resistance the progression of the avirulent pathogen is blocked, and the plant-pathogen interaction is termed incompatible. When the pathogen does not carry an *avr* gene recognized by the plant, defense responses of the host are weak or not activated and the invasion by the pathogen leads to disease development. This interaction is called compatible and the pathogen virulent.

Systemic acquired resistance
After the formation of a necrotic lesion provoked by a HR, or as a result of disease symptoms, a distinct signal transduction pathway refered to systemic acquired resistance (SAR) is activated in local and distant tissue of the plant. SAR activation provides the ability of plants to develop systemic resistance and to defend themselves against a broad spectrum of virulent pathogens (Ryals *et al*., 1996; Sticher *et al*., 1997). At the onset of SAR, synthesis of salicylic acid (SA) occurs in the infected tissues and in systemic non-infected tissue (Malamy *et al*., 1990; Métraux *et al*., 1990), and the expression of a set of genes called SAR genes is associated with the occurence of SAR (Ryals *et al*., 1996; Ward *et al*., 1991). These genes encode a class of SAR proteins called pathogenesis-related (PR) proteins. Several PRs show antimicrobial activities *in vitro*, and some activities like ß-1,3-glucanase (PR-2), chitinase (PR-3) and osmotin (PR-5) are well (Abad *et al*., 1996; Mauch *et al*., 1988; Niderman *et al*., 1995). Therefore PRs are thought to contribute to protection. In Arabidopsis, the SAR marker genes are PR-1, PR-2 and PR-5 (Uknes *et al*., 1992).

SA plays a key role in both SAR signaling and disease resistance (Ryals *et al*., 1996). The level of SA was found to increase in different plant species such as tobacco, cucumber and Arabidopsis after pathogen infection and this increase was shown to correlate with SAR (Cameron *et al*., 1994; Dempsey *et al*., 1993; Malamy *et al*., 1990; Métraux *et al*., 1990; Rasmussen *et al*., 1991; Uknes *et al*., 1993; Yalpani *et al*., 1993; Yalpani *et al*., 1991). Furthermore application of exogenous SA can induce SAR and SAR gene expression (Uknes *et al*., 1992; Vernooij *et al*., 1995; Ward *et al*., 1991), leading to the suggestion that SA is involved in SAR signaling.
Experiments with transgenic plants expressing a bacterial salicylate hydroxylase encoded by the *nahG* gene, which degrade SA into catechol, have shown that SA is required as a signal for SAR (Delaney *et al*., 1994; Gaffney *et al*., 1993), because *NahG* plants fail to develop SAR after inoculation with a pathogen or treatment with SA (Lawton *et al*., 1995).
SA is required for PR expression in systemic tissue (Ryals *et al*., 1996), and therefore has been proposed to be the translocated signal for SAR. To support this hypothesis, it has been shown that in TMV-infected tobacco or in TNV-infected cucumber, SA produced in the infected leaves is transported throughout the plant and accumulates in uninfected tissues (Molders *et al*., 1996; Shulaev *et al*., 1995). However, grafting experiments with wild-type and *NahG* tobacco plants indicate that this transport is apparently not essential for full induction of SAR in tobacco (Vernooij *et al*., 1995; Willits and Ryals, 1998). Thus, the specific role of SA in disease resistance and the identity of the systemic signal should be further investigated.

Genes involved in SAR

In order to identify steps in the SAR signal transduction pathway, several groups have taken a genetic approach. The analysis of mutagenized populations of Arabidopsis seeds has led to the characterization of mutants that are constitutively activated for SAR or mutants compromised in SAR activation and disease resistance. The first class of mutants includes *lsd* (lesion simulating disease; Dietrich *et al*., 1994) *acd* (accelerated cell death; Greenberg and Ausubel, 1993; Rate *et al*., 1999), *cpr* (constitutive expresser of *PR* genes; Bowling *et al*., 1997; Bowling *et al*., 1994; Clarke *et al*., 1998) and more recently *mapk4* (loss of MPK4 activity; Petersen *et al*., 2000), as well as *edr1* (enhanced disease resistance; Frye *et al*., 2001). All these mutants exhibit an elevated level of SA, a constitutive expression of *PR-1*, *PR-2*, *PR-5* and are resistant to bacterial and fungal pathogens. In *cpr1/NahG* and *mpk4*/*NahG* double mutants, the resistance to *P. syringae* and the expression of *PR-1* gene are lost. These findings suggest that *cpr1* and *mpk4* are fully dependent upon SA, and that CPR1 and MPK4 function upstream of SA in SAR signaling.
Mutant screens have also identified recessive Arabidopsis mutants affected in SA signaling and highly susceptible to pathogens. For instance the *sid1* (salicylic acid induction deficient; Nawrath and Metraux, 1999), allelic to *eds5* (enhanced disease susceptibility; Rogers and Ausubel, 1997), the *sid2* and the *pad4* (phytoalexin deficient; Zhou *et al*., 1998) mutations are compromised in SA accumulation and *PR-1* expression upon pathogen infection. Whereas *eds5* and *sid2* are still able to accumulate the antimicrobial compound phytoalexin (Nawrath and Metraux, 1999), *pad4* is not. In addition, the *eds1* mutation also operates upstream of SA-mediated plant defense (Falk *et al*., 1999). In contrast, *npr1* (nonexpresser of *PR* genes; Cao *et al*., 1994)) mutants are able to accumulate SA, but fail in their SAR response after pathogen infection or application of SA. This indicates that NPR1 acts downstream of the SA signal transduction pathway. Whereas PAD4 and EDS1 encode for lipase-like proteins (Falk *et al*., 1999; Jirage *et al*., 1999), NPR1 encodes an ankyrin repeat protein (Cao *et al*., 1997). NPR1 interacts with a basic leucine zipper transcription factors that binds to *PR-1* promoter

elements, suggesting a direct link between NPR1 activity and regulation of *PR* gene expression (Zhang *et al*., 1999).
Further analysis have shown that constitutive *PR* gene expression in *cpr1* and *cpr6* requires SA (Bowling *et al*., 1997; Clarke *et al*., 1998; Clarke *et al*., 2000; Dong, 1998). Although NPR1 is necessary for bacterial resistance, *cpr6* is NPR1 independent for PR genes expression (Clarke *et al*., 1998). This opens new perpspective in finding important regulators of multiple signal transduction pathways involved in plant defense.

Chemical activators of SAR
Various natural or synthetic substances are inducers of SAR (Kessmann *et al*., 1994). To be a SAR inducer the following criteria are required. Neither the compound nor the derived metabolite must present an antimicrobial activity *in vitro* or *in planta*. In addition, the agent must protect a plant against the same range of pathogens as biological SAR, with similar protection at both phenotypic and genetic levels.
Two synthetic compounds, INA (2,6-dichloroisonicotinic acid) and BTH (benzo-(1,2,3)-thiadiazole-7carbothioic acid S-methyl ester, BION®, ACTIGUARD®) are functional analogs of SA and activate the same SA-dependent PR genes (Lawton *et al*., 1996; Uknes *et al*., 1992). They are therefore considered as SAR inducers. INA and its methyl ester are efficient agents and protect plants against a wide spectrum of pathogens, ranging from viruses, bacteria, to fungi (Kogel *et al*., 1994; Nielsen *et al*., 1994; Uknes *et al*., 1992). INA shows a weak antifungal activity *in vitro* and induces the expression of SAR genes (Ward *et al*., 1991). In tobacco and Arabidopsis, INA is still effective on *NahG* plants, indicating that it operates downstream of SA (Vernooij *et al*., 1995).
BTH is active as SAR inducer in many plants such as wheat, rice, tobacco and Arabidopsis (Sticher *et al*., 1997). Like INA, BTH has practically no antifungal effect, leads to the activation of the same SAR genes as SA (Friedrich *et al*., 1996; Gorlach *et al*., 1996; Lawton *et al*., 1996) and acts downstream of SA (Vernooij *et al*., 1995). However, work with *npr1* mutants has revealed that INA and BTH protect only Arabidopsis plants carrying a functional NPR1, indicating that BTH operate through a common signaling cascade downstream of SA perception (Delaney *et al*., 1995; Lawton *et al*., 1996).
The non-protein amino acids ß-aminobutyric acid (BABA) demonstrates biological activity and protects plants against a broad range of pathogens (Cohen, 1994; Cohen and Gisi, 1994; Oka *et al*., 1999; Siegrist *et al*., 2000; Zimmerli *et al*., 2000; Zimmerli *et al*., 2001). BABA does not possess direct antimicrobial activity (Zimmerli *et al*., 2000) and activates SA-dependent and independent host-specific defense mechanisms. For instance, in Arabidopsis SA-, JA- and ethylene-signaling mutants, BABA protects plants against *Peronospora parasitica*, but does not against *Pseudomonas syringae* nor *Botytis cinerea* (Zimmerli *et al*., 2000; Zimmerli *et al*., 2001).

Jasmonate- and ethylene-dependent signaling
Concomitantly to the SA signaling involved in induced disease resistance, plants use a network of signal transduction pathways, some of which are SA-independent. Jasmonic acid (JA) and ethylene play key roles in these SA-independent pathways.
Infection by necrotizing pathogens induces locally and systemically rapid synthesis of jasmonic acid and ethylene. Moreover, exogenous application of these signaling molecules induces a set of defense genes that are also activated upon pathogen infection. For instance, in tobacco the hormone ethylene activates several defense genes and promotes basic PR gene expression in TMV-infected plants (Ohtsubo *et al*., 1999). In Arabidopsis these defense genes encode among others, plant defensin (*PDF1.2*), thionin (*Thi2.1*), and small cysteine-rich basic

proteins with antimicrobial activity (Epple *et al*., 1995; Epple *et al*., 1997; Penninckx *et al*., 1998).
Exogenous application of SA does not induce *PDF1.2* or *Thi2.1* (Epple *et al*., 1995; Penninckx *et al*., 1996; Vignutelli *et al*., 1998). Furthermore, pathogen-induced systemic activation of *PDF1.2* is unaffected in *NahG* plants and *npr1* mutants (Penninckx *et al*., 1996; Thomma *et al*., 1998), indicating that this regulatory pathway is SA- and NPR1-independent. However, the expression of *PDF1.2* gene is blocked in the ethylene-insensitive mutant *ein2* (Guzman and Ecker, 1990) and in the *coi1* (coronatine insensitive; Feys *et al*., 1994) mutant, defective in JA signaling (Penninckx *et al*., 1996). In fact, the expression of *PDF1.2* upon pathogen infection requires concomitant activation of the ethylene and the JA signaling pathways (Penninckx *et al*., 1998). Finally, functional JA and ethylene transduction pathway are also required for resistance to fungal pathogens (Thomma *et al*., 1998; Thomma *et al*., 1999).

Induced systemic resistance by Rhizobacteria
Selected non-pathogenic, rhizosphere-colonizing *Pseudomonas* bacteria trigger a form of induced resistance, phenotypically similar to SAR, called rhizobacteria-mediated induced systemic resistance. (ISR) (Van Loon, 1998). *Pseudomonas fluorescens* strain WCS417r (WCS417r) has been shown to activate ISR in several plant species (Duijff *et al*., 1998; Pieterse *et al*., 1996; van Peer *et al*., 1991). In Arabidopsis, WCS417r-mediated ISR is effective against different types of fungal and bacterial pathogens (Pieterse *et al*., 1996; van Wees *et al*., 1997). Interestingly, SAR and ISR are regulated by distinct signaling pathways. In contrast to SAR, WCS417r-mediated ISR functions independently of SA and *PR* gene activation (Pieterse *et al*., 1996; van Wees *et al*., 1997), but requires JA and ethylene signaling. The JA response mutant *jar1* (Staswick, 1992) and the ethylene response mutant *etr1* (Bleecker and Kende, 1988) do not express ISR upon treatment with WCS417r, indicating that the ISR-signaling pathway requires components of the JA and ethylene response (Knoester *et al*., 1999; Pieterse *et al*., 1998). Although SAR and ISR follow distinct signaling pathways, they are both blocked in the regulatory mutant *npr1* (Cao *et al*., 1994; Pieterse *et al*., 1998). Thus, NPR1 is not only required for the SA-dependent expression of PR genes during SAR, but also for the JA- and ethylene-dependent activation of unidentified defense responses resulting from rhizobacteria-mediated ISR.

Cross-talk between defense signal transduction pathways
The previous sections have illustrated the multiplicity of the signaling processes that affect plant defense. The question is now, how different signaling circuits cross-talk with each other. Recent analyses illustrate the ability of the plant to fine-tune responses to particular pathogens that leads to the activation of the appropriate sets of defense mechanisms (Feys and Parker, 2000; Genoud and Metraux, 1999; Glazebrook, 1999; Pieterse and van Loon, 1999; Reymond and Farmer, 1998).
Whereas the signaling molecules SA and JA are involved in the expression of mostly distinct and separate sets of defense responses, a number of studies have revealed antagonistic effects of SA on JA-induced genes through a possible inhibition of JA synthesis or mode of action (Doares *et al*., 1995; Gupta *et al*., 2000; Pena-Cortes *et al*., 1993). Recent analysis on tobacco plants showed an inverse relationship between the level of phenylpropanoid compounds, including SA, and the induction of JA-mediated systemic resistance to insects (Felton *et al*., 1999). In Arabidopsis, studies in the SAR defective *NahG* plants and *npr1* mutants, have revealed that genes activated by JA or ethylene are highly inducible (Clarke *et al*., 1998; Penninckx *et al*., 1996). Other antagonistic effects have been observed in the two reduced SA level mutants *eds4* and *pad4* that presented higher PDF1.2 expression after treatments with

either JA or rose bengal, an AOS inducer (Gupta *et al*., 2000), supporting the idea that SA interferes with JA-dependent signaling.
However, other studies have revealed that the concomitant activation of SA-, JA- and ethylene-dependent signaling was required for defense response. For instance, the expression of *PDF1.2* upon pathogen infection requires concomitant activation of the ethylene and the JA signaling pathways (Penninckx *et al*., 1998). Interestingly, in the constitutive SAR mutant *cpr6* the resistance pathway independent of NPR1 requires both SA and sensitivity to JA and ethylene (Clarke *et al*., 2000). Indeed, induced disease resistance in Arabidopsis can be enhanced by simultaneous activation of salicylate- and jasmonate-dependent defense pathways (van Wees *et al*., 2000).

Active oxygen species during plant stress responses
Adaptation to environmental changes is crucial for plant growth and survival. However, the signaling pathways involved in the molecular mechanisms of adaptation are still poorly understood. Active oxygen species (AOS), have been proposed as a central component of plant adaptation to both biotic and abiotic stresses. Indeed AOS increase under high light (Foyer *et al*., 1994), drought and salt stress (Smirnoff, 1993), low- and high-temperature exposure (Dat *et al*., 1998; O'Kane *et al*., 1996), heavy metals (Weckx and Clijsters, 1996), UV radiation (Surplus *et al*., 1998), air pollutants (Mehlhorn, 1990), mechanical and physical stress (Orozco-Cardenas and Ryan, 1999) and pathogen attack (Levine *et al*., 1994). AOS, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$) are derived from partial reduction of dioxygen. O_2^- is a by-product of mitochondrial electron transport, photosynthesis and flavin dehydrogenase reactions and can be converted spontaneously or enzymatically to other active oxygen species, of which $^{\cdot}OH$ is the most reactive.
During pathogenesis, AOS are involved in plant disease resistance. Infection by an avirulent pathogen, or specific bacterial or fungal avirulence factors induce the accumulation of O_2^- and/or H_2O_2, initiating the HR (Levine *et al*., 1994). Furthermore, H_2O_2 acts directly on oxidative cross-linking in the cell wall (Bradley, 1992), on defense genes activation (Levine *et al*., 1994) and as a signal for SAR (Bi *et al*., 1995; Neuenschwander *et al*., 1995). The oxidative burst, involves an NADPH-dependent O_2^- generating system which is similar to the O_2^- production catalyzed by a plasma membrane oxidase in neutrophil cells (Auh and Murphy, 1995; Doke, 1985; Doke and Chai, 1985).
The omnipresence of O_2 in the environment and the constant AOS production render oxidant scavengers necessary for plant growth and survival. Plants have several antioxidant enzymes like superoxide dismutases (SODs) and catalases (CATs), as well as the enzyme ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) (Noctor and Foyer, 1998). Catalase and the enzyme of the ascorbate-glutathione cycle are important in H_2O_2 scavenging in different plant cell compartments. Although their properties and requirements are different, they function effectively in parallel being constantly up- and downregulated. For instance a cytosolic APX is transcriptionally suppressed during pathogen-induced programmed cell death in tobacco (Mittler *et al*., 1998), but expressed in Arabidopsis in response to ozone or sulfur dioxide (Kubo *et al*., 1995). Also, catalases are highly expressed in leaves in a light dependent manner (Frugoli *et al*., 1996), and involved in H_2O_2 scavenging during photorespiration. Catalase is photoinactivated in moderate light (McClung, 1997) and its *in vitro* activity is inhibited by SAR inducers (Conrath *et al*., 1995).
Glutathione peroxidase and other peroxidative enzymes destroy AOS and block oxidant-mediated programmed cell death (Hockenbery *et al*., 1993). Also glutathione *S*-transferase (GST), detoxifies lipid hydroperoxides generated by active oxygen species (Berhane *et al*.,

1994), and GST induction in response to low doses of H_2O_2 provides cellular protectants to block oxidant-mediated programmed cell death in cells surrounding the oxidative burst (Levine *et al*., 1994; Tenhaken *et al*., 1995).

Glutathione *S*-transferases

Glutathione *S*-transferases (GSTs), encoded by a large gene family, are very abundant in plants (Noctor and Foyer, 1998) and represent the most important group of glutathione-(GSH) dependent detoxifying enzymes. Gene analysis and genomics projects indicate that plants have more than 40 genes (McGonigle *et al*., 2000) coding for GSTs and that the proteins share as little as 10% amino acid identity. Plant GSTs are categorized in four classes (phi, theta, zeta and tau) according the sequence similarity (Edwards *et al*., 2000). Although individual GST isoenzymes can selectively detoxify specific xenobiotics, their role in endogenous plant metabolism have remained an enigma.

Typically, GSTs (EC 2.5.1.18) catalyze the nucleophilic attack of the thiol group of the tripeptide GSH to various electrophilic molecules, including herbicides. The GSH-conjugated metabolites are tagged for vacuolar import by ATP binding cassette (ABC) transporters, which selectively transport glutathione conjugates (Martinoia *et al*., 1993; Rea, 1999).

Recent research has demonstrated through expression profile and *in vitro* activity, that gene regulation is highly variable within GSTs and that the GST substrate specificities seem to be overlapping. Moreover, GST function does not correspond to the classifications which are based on sequence identity (McGonigle *et al*., 2000). Indeed, the enhancement of GST expression has become a marker for plant response to stress, although the functional significance of selective GST expression is only just emerging.

In addition to GSH conjugation, GSTs catalyze also GSH-dependent peroxidase and isomerase reactions, or act as ligandin for anthocyanins sequestration. By catalyzing the nucleophilic attack of GSH on hydroperoxides, GSTs reduce organic hydroperoxides to the less-toxic monohydroxy alcohols. Tobacco seedlings constitutively expressing a tobacco tau GST with a high glutathione peroxidase activity are more tolerant to drought and cold stress than wild-type plants (Roxas *et al*., 1997). GSTs are also essential for the isomerization of specific metabolites. In plants, the isomerase activity of GSTs has been demonstrated using thiadiazolidine herbicides. These are bioactivated by GST-mediated isomerization to triazolidines, which are potent inhibitors of protoporphyrinogen oxidase (Jablonkai *et al*., 1997).

Molecular genetics and biochemistry have revealed the surprising complexity in the variety of GST genes and enzymes in plants. The diverse physiological functions performed by GSTs have direct cytoprotective activities and they might be essential to preserve plants during environmental stress and disease, as well as supporting normal development. Because of the extreme divergence among GSTs, sequence analysis alone cannot uncover function. Manipulation of the GSTs expression in plants will allow future investigations to test these models of GST function *in vivo*.

Outline of this thesis

As mentioned above, local and systemic resistance are important adaptative mechanism developed by plants to cope with pathogens. The regulation of the oxidative burst is crucial to contain the oxidative stress and trigger resistance, as well as build the signals for systemic induction of resistance genes. However, many questions concerning the mechanisms involved in resistance remain elusive and need to be further characterized. Therefore, I have analyzed the regulation of the expression of GSTs involved in the cellular protection during the hypersensitive response in *Arabidopsis thaliana*. I also studied in more detail a SAR gene isolated previously from cucumber by mRNA differential display (Marro, 1997).

In Chapter 2, I have analyzed the expression of two pathogen-induced GSTs, *At*GSTF2 and *At*GSTF6 in *Arabidopsis thaliana* inoculated with an avirulent strain of *Pseudomonas syringae*. The induction of the GST genes was rapid, in the same range as the oxidative burst, appeared before the expression of the pathogenenesis-related gene *PR-1*, and was dependent on the accumulation of SA and ethylene. Moreover, both GSTs required a functional SA defense signaling pathway. Whereas the expression of *AtGSTF6* was only dependent on SA, the expression of *AtGSTF2* required the combination of both SA- and ethylene-dependent signaling pathways. Interestingly, *E.coli* expressing AtGSTF2 were able to detoxify substantial quantities of fatty acid hydroperoxides *in vitro*. These results indicate that *At*GSTF2 might play a positive role in the reduction of damage caused by pathogens or generated in surrounding cells during the process of HR.
Using mRNA differential display, Marro (1997) has isolated novel genes that are expressed during SAR in cucumber after inoculation with *P.s. lachrymans*. These genes included chitinase, peroxidase, as well as novel gene called cucumber pathogen-induced 1 (*CuPi1*). Molecular and functional aspects of CuPi1 were further investigated and results are presented in Chapter 3. The single copy gene*CuPi1* is constitutively expressed in fruit, induced by chemical SAR activators and by various pathogens in local and systemic tissue of infected cucumber. CUPI1 is a small protein of 87 amino acids with homology to ω-conotoxin of a sea snail (*Conus geographus*) venom toxin. In particular, the position of the cysteine residues is conserved between the two sequences. The expression of CuPi1 was toxic for procaryotic expression systems, and the protein was obtained in an *in vitro* expression system.
Finally, Chapter 4 presents a general synthesis of the results obtained during this work.

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CHAPTER 2

Synergistic activity of salicylic acid and ethylene in the induction of glutathione *S*-transferase *AtGSTF2* by *Pseudomonas syringae*

Damien Lieberherr, Ulrich Wagner, Jean-Pierre Metraux and Felix Mauch

To be submitted

Summary

Inoculation of wild-type Arabidopsis plants with an avirulent strain of *Pseudomonas syringae* results in a hypersensititve response (HR), comprising an oxidative burst characterized by the production of toxic active oxygen species (AOS), as well as an accumulation of salicylic acid (SA) and an induction of the pathogenesis-related gene *PR-1*. Before the significant expression of *PR-1*, two members of the glutathione *S*-transferase (GST) multigene family, *AtGSTF2* and *AtGSTF6*, were highly expressed early in the infection. *AtGSTF2* and *AtGSTF6* induction correlated with the production of ethylene and the accumulation of SA after pathogen attack, and both genes were SA- and ethylene-inducible. Expression studies in *NahG* plants, *cpr1*, *npr1*, and *etr1* mutants revealed that expression of both GSTs were SA-dependent, but not under the control of NPR1. Interestingly, *AtGSTF2* expression was also repressed in *etr1*. Furthermore, *At*GSTF2 expressed in *E.coli* was able to detoxify fatty acid hydroperoxides *in vitro*. Our data support strongly the hypothesis that full expression of the pathogen-induced *AtGSTF2* results from a crosstalk between components of SA and ethylene signaling pathways and that *At*GSTF2 plays a positive role in the reduction of damage caused by the pathogen or generated in surrounding cells during the process of HR.

Introduction

Infection of plants by avirulent pathogens induces a battery of defense responses in both the challenged and the surrounding cells. This includes a reinforcement of cell wall, a production of antimicrobial metabolites and proteins and a form of programmed cell death called hypersensitive response (HR) (Dixon and Harrison, 1994) leading to the rapid collapse of the challenged host cells. Early events in the HR include an oxidative burst characterized by the generation of superoxide (O_2^-) and a subsequent accumulation of hydrogen peroxide (H_2O_2). These active oxygen species (AOS) might be involved in oxidative cross-linking of the cell wall (Lamb and Dixon, 1997). However, H_2O_2 seems not to be the primary signal for the activation of defense genes involved in phytoalexin and lignin synthesis, but plays a role in triggering the HR (Levine *et al*., 1994). Moreover, H_2O_2 functions in the surrounding cells as a rapid diffusible molecule for induction of genes of cellular protection such as glutathione *S*-transferase (GST) (Levine *et al*., 1994) and contributes to the non-enzymatic formation of a significant amounts of nucleic acid or fatty acids hydroperoxides (Porter *et al*., 1995), or to the activation of a lipoxygenase (Rusterucci *et al*., 1999). The direct consequences of hydroperoxides formation are membrane damage and formation of toxic compounds such as alkenals and hydroalkenals (Deighton *et al*., 1999; Porter *et al*., 1995). Alternatively hydroperoxides can act as precursors of diverse signaling compounds implicated in the induction of defense genes during pathogen attack (Reymond and Farmer, 1998).

The GSTs are encoded by a multigene family showing an amazing diversisty of sequences. As an example, the Arabidopsis genome contains more than 40 GST genes distributed in four classes, Phi (F), Zeta (Z), Tau (U), and Theta (T) with two, one, six, and nine introns respectively (Edwards *et al*., 2000). GSTs have been extensively studied for their potential to detoxify herbicides (Dixon *et al*., 1998). However, the primary function of GSTs is the endogenous detoxification of various metabolites. Also, stress-inducible GSTs conjugate metabolites arising from oxidative stress. For example, 4-hydroxynonenal, a toxic alkenal released following oxidative damage of membranes is actively detoxified by GSTs in Sorghum (Gronwald and Plaisance, 1998) and in wheat (Cummins *et al*., 1997). Recent research on the multigene family encoding soybean and maize GSTs have demonstrated through expression profile and in vitro activity that GST genes regulation is highly variable and that GST function does not correspond to the classifications based on sequence identity (McGonigle *et al*., 2000).

Local infection by incompatible pathogens leads to the induction of a broad spectrum disease resistance in distant parts of the plant, termed systemic acquired resistance (SAR) (Ryals *et al*., 1996). Salicylic acid (SA) has been shown to play a key role in the signal transduction pathway leading to SAR and the expression of SAR genes, of which several encode pathogenesis-related (PR) proteins, (Lawton *et al*., 1995; Métraux *et al*., 1990; Nawrath and Metraux, 1999; Uknes *et al*., 1992; Ward *et al*., 1991). Infection with *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 (*Pst*DC3000) carrying the *avrRpt2* avirulence gene causes a macroscopic HR on leaves of *Arabidopsis thaliana* ecotype Col-0, having the corresponding *RPS2* resistance gene (Bent *et al*., 1994; Mindrinos *et al*., 1994), with a simultaneous accumulation of SA followed by the expression of defense-related genes, such as *PR-1* (Lawton *et al*., 1995), and of genes of cellular protection such as GSTs (Greenberg *et al*., 1994). Plants expressing a bacterial salicylate hydroxylase that converts SA to catechol (encoded by the *NahG* gene) exhibit a higher susceptibility and a decreased PR gene expression. SA has also been implicated in gene-for-gene interaction. (Delaney *et al*., 1994).

Recent studies have indicated the importance of plant hormones like ethylene and jasmonic acid in SA-independent resistance responses to wounding, to non-pathogenic rhizobacteria or to pathogen attack (O'Donnell, 1996; Penninckx *et al*., 1996; Penninckx *et al*., 1998; Pieterse and van Loon, 1999; Pieterse *et al*., 1996; Pieterse *et al*., 1998; Schweizer *et al*., 1998). For instance, infection by necrotizing pathogens induces locally and systemically rapid synthesis of jasmonic acid, ethylene and systemically the expression of SA-independent defense genes encoding plant defensin (*PDF1.2*) and thionin (*Thi2.1*), which are basic proteins with antimicrobial activity (Epple *et al*., 1995; Epple *et al*., 1997; Penninckx *et al*., 1996; Penninckx *et al*., 1998). Studying stress responses in signaling pathway-deficient mutants, it has become obvious that plants use multiple pathways to transduce stress signals to activate cell death (Overmyer *et al*., 2000; Rao and Davis, 1999) or other resistance responses (Penninckx *et al*., 1996; Penninckx *et al*., 1998; Schenk *et al*., 2000), and that crosstalk exists in plant cell signaling that leads to the activation of a particular pathway (Gupta *et al*., 2000), or several pathways concomitantly(Penninckx *et al*., 1998). Experiments with spontaneous lesion- forming mutants *cpr5* and *acd2* (Bowling *et al*., 1997; Penninckx *et al*., 1996) suggest that a common early step in both SA-dependent and SA-indepedent pathways could initiate the HR-lesion formation.

Including a new nomenclature of the GSTs studied in the present work (Table 1), we report that inoculation of Arabidopsis leaves with an avirulent strain of *Pseudomonas syringae* rapidly induces two members of the GST multigene family, *AtGSTF2* and *AtGSTF6*, before the expression of the *PR-1* gene. Both *GSTs* are strongly SA- and ethylene-inducible, and full expression of the pathogen-induced *AtGSTF2* results from a crosstalk between components of SA and ethylene signaling pathways. Furthermore, *At*GSTF2 is able to detoxify substantial quantities of hydroperoxides *in vitro* (Zettl *et al*., 1994). For this reason, it is supposed to play a role in the reduction of damage caused by pathogens or generated in surrounding cells during the HR.

GST Class	Old names	cDNA Accession	New names	Reference
PHI (F)	GST2 GSTPM24	X75303 LL11601	AtGSTF2	Zhou and Goldsbrough, 1993
PHI (F)	GST16	AF288181	AtGSTF3	Wagner and Mauch, unpublished
PHI (F)	ATHERD11 ATGST1	D17672 L12057	AtGSTF6	Kiyosue et al., 1993
PHI (F)	ATGST11	AF288177	AtGSTF7	Yang et al., 1999

Table 1. New nomenclature applied to Arabidopsis GSTs.
The GSTs names used in this work are listed in the table, with old name, accession number, reference and GSTs new names based on plant species, gene-class and a number corresponding to sequence similarity.

Results

SA is required for the rapid local induction of AtGSTF2 and AtGSTF6 after infection by Pseudomonas syringae

Arabidopsis Col-0 and *NahG* plants were syringe-inoculated with $MgCl_2$ (mock) or with the *Pst*DC3000(*avrRpt2*) strain. Control Col-0 plants showed a weak and transient expression of *AtGSTF2*, *AtGSTF6* and *PR-1* genes, as a consequence of mechanical stresses induced by the injection (Figure 1A). However, 4 hours, respectively 2 hours after the inoculation of Col-0 plants with the pathogen, the *AtGSTF2* and *AtGSTF6* genes were strongly upregulated, whereas *PR-1* mRNA transcripts accumulated later, between 8 and 12 hours postinoculation (Figure 1A). Both *GSTs* reached a maximum induction during the first 12 hours, and decreased in expression from 1 to 2 days after *Pseudomonas* infection. In contrast, the *PR-1* transcripts remained evenly high over three days. The first important accumulation of free SA in Col-0 plants was observed 4 hours after *Pseudomonas* infiltration (Figure 1B). At this time point, both *AtGSTF2* and *AtGSTF6* genes were already induced. Free SA decreased after 12 hours in the infected leaves correlating with the accumulation of conjugated SA (data not shown), while $MgCl_2$-treated leaves revealed no significant increase in SA. Inoculation of the pathogen on the leaves of *NahG* plants was not followed by an accumulation of SA or by the expression of the *PR-1* gene. Interestingly, *AtGSTF2* was not expressed in *NahG* plants, showing a full dependence on the SA signaling pathway. *AtGSTF6* shared the same expression profile, except for the first 4 hours of infection (Figure 1A). These results indicate that both the SA signaling pathway and the accumulation of SA are required for the expression of *PR-1*, *AtGSTF2* and *AtGSTF6* upon *Pseudomonas* infection. Furthermore, the rapid response of *GSTs* after pathogen attack, suggests that another signaling compound might be involved in the enhancement of the SA effect.

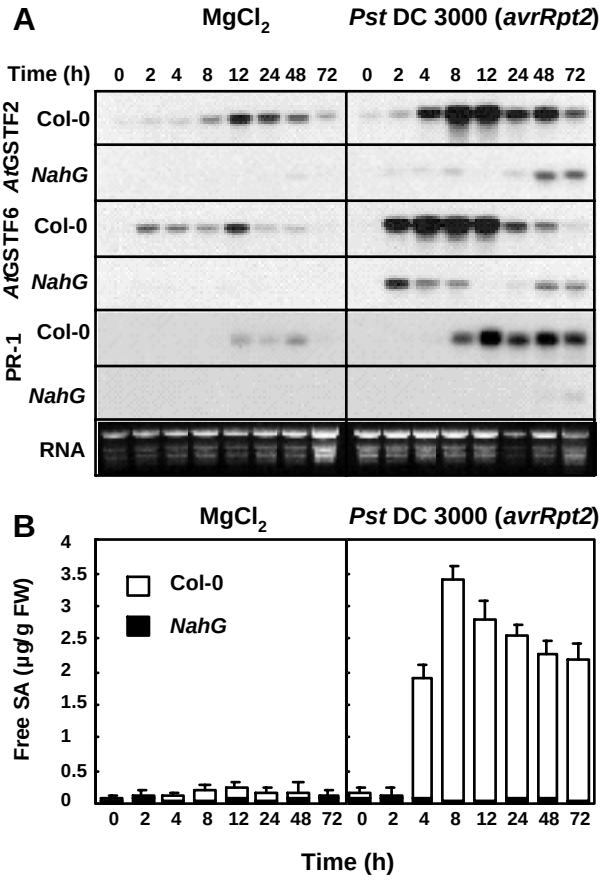

Figure 1. Induction of *AtGSTF2*, *AtGSTF6*, *PR-1* and level of endogenous free SA in leaves of Arabidopsis Col-0 plants and *NahG* plants after inoculation with *Pst*DC3000 (*avrRpt2*).
(A) Leaves were syringe-infiltrated with either 10mM $MgCl_2$ or a bacterial suspension of 10^7cfu/mL and analyzed at different time points. Leaf material of 5 different plants was pooled and RNA samples of 6 µg were analyzed. Membranes were probed with *AtGSTF2*, *AtGSTF6* and *PR-1*, respectively. Equal loading was monitored by ethidium bromide staining of total RNA.
(B) The same leaf material was analyzed for free SA in Arabidopsis Col-0 and *NahG*. Each bar represents the average 3 measurements (±SD). Similar results were obtained in two independent experiments. FW, fresh weight.

Induction of AtGSTF2 requires components of the SA and ethylene response pathways

In order to determine whether additional signaling pathways were required for *GST* induction, *Arabidopsis* Col-0 plants, *NahG*, *npr1* (non-expresser of PR genes) (Cao *et al*., 1994), *cpr1* (constitutive expresser of *PR* genes) (Bowling *et al*., 1994) mutants, and the ethylene response mutant *etr1* (Bleecker and Kende, 1988) were inoculated with *Pst*DC3000(*avrRpt2*). The induction of marker gene *PR-1* was then analyzed at 0, 4 and 12 hours post-inoculation. The results we obtained with Col-0 and *NahG* infected plants (Figure 2) were similar to those previously observed (Figure 1). However, the SAR mutant *npr1* did not expressed *PR-1* after treatment with chemical inducers (SA or INA) of defense gene (Cao *et al*., 1994), nor after inoculation with *Pseudomonas* (Figure 2). Interestingly, the response of *GSTs* to the pathogen was slightly affected in the mutant, but not abolished, indicating that the presence of NPR1 may interfere with *AtGSTF2* and *AtGSTF6* induction.
In contrast, the *cpr1* mutant, which contains an elevated endogenous level of SA (Bowling *et al*., 1994), expressed constitutively the *PR-1*, *AtGSTF2* and *AtGSTF6* genes (Figure 2). Furthermore, the *Pseudomonas*-inoculated *cpr1* plants, showed a higher expression level of *AtGSTF2*, *AtGSTF6* and *PR-1* genes.
The ethylene signaling pathway is known to be required for the activation of certain classes of defense genes (Penninckx *et al*., 1996). Moreover, ethylene promotes the expression of basic PRs in TMV-infected tobacco, (Ohtsubo *et al*., 1999) but is not required for SA-mediated resistance in Arabidopsis (Lawton *et al*., 1995). Whereas the *etr1* mutant of Arabidopsis is insensitive to ethylene (Bleecker and Kende, 1988) it can however accumulate SA after pathogen attack (data not shown). The response of *AtGSTF6* and *PR-1* to *Pseudomonas* was delayed in this mutant supporting the observation that ethylene functions to enhance the SA-dependent signaling pathway (Lawton *et al*., 1994). The expression of *AtGSTF2* after pathogen attack, was almost blocked in *etr1*, confirming the role of ethylene in the induction of *AtGSTF2* (Zhou and Goldsbrough, 1993). Thus, *AtGSTF2* expression upon *Pseudomonas syringae* infection requires both SA and ethylene transduction pathways.

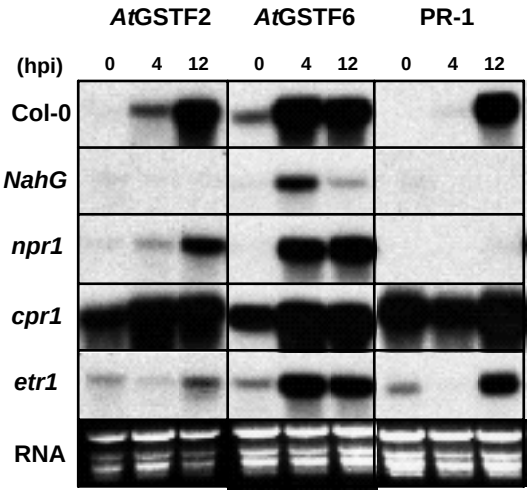

Figure 2. Induction of *AtGSTF2*, *AtGSTF6* and *PR-1* in leaves of Arabidopsis Col-0 plants, *NahG* plants, and *npr1*, *cpr1*, *etr1* mutants after inoculation with *Pst*DC3000 (*avrRpt2*).
Total RNA was extracted after local treatments with *Pst* DC3000 (*avrRpt2*) from a pool of 3 treated plants and samples of 6 µg were submitted to RNA-gel blot analysis. Equal loading was monitored by ethidium bromide staining of total RNA. The experiment was repeated once with similar results. (hpi), hours post inoculation

AtGSTF2 and AtGSTF6 are induced by exogenous SA and ethylene

SA accumulates in plants in response to pathogen attack and functions in signal transduction for SAR (Ryals *et al*., 1996). In order to test the sensitivity of *PR-1* and GSTs to SA, increasing concentrations of exogenous SA were applied on Arabidopsis plants. As shown in Figure 3A, the expression of both *GSTs* presents a high sensitivity to SA. Expression occured after a treatment with 0.1 mM SA, whereas *PR-1* expression started above 0.2 mM SA, reaching a maximum induction around 0.3 mM SA. In *NahG* plants, SA treatment had an effect on gene activation, when concentration above 0.5 mM was used. The response of *PR-1* and *AtGSTF6* to pathogens was affected in *etr1*, but only *PR- 1* expression was reduced after a treatment with exogenous SA. Surprisingly the expression of *AtGSTF2* , which was blocked in the *etr1* mutant after *Pseudomonas* inoculation, was slightly induced in *etr1* upon application of exogenous SA, indicating that the perception of SA may depend on ethylene.
To test this hypothesis, the same plants were exposed to a treatment with ethylene. An ethylene concentration of 0.1 µl/L was sufficient to induce both *AtGSTF2* and *AtGSTF6*, with an expression increasing at higher hormone concentrations (Figure 3B). Interestingly, the response of *GSTs* towards ethylene was affected in *NahG* plants, while no expression could be detected in *etr1*. This suggests that SA accumulation may be important for the regulation of ethylene perception. *PR-1* was not expressed in Arabidopsis plants after most ethylene concentrations.

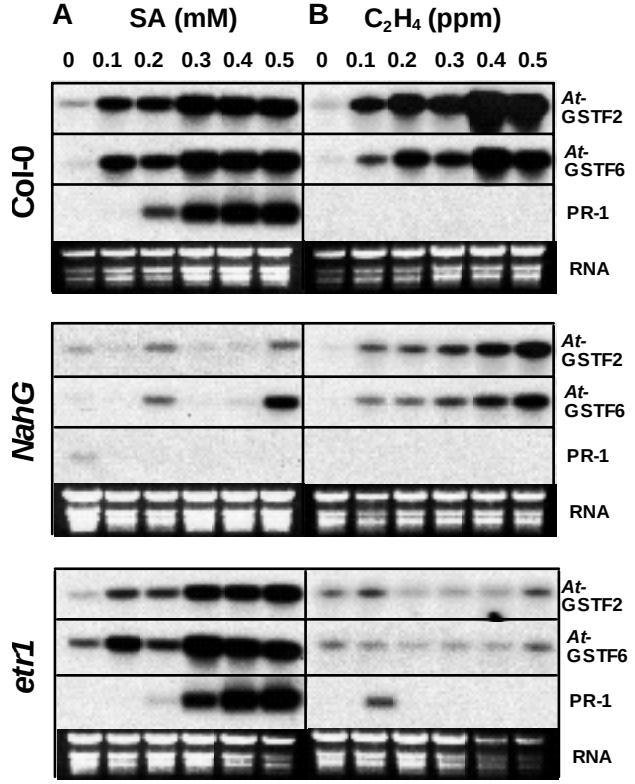

Figure 3. Expression of *AtGSTF2*, *AtGSTF6* and *PR-1* in Arabidopsis Col-0 plants, *NahG* plants and *etr1* mutants after the application of exogenous SA or ethylene.
(A) SA treatment
(B) Ethylene treatment
Soil was drenched with water or SA (0.1-0.5 mM final) for 1 day, or plants treated with ethylene (0-50 ppm) for 6 hours before harvesting the leaf material. Total RNA was extracted as described in figure 2. The experiment was repeated once with similar results.

ATGSTF2 and ATGSTF6 genes are specifically regulated

In Arabidopsis, *AtGSTF2* and *AtGSTF6* have very close relatives, respectively the *AtGSTF3* and *AtGSTF7* genes. The Northern blot hybridization technique does not allow the detection of their specific expression, because the two GST homologs share more than 97% of identity in the coding sequence. To solve this problem, we designed pairs of specific primers and perform RT-PCR analysis of the different GSTs.
In the first series of PCR reactions we tested the sensitivity of amplification by using the full coding region of *GSTs* cloned into plasmid vectors. The absence of cross-amplification among the considered plasmidial *GSTs* (Figure 4A) confirmed that each pair of primers used was specific to the correspondent *GST* gene.
The second series of RT-PCR reactions with gene-specific primers were carried out using DNA from Col-0 plants treated with inducers of *GSTs* (*Pst*, SA, ethylene, auxin). As the *AtGSTF2*, *F3*, *F6*, *F7*, (PHI class) contain 2 introns in the coding region, the amplification with genomic DNA gave the expected product size of approximately 850 bp, whereas the amplified RT reaction product has a size of about 650 bp (Figure 4B). This difference in size indicated that a genomic DNA contamination has not occured in the RT samples.

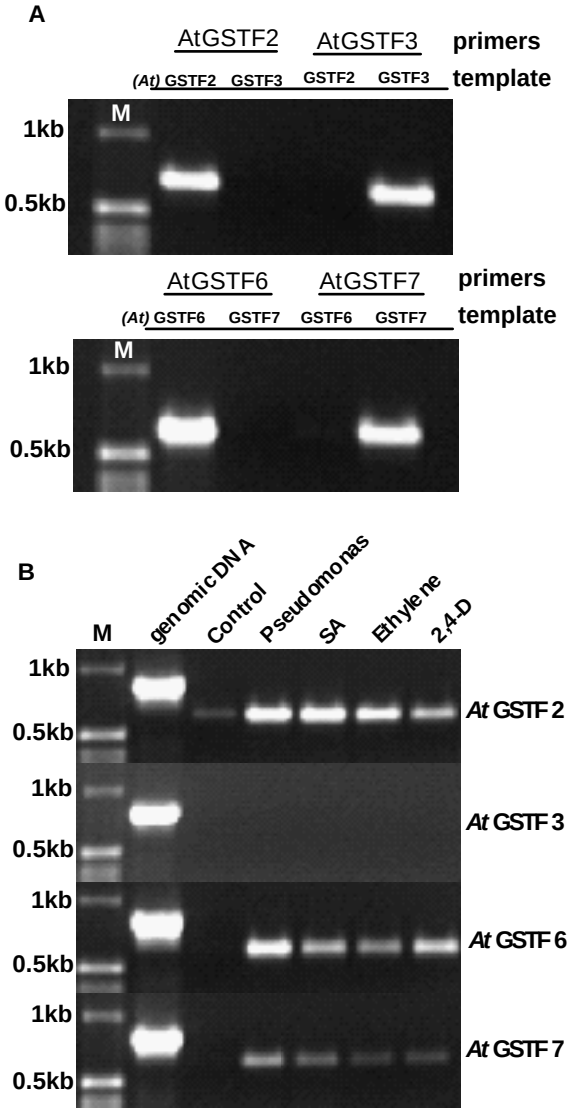

Figure 4. Specific PCR amplification of GSTs genes and gene product.
(A) Primer specificity with cDNA as a template
(B) Amplification of genomic DNA and RT products
Genomic DNA and cDNA were amplified by PCR using specific primers of *At*GSTF2, *At*GSTF3, *At*GSTF6 and *At*GSTF7. PCR reaction was performed with the same primers on RT products of plant material control, inoculated with *Pseudomonas*, treated with SA, ethylene and 2,4-D.
The product of the reaction was loaded equally and separated on an agarose gel, and stained with ethidium bromide. M, molecular weight marker.

AtGSTF2 was highly induced by the treatments, while *AtGSTF3* did not respond to any of the stimuli. Interestingly, both *AtGSTF6* and *AtGSTF7* genes were induced by the treatments at different levels. However, the expression of *AtGSTF6* was higher than its homolog. Since the RT-PCR technique gives semi-quantitative data, it was not possible to determine the the accumulation ratio of *AtGSTF6* and *AtGSTF7* transcripts. The data of RNA-gel blot analysis with *AtGSTF2* probe are specific to the corresponding gene, whereas hybridization with *AtGSTF6* probe is less representative.

Ethylene production is associated with HR upon Pseudomonas infection

Since rapid SA synthesis occurred in Arabidopsis plants infected by *Pseudomonas syringae*, we measured ethylene production in Arabidopsis Col-0 plants, *NahG* and *etr1* mutants inoculated with 10 mM $MgCl_2$ (Figure 5A) or with the necrotizing pathogen *Pst*DC3000(*avrRpt2*) (Figure 5B). A low production of ethylene (between 3 and 95 nmoles ethylene$\cdot h^{-1}g^{-1}$fresh weight) is induced after syringe inoculation of $MgCl_2$.
In the presence of the pathogen, ethylene increased rapidly during the first day of infection with a peak of production at 12 hours post-inoculation corresponding in Col-0 plants to approximatively 400 nmoles ethylene$\cdot h^{-1}g^{-1}$fresh weight. Thus, 2 hours after *Pseudomonas* inoculation, Arabidopsis Col-0 plants produced already 6 times more ethylene than the control plants. At this level, ethylene production is sufficient for GSTs induction. In *etr1* mutants, ethylene was produced in both control and infected plants in the same proportion as in Col-0. Surprisingly, *NahG* plants synthesized a lower amount of ethylene, corresponding to about one half of the wild type level. Inoculation with *Pst*DC3000(*avrRpt2*) and non-pathogenic ethylene inducing treatments (wounding, salt, α-aminobutyric acid) revealed that at least three independent lines of Arabidopsis *NahG* plants produced less ethylene than the wild type plants (data not shown).

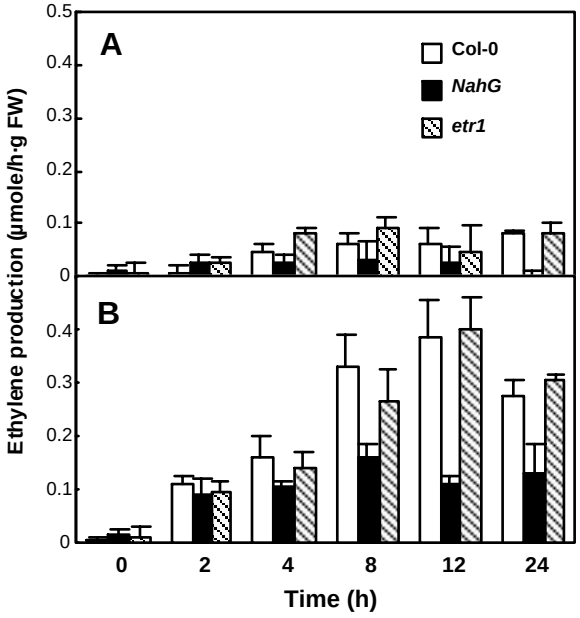

Figure 5. Ethylene accumulation in leaves of Arabidopsis Col-0 plants, *NahG* plants and *etr1* mutants after infection with *Pseudomonas syringae*
(A) $MgCl_2$
(B) *Pst*DC3000(*avrRpt2*).
Three leaves of treated plants were placed in 8ml vials 2 hours before sample harvesting and GC measurement. Each bar represents the average of 6 plants (±SD). The experiment was repeated once with similar results. FW, fresh weight.

Recombinant AtGSTF2 is active as glutathione peroxidase towards fatty acid hydroperoxides

Functional expression of full-length cDNA in *E.coli* revealed that the protein encoded by the *AtGSTF2*- gene possess diverse glutathione *S*-transferase (GST) and glutathione peroxidase (GPOX) enzyme activities (Zettl *et al*., 1994). To further characterize the specificity of the GST encoded by *AtGSTF2*, we cloned the cDNA into expression vector pET11d. The resulting plasmid, p*AtGSTF2*, allowed the expression of the protein in *E.coli* under the control of an IPTG- inducible promoter.
SDS-PAGE analysis of extracts from *pAtGSTF2*-containing bacteria showed that a protein with apparent molecular mass of 26 kD accumulates in a IPTG-dependent manner (Figure 6A). Calculations of the size of the *AtGSTF2*-gene product predicted a polypeptide of approximately 24 kD.
Then bacterial extracts were subsequently analyzed for GST- and GPOX-activity with different selected substrates. The background bacterial activity was substracted from all other AtGSTs measurments.
The highest GSTenzymatic activity has been measured in presence of the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Figure 6B). Unlike results obtained with the closely related structure of p-nitrobenzyl chloride (pNBC), no or little activity was detected using 1,2-epoxy-3(nitrophenoxy)-propane (ENPP) as substrate (data not shown). The measured activity for the conjugation of benzyl isothiocyanate (BITC), a naturally occurring compound, was shown to be one half lower than the activity of this enzyme for CDNB conjugation.
In comparison, the bacterial extracts exhibited much higher GPOX activities. This was particularly apparent when the natural compound linoleic acid hydroperoxide (LinAHPO) was used as a substrate for the measurement of GPOX activity This activity was three times higher than the activities measured with the artificial substrates CDNB or cumene hydroperoxide.

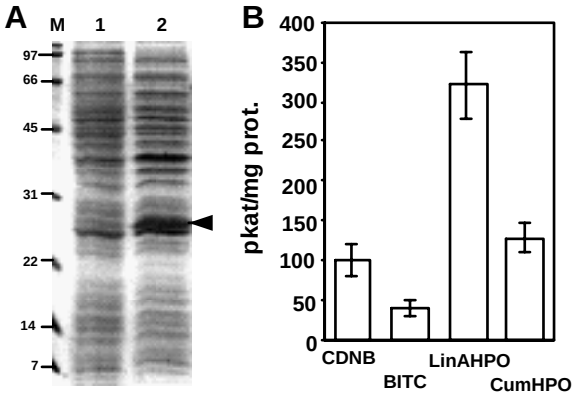

Figure 6. Enzymatic activity of the *At*GSTF2 protein expressed in *E.coli*.
(A) 10 µL of the soluble fraction of bacteria containing the empty vector pET11d (1) and p*At*GSTF2 (2) after incubation for 3 hours with IPTG were submitted to SDS-PAGE. The gel was stained with Coomassie blue. The molecular masses of the marker bands (M) are indicated in kDa and the band corresponding to *At*GSF2 is indicated by the arrow.
(B) GST activity (first two bars from the left) and GPOX activity (last two bars from the left) profile from bacterial extracts containing p*At*GSTF2. Activity values were corrected with the values of the extracts from bacteria transformed with pET11d. The measurements represent the mean value of three independent experiments (±SD).

Discussion

In the present work, we characterized the expression of *AtGSTF2* and *AtGSTF6* after Arabidopsis infection with an avirulent strain of *Pseudomonas syringae*. The rapid induction of the *GSTs* that has been observed results from a combined effect of SA and ethylene that are synthesized in the infected leaves during the infection process. In addition, the *AtGSTF2 in vitro* activity towards reactive compounds such as linoleic hydroperoxides indicates that GSTs may play an important role in the detoxification of such products during the pathogen-mediated HR.

GSTs are a large gene family

GSTs have been mostly studied for their potential to detoxify specific xenobiotics (Dixon *et al*., 1998). However, their potential role in plant metabolism remains unclear. Gene analysis indicate that GSTs are present in maize, wheat, tobacco and Arabidopsis. Expression pattern in Arabidopsis have revealed that GSTs are often regulated in a stress-dependent manner (Alvarez *et al*., 1998; Chen and Singh, 1999; Greenberg *et al*., 1994; Yang *et al*., 1999; Zhou and Goldsbrough, 1993). At least three *GSTs* are rapidly and highly upregulated following the inoculation of *Arabidopsis thaliana* Col-0 plants with *Pseudomonas syringae* (data not shown). We focused our research on two pathogen-induced *GSTs*, *At*GSTF2 and *At*GSTF6 (Zhou and Goldsbrough, 1993) (Greenberg *et al*., 1994), previously described as strongly inducible by ethylene treatment and by pathogen-mediated HR respectively. Using a RT-PCR method and GST-specific primers, we were able to show that *AtGSTF2* gene was expressed after various induction treatments, whereas *AtGSTF3* was not induced. In the case of *AtGSTF6* and *AtGSTF7*, both expressions could be detected by RT-PCR. These two genes are tandemly arranged, but *AtGSTF7* is not induced by environmental factors (wounding, low temperature, high salt) (Yang *et al*., 1999). Interestingly, this result coincides with the low signal of *AtGSTF7* detected when auxin, a stress response hormone, was applied on Arabidopsis leaves. However, in plants inoculated with pathogens and treated with SA and ethylene, both genes were induced, but with a higher expression observed for *AtGSTF6*. These results are globally confirmed by the corresponding RNA-gel blot analysis.

Induction of AtGSTF2 and AtGSTF6 during pathogen-mediated HR is associated with SA accumulation and ethylene production

An early response of plants undergoing HR is a rapid H_2O_2 accumulation at the cell surface (Mehdy, 1994), followed by the synthesis of SA, which plays a central role for the occurence of HR, the induction of PR-1 protein, and the establishment of subsequent plant immunity (Ryals *et al*., 1996). Interestingly, *GSTs* are induced during the early phase of HR, before the expression of *PR-1*. Plant cells may have developed mechanisms of protection to minimize the damaging effect of reactive oxygen species (ROS) produced during HR, and to reduce the effect of toxins released by the pathogens. SA seems essential not only for SAR but also for the rapid local induction of enzymes of cellular protection, such as GSTs (Alvarez *et al*., 1998) (this work). In *NahG* plants, the induction of *AtGSTF2* and *AtGSTF6* by pathogen infection is abolished, indicating that an SA accumulation is required for the expression of both *GSTs*. However, the regulation of *AtGSTF6* expression is partially independent of SA until four hours post-inoculation. Although H_2O_2 is described as a signal for the induction of *AtGSTF6* (Levine *et al*., 1994), evidence presented in this work suggests that a low level of

ethylene (0.1 µL/L) may also induce *AtGSTF2* and *AtGSTF6* genes, whereas *PR-1* is not expressed. Also, both ethylene and SA are produced after 2 hours and 4 hours respectively following the inoculation with *Pseudomonas syringae*, and *GST* induction occurs at lower level of SA than *PR-1* induction. This indicates that the induction of *AtGSTF2* and *AtGSTF6* is the result of the combined effect of at least two signaling compounds, SA and ethylene, whereas *PR-1* expression is only dependent on SA.

AtGSTF2 gene induction by Pseudomonas syringae is the result of a cross-talk between SA and ethylene transduction pathways

Whereas the regulation and the expression of *PR-1* in *Arabidopsis thaliana* have been well characterized in SAR mutants and in mutants defective in ethylene and jasmonic acid signaling pathways (Bowling *et al*., 1994; Cao *et al*., 1994; Penninckx *et al*., 1996), little is known concerning the regulation of the pathogen-induced GSTs. In this study, we characterize the expression of *AtGSTF2* and *AtGSTF6* in the SAR mutants *npr1* and *cpr1*, and in the ethylene insensitive *etr1* mutants inoculated with *Pst*DC3000(*avrRpt2*).
Interestingly, the induction of *AtGSTF2* and *AtGSTF6* is found to be slightly affected in leaves of *npr1* mutant challenged with the pathogen. NPR1 activity is SA-dependent and acts as a positive regulator of SAR, it contains ankyrin repeats and therefore may interact with other protein(s) such as transcription factors (Cao *et al*., 1997; Ryals *et al*., 1997; Xiang *et al*., 1997; Zhang *et al*., 1999). Thus, NPR1 might mainly regulate other SA-dependent defense genes than *AtGSTF2* and *AtGSTF6* during plant-pathogen interaction.
PR-1, as well as the *AtGSTF2* and *AtGSTF6* genes were constitutively expressed in the SAR mutant *cpr1*. CPR1 is a negative regulator of SAR and acts upstream of SA (Bowling *et al*., 1994). Consequently, the level of SA is elevated in the *cpr1* mutant and the SA-dependent genes must be expressed constitutively. This is the case for the pathogen-induced *AtGSTF2* and *AtGSTF6*; indicating that these genes are placed under the control of SA.
Ethylene is a unique signal molecule controlling many aspects of plant development, however it has not been shown to play a role as signal for SAR in Arabidopsis (Lawton *et al*., 1995; Lawton *et al*., 1994). The ethylene receptor mutant *etr1* still expresses *PR-1* and *AtGSTF6* genes after pathogen attack. However, the reduced level of gene expression compared to the wild type indicates that ethylene functions as an enhancer of the SA signaling pathway (Lawton *et al*., 1995) (this work). Interestingly, *AtGSTF6* is strongly induced by ethylene, but does not require a functional ethylene transduction pathway to show expression during pathogenesis. In contrast, *AtGSTF2* is weakly expressed in *etr1* during infection with *Pseudomonas syringae*, indicating that this gene is regulated by ethylene (Zhou and Goldsbrough, 1993) (this work). Because *AtGSTF2* was not expressed in *NahG* plants after pathogen inoculation, ethylene is therefore not the only factor that may regulate *AtGSTF2*. The expression of *AtGSTF2* is slightly reduced in *etr1* after application of SA and fairly reduced in *NahG* plants gazed with ethylene. Also, a low amount of ethylene is measured in three independent lines of *NahG* plants treated with ethylene inducers indicating that the expression of *NahG* could interfere with ethylene production. These results point out that the regulation of the pathogen-induced *AtGSTF2* gene is directly under the control of a cross-talk element integrating the SA and ethylene transduction pathways.
Finally, an experiment with the *jar1* (Staswick, 1992) mutant (data not shown) suggests that the *PR-1*, *AtGSTF2* and *AtGSTF6* genes do not require a functional JA signaling pathway to be expressed after inoculation with *Pseudomonas syringae*.

AtGSTF2 activity is involved in lowering cellular stress during pathogenesis

Recombinant *At*GSTF2 proteins revealed considerable GST activity for the artificial substrate CDNB, and for BITC which is found endogenously in Brassicaceae (Rask *et al*., 2000). However, the same protein presents no activity with the artificial chemicals pNBC and the epoxide ENPP (data not shown). Among the tested GSTs, the recombinant *At*GSTF2 has only a low kinetic with the choosen substrates, indicating that the specific transfer target may belong to another class of chemicals.
We found that this protein has three times more GPOX activity with the natural compounds linoleic hydroperoxides than GST activity. Hence, fatty acid hydroperoxides might represent the natural substrates of *At*GSTF2 *in vivo*. This could explain the low GPOX activity of *At*GSTF2 in presence of the artificial substrate cumene hydroperoxide. Linoleic acid hydroperoxide being an equal mixture of 9- hydroperoxy-10,12(Z,E)-octadecadienoic acid and 13-hydroperoxy-9,11(Z,E)-octadecadienoic acid (Rusterucci *et al*., 1999), it is not possible to determine which is the best substrate for this enzyme.
In plants, fatty acid hydroperoxides accumulate to substantial amounts during pathogen attack (Rusterucci *et al*., 1999), leading to tissue damage and thus weakening the defense of the plant. Evidence for this hypothesis was provided by von Tiedemann (1997) in the interaction between *Phaseolus vulgaris* and *Botrytis cinerea*.
During an incompatible plant-pathogen interaction, cells that undergo HR produce many reactive oxygen species (Mittler *et al*., 1996). This can lead to a non-enzymatic formation of significant amounts of nucleic acid or fatty acids hydroperoxides (Porter *et al*., 1995). Large quantities of these reactive compounds are also produced during HR through the action of a lipoxygenase (Rusterucci *et al*., 1999). The hydroperoxide molecules are then broken down by lyases, a process leading to direct damages of membranes and to the formation toxic compounds such as alkenals and hydroxyalkenals (Takamura and Gardner, 1996). Alternatively, hydroperoxides are also known as precursors for diverse signaling compounds, such as JA which plays a role in the induction of diverse genes during pathogen attack (Farmer *et al*., 1998; Reymond and Farmer, 1998).
As the pathogen-induced *At*GSTF2 is able to detoxify substantial quantities of hydroperoxides, it might be an important factor helping the plant to reduce cell damage during pathogen attack. The detoxifying potential of *At*GSTF2 may counteract the toxicity of endogenously produced harmful compounds, and therefore control the suicide defence mechanism (Mauch and Dudler, 1993). Finally, *At*GSTF2 might play a role in the regulation of the oxylipin signaling pathways for defense, by metabolizing the precursors of the signaling molecules and limiting the formation of fatty acid hydroperoxides derivatives.

Experimental procedures

Growth conditions for plants and bacteria

Arabidopsis thaliana plants were grown on a pasteurized soil mixture of humus/perlite (3:1) under a 12-hr-light cycle with a night temperature of 18°C and a day temperature of 20 to 22°C (60 to 70% relative humidity).
Arabidopsis accession Columbia (Col-0) were obtained from the Arabidopsis Biological Research Center (Columbus, OH), *NahG* plants were obtained from J. Ryals (Novartis, Research Triangle Park, NC). The Columbia (Col-0) ecotype mutants *cpr1*, *npr1*, *jar1* and

etr1 were provided by X. Dong (Duke University, Durham, NY), P. E. Staswick (University of Nebraska, Lincoln, NE) and the Nottingham Arabidopsis Stock Center, respectively.
Pseudomonas syringae pv *tomato* strain DC3000 carrying the avirulence gene *avrRpt2* (Wahlen et al., 1991) were grown at 28°C and 220 rpm in King's B medium (pH 7.0, 10 mg/mL protease peptone, 15 mg/mL glycerol, 1.5 mg/mL KH_2PO_4, and 4mM $MgSO_4$) containing the appropriate antibiotic.

Inoculation with Pseudomonas syringae

Four leaves of 5 to 6-week-old plants were syringe-inoculated with *Pst* DC3000(*avrRpt2*) at a titer of ~10^7 cfu per mL (OD_{600}=0.02) in 10 mM $MgCl_2$ by infiltrating ca. 35% of the leaf surface. Then, the leaves were detached from plants at different time-points after inoculation, and either used directly for ethylene measurements, or frozen and ground in liquid nitrogen for the analysis of gene expression and SA content.

RNA gel blot analysis

RNA was extracted from approximatively 0.15 g of ground leaf tissue with 0.5 mL of 2 M Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, and 20% SDS (1:2:1). The aqueous phase was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (50:49:1), precipitated with 1 volume of 6 M LiCl overnight at 4°C, and resuspended in DEPC-treated H_2O. Six micrograms of RNA was separated on a formaldehyde/agarose gel, visualized under UV light, and transferred to a Nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was probed with the cDNA of *At*GSTF2 and *At*GSTF6 provided by P. Goldsbrough (Purdue University, Indiana, USA) and K. Shinozaki (RIKEN, Tsukuba, Japan) respectively, and with the cDNA of the PR-1 gene (Uknes *et al*., 1992). RNA loading was estimated on ethidium bromide stained gel under UV light.

Measurements of SA

For the analysis of free and conjugated SA, 0.2 to 0.3 mg of ground leaves were extracted once with 0.5 mL of 70% methanol and once with 0.5 mL of 90% methanol in the presence of 100 ng of *o*-anisic acid as internal standard (Meuwly and Metraux, 1993). After evaporation of the methanol in a vacuum centrifuge, the proteins were precipitated with 0.1 mL of 5% trichloroacetic acid and the free phenols were partitioned twice against 0.5 mL of cyclohexane/ethyl acetate (1:1). Fuming hydrochloric acid was added to the aqueous phase to a final concentration of 4M, heated at 80°C for 1 hr and phenolic compounds were extracted as above.

Measurments of ethylene

Three detached leaves (ca. 150mg) were placed 2 hours before analysis in 8 mL-serum cap vials closed with rubber seals. At appropriate time points, samples (250 µL) of gas were then taken and analyzed using a Hewlett Packard 5890 GC fitted with a FID detector. Separations were carried out on a PLOT capillary column (10m x 0.5mm) of Poraplot Q (Chrompack, Antwerp, Belgium) at 50°C with a helium flow rate of ca. 1 mL/min. The detector was calibrated with a gas mixture containing 105 ppm ethylene in helium (Supelco, Bellafonte, USA).

Treatments with SA and ethylene

Five-week-old plants were soil-drenched for 24 hours with a final concentration of 0 to 0.5 mM SA for SA uptake experiments. To analyze the effect of ethylene, 5-week-old plants were gazed for 6 hours in 100 L chamber with ethylene at a concentration of 0 to 50 ppm.

RT-PCR

For the RT reaction, first cDNA strand was synthetized at 37°C for 2 hours in a final volume of 50 µl containing 10 µg of total RNA, 7 µM of oligo-dT primer, 400 µM of dATP, dCTP, dGTP, dTTP, and 200 units of M-MLV (Promega, Madison, USA). DNA was amplified in a 20-cylces PCR reaction using 1 µl of RT reaction, or 1 µg of genomic DNA, or 1 ng of cDNA, and the specific primers for *At*GSTF2 (5'-ATGGCAGGTATCAAAGTTTTCGG-3', 5'-ATTTCTCACTGAACCTTCTCG-3'), *At*GSTF3 (5'-ATGGCAGGTATCAAAGTTTT-CGG-3', 5'-CTGAAGCAAAACCACTTTTG-3'), *At*GSTF6 (5'-ATGGCAGGAATCAA-AGTTTCGGTC-3', 5'-GAGATTCACTTAAAGAACCTTCTG-3'), and *At*GSTF7 (5'-ATGGCAGGAATCAAAGTTTCGGTC-3', 5'-GTGAGTCACTTAAAGAACCTTCTT-3'). The reaction products were analyzed on agarose gels stained with ethidium bromide.

AtGSTF2 expression in E.coli

The *At*GSTF2 cDNA was a gift from P. Goldsbrough (Purdue University, USA). The insert has been amplified by PCR using a set of primers designed to contain Nco I and BamHI sites. The amplification product was digested with the appropriate restriction enzymes, and cloned into pET11d vector (Novagen, Madison,USA). The identity of the pAtGSTF2 construct was confirmed by sequencing.
E.coli BL21(DE3)pLysS (Novagen, Madison, USA) transformed with pET11d containing pAtGSTF2 were precultured overnight in LB medium containing 100 µg/mL ampicillin at 37°C. Fresh LB medium (100 mL) containing ampicillin was inoculated with 1 mL of the preculture under shaking 37°C. Once the OD_{600} reached 0.5, isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM. Three hours later, the cells were harvested by centrifugation, resuspended in 2 mL of ice-cold 20 mM Tris-Cl, pH 7,8, lysed by freezing and thawing followed by two rounds of sonication of 10 s at maximum power (MSE-Gallenkamp, Loughbourough, UK). Debris were removed by centrifugation (15000g, 5 min), and the soluble fraction (crude extract) was used directly to measure the enzyme activities. Protein bands were separated by SDS-PAGE (Laemmli, 1970), and visualized by staining with Coomassie brilliant blue. The molecular mass of the protein was estimated with a broad range molecular weight marker (Bio-Rad, Hercules, USA)

Enzyme assays

Benzyl isothiocyanate (BITC) and p-nitrobenzyl chloride (pNBC) were purchased from FLUKA Chemie (Buchs, Switzerland), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3(nitrophenoxy)-propane (ENPP), cumene hydroperoxide and linoleic acid were obtained from SIGMA Chemicals, (St.Louis, USA). Linoleic acid hydroperoxides (being an equal mixture of 9- hydroperoxy-10,12(Z,E)-octadecadienoic acid and 13-hydroperoxy-9,11(Z,E)-octadecadienoic acid) were produced according to a method described by Graff et al. (Graff *et al*., 1990) using soybean lipoxygenase I.

Spectrophotometric assays were used to determine GST activities (Mannervik and Guthenberg, 1981), ENPP, pNBC (Habig *et al*., 1974) and BITC (Kolm *et al*., 1995). All GST activities were expressed in katal after correction with the background activities of the extracts obtained from bacteria containing an empty pET11d vector. The glutathione peroxidase activity was determined by a coupled glutathione reductase assay according to a protocol from Wendel (Wendel, 1981). Assays were repeated 3 times for each experiment.

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CHAPTER 3

Analysis and expression of *CuPi1*, a pathogen-induced gene in cucumber

Summary
Using mRNA differential display, Marro (1997) has isolated novel genes that are expressed during SAR in cucumber after inoculation with *P.s. lachrymans*. These genes included chitinase, peroxidase, as well as novel gene called cucumber pathogen-induced 1 (*CuPi1*). The single copy gene *CuPi1* is constitutively expressed in fruit, induced by chemical SAR activators and by various pathogens in local and systemic tissue of infected cucumber. *CuPi1* encodes for a small protein of 87 amino acids with homology to ω-conotoxin of a sea snail (*Conus geographus*) venom toxin. In particular, the position of the cysteine residues is conserved between the two sequences. The expression of CuPi1 was toxic for procaryotic expression systems, and the protein was obtained in an *in vitro* expression system. Since experimental results tend to indicate that CUPI1 is toxic for bacteria, it is hypothesized that CUPI1 functions as a toxic polypeptide.

Introduction

Upon recognition of an attacking pathogen, plants have developed sets of induced defense mechanisms, depending on the activation of specific signal transduction pathways. Infection with necrotizing pathogens induces a form of programmed cell death called hypersensitive response (HR) (Dixon and Harrison, 1994) leading to the rapid collapse of the challenged host cells. Furthermore, resistance develops in distant part of the infected cells, protecting the plant to subsequent attack by a broad range of virulent pathogens (Ryals *et al*., 1996). This form of induced resistance is termed systemic acquired resistance (SAR) (Sticher *et al*., 1997) and is dependent on the presence of salicylic acid (SA) (Malamy *et al*., 1990; Metraux *et al*., 1990). The expression of a number of genes (Uknes *et al*., 1992; Ward *et al*., 1991), encoding pathogenesis-related (PR) proteins correlates with SAR (Uknes *et al*., 1992; Ward *et al*., 1991). Concomitantly to SA, the signal molecules ethylene and jasmonate play a key role in the SA-independent defense responses through the expression of protease inhibitors (Farmer *et al*., 1992; Farmer and Ryan, 1990), thionin (Epple *et al*., 1995) and defensin (Penninckx *et al*., 1996).

Besides pathogens, several chemicals including SA, arachidonic acid, 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) can induce acquired resistance as well as the expression of SAR genes (Malamy and Klessig, 1992; Uknes *et al*., 1992; Ward *et al*., 1991; White, 1979), whereas exogenous application of ethylene and jasmonate induces a set of SA-independent defense genes that are also activated upon pathogen infection (Epple *et al*., 1995; Penninckx *et al*., 1996; Vignutelli *et al*., 1998).

In plants several PRs, including PR-1, ß-1,3-glucanases (PR-2), chitinases (PR-3), PR-4 and osmotin (PR-5) show antimicrobial activity in vitro (Abad *et al*., 1996; Caruso *et al*., 1996; Liu *et al*., 1994; Mauch *et al*., 1988; Niderman *et al*., 1995; Ponstein *et al*., 1994; Woloshuk *et al*., 1991). Moreover, it was reported that small disulfide-containing antimicrobial peptides were induced by pathogen infection together with proteins such as glucanases and chitinases, zeamatins, osmotins, and thaumatin-like and ribosome-inactivating proteins (Broekaert *et al*., 1997; Shewry and Lucas, 1997). Another group of cysteine-rich plant peptide families such as thionins, defensins, hevein-like and knottin-like peptides, and the so-called lipid-transfer proteins (LTPs) are active *in vitro* against pathogens (Broekaert *et al*., 1997; Garcia-Olmedo *et al*., 1992; Garcia-Olmedo *et al*., 1995; Shewry and Lucas, 1997).

Overexpression in transgenic plants of genes encoding some of these peptides, such as thionins (Carmona *et al*., 1993; Epple *et al*., 1997), defensins (Terras *et al*., 1995), or LTPs (Molina and Garcia-Olmedo, 1997) has been shown to confer enhanced tolerance to different pathogens, whereas a marked decrease in virulence has been observed for peptide-sensitive mutants of some pathogens (Lopez-Solanilla *et al*., 1998; Titarenko *et al*., 1997).

Cucumber has been used as a model system for the study of SAR induced by different pathogens as well as by SA, INA or BTH (Feussner *et al*., 1996; Schneider and Ulrich, 1994; Siegrist *et al*., 1994). Infection by *Colletotrichm lagenarium*, tobacco necrosis virus (TNV), *Pseudomonas syringae* pv. *syringae*, or *P.s.* pv. *lachrymans* induce local and systemic increase in chitinase, peroxidase, ß-1,3-glucanase and lipoxygenase (Boller and Métraux, 1988; Hammerschmidt *et al*., 1982; Ji and Kuc, 1995; Metraux *et al*., 1989; Rasmussen *et al*., 1995). Among these proteins, class III chitinase has been used as SAR marker of cucumber, since its expression is absent in control tissue and strongly increased locally and systemically after inoculation by pathogens, and after treatments with SA, INA or BTH (Lawton *et al*., 1994; Metraux *et al*., 1989).

To better understand the mechanisms of induction and establishment of SAR, Marro (1997) searched for novel genes expressed in systemic tissue of cucumber plants during SAR induced by *P.s.* pv. *lachrymans*, using mRNA differential display (Liang and Pardee, 1992;

Sharma and Davis, 1995; van der Knaap and Kende, 1995). A PCR fragment called Did-1 was sequenced and showed no homology to known plant sequences in the database. Using the Did-1 fragment, a novel cucumber cDNA, *CuPi1* was isolated and characterized. *CuPi1* expression is induced in cucumber tissue showing SAR, after infection by several pathogen including *P.s.* pv. *syringae*, *P.s.* pv. *lachrymans*, *C. lagenarium* and TNV, or after treatment with SA and INA (Marro, 1997). Sequence analysis of the 568 bp *CuPi1* cDNA reavealed an open reading frame (ORF) of 278 bp capable of coding for a 87 amino acid protein with a molecular mass of 9670 Da (Figure 1). Hydropathy analysis (Kyte and Doolittle, 1982) of the amino acid sequence indicated that the protein is highly hydrophilic except for the N-terminal part that has the features of a potential signal sequence (von Heijne *et al*., 1988), containing 28 amino acids. The mature form of the protein has a calculated molecular mass of 6668 Da and a calculated pI of 10.4. Additional analysis of the amino acid sequence of CUPI1 protein with the PrositeScan revealed a potential cdc2 kinase phosphorylation site on Thr-46, and a putative prenylation site on Cys-84 (Figure 1).
We report here, the further molecular characterization of *CuPi1*, an attempt of the expression of the corresponding protein with the goal to find out the putative function of CUPI.

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cgataggaataaga ATG AGT AGT GGA AAG GGA CGT GGT TAT TCA TTG CTG GCT TTT        56
               M   S   S   G   K   G   R   G   Y   S   L   L   A   F        14

   TTC TTC TTC TTC GTT CTC TTC ATC TCC TCG GAG GTG ACT GCA GCA AGG AAG CTG   110
   F   F   F   F   V   L   F   I   S   S   E   V   T   A   A   R   K   L    32

   AAG ATG CAC CAA AAC AAC GAA AAG GGA ATT GTT TAC TTG ACT CCG AGA ACA GCA   164
    K   M   H   Q   N   N   E   K   G   I   V   Y   L   T   P   R   T   A    50

   ATT CGC AAC AAA CCA ATC TGC GAT GGT AGC GGT CCT TAT AGC CGT TGC ATC CCT   218
    I   R   N   K   P   I   C   D   G   S   G   P   Y   S   R   C   I   P    68

   AGA AGT AAG CCA CCA AAG GAA AAG TGC AAT CCT TAT GTT AGA GGA TGC AGC TTG   272
    R   S   K   P   P   K   E   K   C   N   P   Y   V   R   G   C   S   L    86

   CCG TAG  actgaggactatcacctgtttttcaaaaaatgaataaatgattccgaaatcacatggatttaa  341
    P   *                                                                   87

   atcctattttaaagtttttgtgtattaatttatcctttcaaaaaaagtgtgtgtaattaatatatatagtag  413

   tttatatgatatatgtcagtttggagtttagtatgttttattgtatggttatctctatgtattcagaatcaa  485

   tttggactttccatatatatatattaccaaacatttggactaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  557

   aaaaaaaaaaa                                                             568
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Figure 1. Nucleotide sequence of *CuPi1* cDNA and deduced amino acid sequence.
The underlined amino acid sequence corresponds to a potential signal peptide, the dotted underlined to a potential cdc2 kinase phosphorylation site, the double-underlined to a potential prenylation site and the star to the stop codon.

Results

Southern blot analysis and PCR amplification of cucumber genomic DNA

Southern blot analysis of DNA prepared from cucumber leaves and hybridized with *CuPi1* cDNA showed an unique band after digestion with EcoRI, EcoRV, HindIII and DdeI, and two bands after digestion with PstI, which cuts once in the *CuPi1* sequence (Figure 2A). This reveals that the cucumber *CuPi1* gene is a single-copy gene within the cucumber genome. Additional analysis of Arabidopsis genomic DNA probed with the *CuPi1* cDNA did not show any hybridizing band, even at low (45°C) stringency (data not shown). PCR amplification of the *CuPi1* coding sequence using *CuPi1* cDNA as well as cucumber genomic DNA as template, and specific primers for CuPi1, gave one single amplification product of about 290 bp (Figure 2B), indicating that no intron is present within the coding region of *CuPi1* gene.

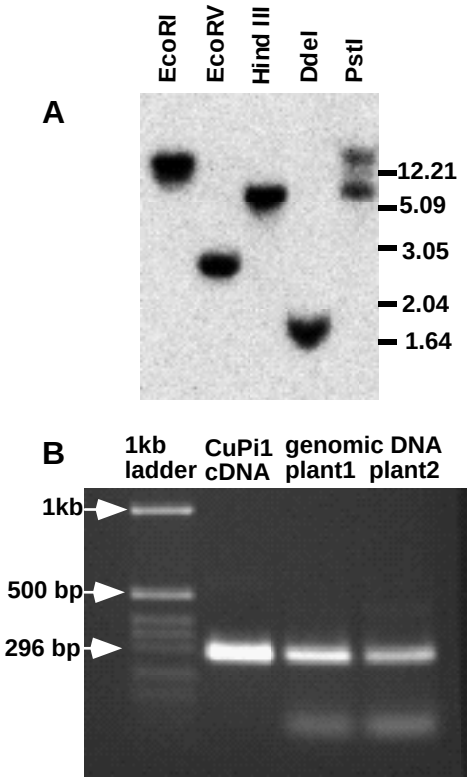

Figure 2. Gene analysis of CuPi1.
(A) Southern blot analysis of genomic DNA extract from cucumber plants. Ten µg of DNA were digested with the corresponding restriction enzyme. Blot was hybridized with the cDNA of *CuPi1*. Molecular marker bands are indicated in kb.
(B) PCR amplification of *CuPi1* cDNA and genomic DNA. 1ng of plasmid containing *CuPi1* cDNA and 1µg of genomic DNA isolated from 2 different plants were PCR amplified using CuPi1 border primers. Amplification products were separated on agarose gel and stained with ethidium bromide.

Expression of Cupi1 mRNA in plant organs and after treatments with chemicals

The level of *CuPi1* transcripts increases after treatment with different pathogens which induce SAR in cucumber (Marro, 1997). The effect of the chemical activators of SAR genes (SA and BTH), and the inducers of SA-independent defense genes, ethylene and methyl jasmonate (MeJA) was tested on the expression of *CuPi1* (figure 3A). The trancripts accumulated after

treatment with SA and BTH, but did not in the control plants, in the plants treated with the wetting powder (WP) of BTH, with ethylene, or with MeJA. Furthermore, the expression level of *CuPi1* was constitutive in fruit (Figure 3B), and particularly high in the fruit skin (data not shown), but was absent in other organs (leaves, roots, flowers).

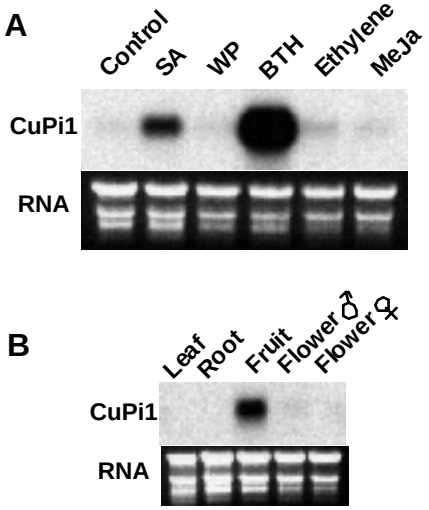

Figure 3. Expression of *CuPi1* after treatments with chemicals and in plant organs.
(A) Expression of *CuPi1* in leaves after treatment with chemicals activators of SAR and the hormones ethylene and MeJA.
(B) Expression of *CuPi1* in plant organs.
Total RNA was extracted from the leaves of the treated plants, or from the different organs, and 6 µg were loaded on agarose-formaldehyde gel. Blot was hybridized with the cDNA of *CuPi1*. Equal loading was monitored by ethidium bromide staining.

Sequence homology to CUPI1

CUPI1 encodes a 87 amino-acids protein showing no signigficant homology to known plant sequence. However, after alignement of small sequence portion of CUPI1, Dr. Jakab was able to find some sequence motifs in common with ω-conotoxin (Figure 4), present in the venom of a sea snail (*Conus geographus*). The ω-conotoxin is divided in three parts, a potential signal peptide (1-22), a propeptide (23-45) and a peptide (46-73). Three toxin peptides are found in the venom, ω-conotoxin GVIB (46-73), ω-conotoxin GVIA (46-72), and ω-conotoxin GVIC (46-71), which are all N-type calcium channel blockers. The toxins are amidated, positively charged, have three disulfide bridges, contain hydroxyproline and many hydroxylated amino acids (Gray and Olivera, 1988; Olivera *et al*., 1987; Olivera *et al*., 1985; Olivera *et al*., 1984). Secondary and tertiary structure of ω-contoxin is essential for the toxicity. This was illustrated by a lack of activity from GVIA analog where two cysteine residues were replaced by serine (Flinn *et al*., 1999).
Amino acid sequence alignment between CUPI1 and ω-conotoxin (Figure 4) revealed that both proteins are basic, contain positively charged amino acids (H, K, R), a potential signal peptide, an intermediate portion, and an almost 40% homologous cysteine-rich C-terminus, where four out of the six cysteine residues of ω-conotoxin were conserved in CUPI1.

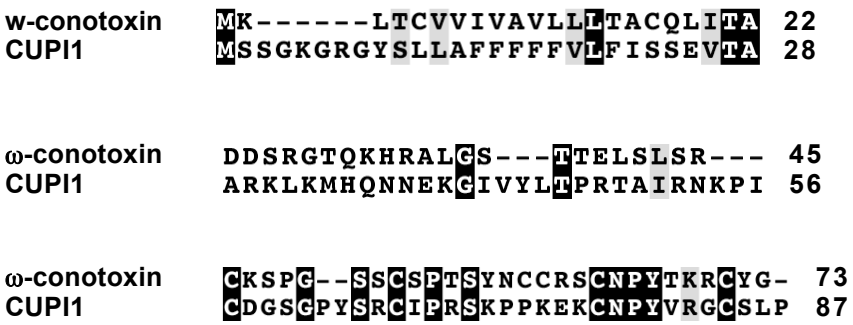

Figure 4. Sequence alignment of CUPI1 and ω-conotoxin.
Identical residues are shaded in black and conserved residues in grey. Number of residues of each polypeptides are indicated.

Expression of the mature protein in E.coli

The nucleic acid sequence corresponding to the mature protein of CuPi1 was cloned into the bacterial 6 histidines-tag-expression vector, pQE30. Two different constructs were obtained, pQE-Did1 (Marro, 1997) and pQE-CuPi1. The *E.coli* strain M15 carrying the plasmid pQE30, pQE-Did1 or pQE-CuPi1 were grown on liquid medium at 37°C and expression was induced by addition of 1mM isopropyl-ß-D-thiogalactopyranoside (IPTG) when absorbance at 600 nm reached 0.5. The growth rate was measured with a spectrophotometer at 600nm one hour before, and during 4 hours after the induction. The bacteria containing the empty vector pQE30 could grow to an OD_{600} of 3.5 independently of the presence of IPTG (Figure 5A). However, the bacterial clone (15) containing the plasmid PQE-Did1 stopped growing as soon as IPTG was added, and the total inhibition of growth lasted at least 4 hours. Interestingly, no inhibition of growth was observed after induction of another clone (pQE-Did1 14), containing an identical CuPi1 sequence, or after induction of the bacterial culture containing pQE-CuPi1 (clone 4.1) with a correct ORF for CuPi1.

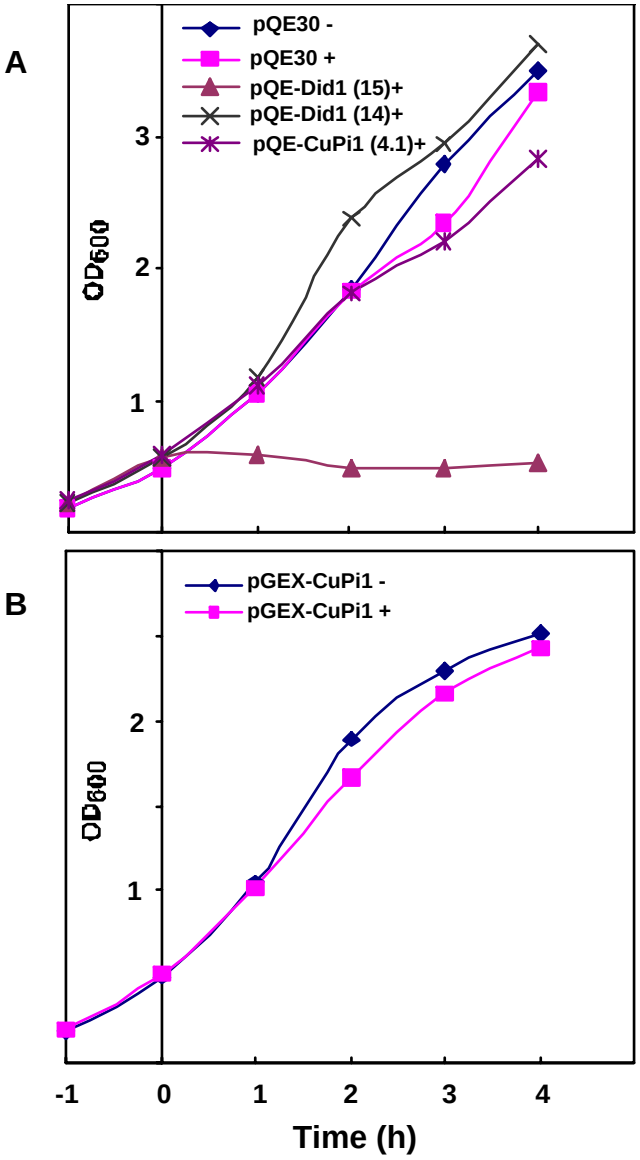

Figure 5. Comparison of the growth of individual bacterial cultures containing an expression vector with CuPi1.
(A) pQE vector with CuPi1. Two constructs were used: pQE-Did1 (Marro, 1997) and pQE-CuPi1.
(B) pGEX vector with CuPi1.
Growth was monitored by measuring the optical density at 600 nm. 1 mM IPTG was added at time zero. - no IPTG; + 1 mM IPTG.

Total proteins were extracted from IPTG-induced bacteria harboring the pQE30 empty vector, pQE-Did1 (14) or pQE-Did1 (15), separated on SDS-PAGE and coomassie-stained (Figure 6A, lane 1 to 3). However it was not possible to visualize a band corresponding to the expected size for CUPI1 (8.5kDa) in the induced bacteria containing pQE-Did1.
To resolve the problem of growth inhibition due to CUPI1 expression, a modified version of the bacterial vector pGEX-6P-1 was used in order to expressed the mature CUPI1 protein fused to the glutathione *S*-transferase (GST). The pGEX-6P-1 vector was slightly modified in order to contain a bacterial LOX recombination site before the cloning site and 6 histidine residues at the C-terminus of the fusion protein. The bacterial strain BL21(DE3) carrying the plasmid pGEX-CupPi1 was grown at 37°C, and protein expression was induced as described above. In this case, no inhibition of the growth was observed after addition of IPTG (Figure 5B). Total proteins were isolated from non-induced and induced bacterial culture and samples run on SDS-PAGE (Figure 6A, lanes 4 and 6). The soluble protein extract of the induced

bacteria was loaded on a column of Glutathione Sepharose® 4B beads for GST fusion protein isolation. Bound proteins were eluated from the column with 10 mM glutathione and analysed on coomassie-stained protein gel (Figure 6A, lane 8). Two stained protein bands, with an apparent molecular mass of 28 and 26 kDa respectively, were visible in the eluate, corresponding to the predicted molecular size of the GST protein, but not to the molecular mass of CUPI1 fused to GST.
Additional protein extractions under denaturing conditions (8M urea) was performed on the cellular debris of bacteria initially containing pGEX-CuPi1, and samples were separated by SDS-PAGE (Figure 6A, lanes 5 and 7). The pellet extract of induced bacteria was loaded on a column containing a nickel-nitrilotriacetic acid (Ni-NTA) matrix for binding of 6 histidine-tag proteins. The eluted fraction, analysed on coomassie-stained protein gel, presented one single low intensity band of approximatively 36 kDa (Figure 6A, lane 9), corresponding to the estimated size of the CUPI1 fused to GST. In order to confirm the identity of the protein to GST-CUPI1, I had the possibility to cleave the fusion protein into GST and CUPI1, using the specific PreScission® enzyme. For this purpose, I attempted first to concentrate the extract using centrifugal filter devices. Unfortunately, protein recovery was very low after centrifugation (data not shown) and I was unable to use the extract for further analysis.

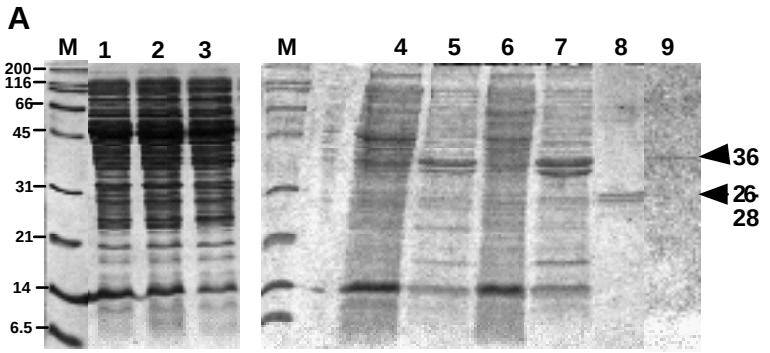

Figure 6. Analysis of the protein extracts from expression systems for CuPi1.
(A) Coomassie stained gel of bacterial protein extract. Lane 1, pQE30 induced (+); lane 2, pQE-Did1 14 (+); lane 3 pQE-Did1 15 (+); lane 4 and 5, soluble extract, respectively pellet of pGEX-CuPi1 non-induced (-); lane 6 and 7, soluble extract, respectively pellet of pGEX-CuPi1 (+); lane 8, elution of glutathione sepharose; lane 9, elution of Ni-NTA. Molecular mass is indicated in kDa. M, molecular weight standards.

Expression of CUPI1 in non-bacterial expression systems

An expression system based on a baculovirus vector was used by Marro for CUPI1 expression. However, an induced protein of the expected size could not be detected in the cells containing the expression vector with CuPi1 sequence (Marro, 1997).
We have used a potato virus X (PVX)-based vector (Chapman *et al*., 1992) for CUPI1 expression. PVX is a single-stranded RNA virus (Koenig and Lesman, 1989), containing five large ORFs (Huisman *et al*., 1988). These ORFs code for one protein involved in replication, three movement proteins and a coat protein (CP). The derived vector (pP2C2S), containing a full length cDNA of PVX with a duplication of the CP promoter followed by a cloning site was used to introduce the full sequence of CuPi1 with a 6 his-tag at the 3'-end (PVX-CuPi1). The DNA vector was then transcribed *in vitro* to produce infectious RNA. The RNA was directly used to infect six weeks-old *Nicotiana benthamiana* plants. Infection developed after

2 weeks and the presence of the CuPi1 transcript in infected leaves was size-estimated after amplification with PVX sequencing primers of RT products (Figure 6B). Total proteins were extracted under denaturing conditions from PVX-, and PVX-CuPi1-infected plants, respectively and loaded on a Ni-NTA column. The eluted proteins were then analyzed on silver-stained SDS-PAGE (Figure 6C). However, elution profile of the proteins produced in PVX- and PVX-CuPi1-infected plants were very similar and no induced protein of the expected size of about 7 kDa was detected.

Finally, the sequence of the mature CUPI1 was cloned into pCITE4a for *in vitro* transcription/translation using reticulocyte lysate. The reaction mix was incubated with plasmid DNA and [^{35}S]-L-methionine; total proteins were separated on SDS-PAGE, and the dried gel was exposed to autoradiography (Figure 6D). Whereas no radiolabeled methionine was incorporated in the reaction mixture without DNA (blank), the bands revealed on the film corresponded, as expected, to the empty pCITE4a, which encodes a predicted polypeptide of 6.8 kDa, and to the pCITE4a-CUPI1 which have a predicted molecular mass of 13.5 kDa.

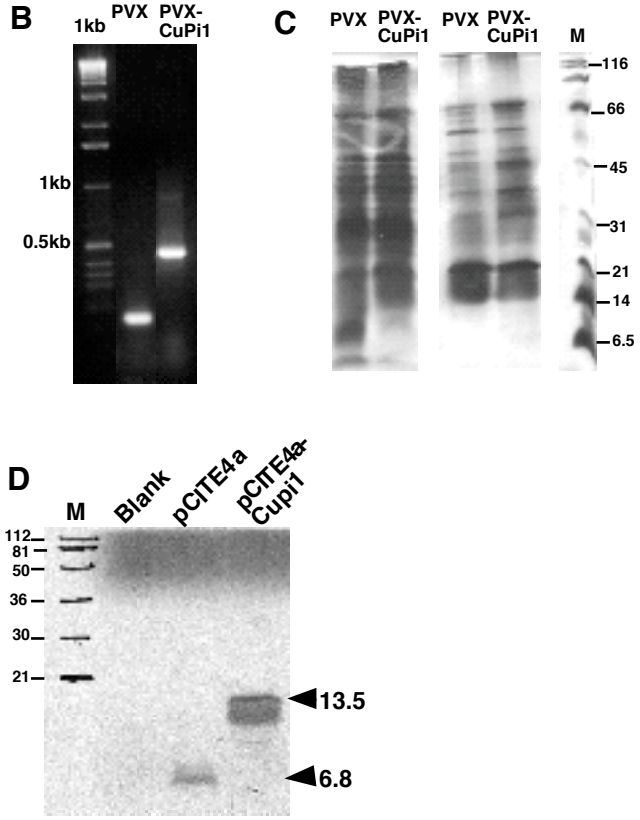

Figure 6. Analysis of the protein extracts from expression systems for CuPi1.
(B) RT-PCR on PVX infected leaves.
(C) Silver-stained gel of proteins extracted from PVX-infected plants. Lanes 1 and 2, total extract, lanes 3 and 4, NiNTA elution fraction. M, molecular weight standards. Molecular mass is indicated in kDa.
(D) Autoradiography from the *in vitro* transcription-translation reaction. One µl of the reaction was loaded on SDS-PGE and exposed with Biomax X-ray film. Molecular mass is indicated in kDa.

Antibody recognition of CUPI1

A synthetic peptide of 11 amino acids corresponding to the **CIPRSKPPKEK** sequence of CUPI1 was injected in rabbit to produce polyclonal IgG. The serum of the rabbit was then used at high concentration (diluted 1: 200) to detect the presence of CUPI1 on protein gel blot analysis. First, in the extracts of the induced bacteria containing pQE30, or pQE-Did1, the antibody recognized two proteins with an apparent molecular mass of 38 and 25 kDa respectively (Figure 6E, lane 1 to 3), independently of the presence of CuPi1 sequence in the plasmid. No protein of about 8.5 kDa (the predicted apparent size of CUPI1 produced in pQE30) was detected by the antibody. Moreover, the same two bands were detected in the soluble extract obtained of the non-induced and induced bacteria containing pGEX-CuPi1 (Figure 6E, lanes 4 and 6), In addition, it was not possible to detect protein, neither in the pGEX-CuPi1 insoluble fraction (Figure 6E, lanes 5 and 7), nor in the sample from the eluate of the Ni-NTA column (Figure 6E, lane 9). Even more, in the *in vitro* reaction samples, the antibody recognized one band of approximatively 33 kDa independently of the presence of CuPi1 sequence in the plasmid, whereas no band was visible at the expected size. Finally the only polypeptide specifically recognized by the antibody was the synthetic peptide (Figure 6E lane P).

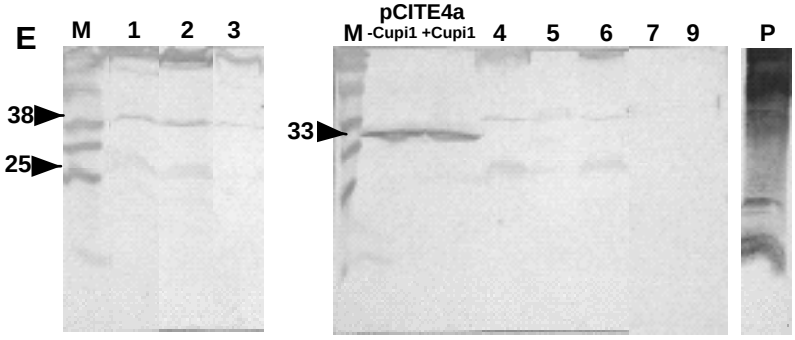

Figure 6. Analysis of the protein extracts from expression systems for CuPi1.
(E) Western gel blot analysis of bacterial extract using an anti-CUPI1 antibody. Molecular mass is indicated in kDa. P, synthetic peptide. M, prestained molecular weight standards, 112, 81, 50, 36, 30, 21.

In vitro antimicrobial activity of CUPI1

Although inhibition of bacterial growth *in vitro* could not be tested with a purified recombinant CUPI1, we observed that total proteins, extracted from an induced culture containing pQE-Did1 15, inhibited the growth of *E.coli* wild type in a microtiter plate assay, whereas no effect was observed with the non-induced extract or with the normally growing induced bacteria containing pQE-Did1 14 and pQE-CuPi1 4.1 (data not shown).

Discussion

CuPi1 is typically a SAR gene

In a previous study, Marro (1997) isolated by differential display a PCR fragment called Did1 the expression of which was induced in cucumber tissues expressing SAR. Did1 was used to isolate the full cDNA *CuPi1* which showed no significant homology to known sequences in the database, and which encodes a putative 87 amino-acids-protein with a putative signal sequence (Figure 1). The expression of *CuPi1* in local and systemic cucumber tissue after inoculation with *P. s.* pv *lachrymans* occured earlier than the SAR marker class III chitinase (Marro, 1997), and *CuPi1* was found to be expressed after inoculation with several pathogens inducing SAR in cucumber (*P.s.* pv. *lachrymans, P.s.* pv. *syringae*, *C. lagenarium* and TNV). Moreover, the *CuPi1* gene was induced after treatment with chemical inducers of SAR such as SA and BTH, but not after treatment with the MeJA or ethylene, which are activators of different defense responses. Taken together, these data show a strong correlation between *CuPi1* expression and SAR, although *CuPi1* is constitutively expressed in fruit of cucumber. Finally, additional analysis revealed that the cucumber *CuPi1* gene is a single-copy gene within the cucumber genome, and that no intron is present within the coding region of *CuPi1* gene.

CUPI1 has some motifs in common with ω-conotoxin of Conus geographus

Alignment of small sequence portion of CUPI1 allowed Dr. Jakab to find homology with ω-conotoxin, a component of the venom of a fish-hunting marine mollusc, *Conus geographus*. Interestingly, sequence alignment of CUPI1 and ω-conotoxin shows that the sequence motifs in common are potential signal peptide, an intermediate sequence, and a cysteine-rich C-terminus, where 40% of the amino acids are conserved. Post-translational modification of the the C-terminal part of CUPI1 (hydroxylation of proline residues, disulfide bridges) are possible and might be implicated in the putative toxicity of CUPI1, since secondary and tertiary structure of ω-conotoxin is essential for the toxicity (Flinn *et al*., 1999).
In plants, several small cysteine-rich polypeptides like thionins, the basic proteins plant defensins, lipid transfer proteins and Snakin-1 have been found to be toxic against microbes in plants (Broekaert *et al*., 1997; Garcia-Olmedo *et al*., 1992; Segura *et al*., 1999; Shewry and Lucas, 1997). In addition, it was shown that the specific binding of plant defensin from Dahlia to fungal cells was required for antifungal activity (Thevissen *et al*., 2000).

CuPi1 expression in prokaryotic systems is toxic for the bacteria

The expression vector pQE30, containing a 6 histidine-tag was choosen to express the mature CUPI1 in *E.coli*. However, as soon as the bacterial culture transformed with the clone containing pQE-Did1 15 was induced by IPTG, complete inhibition of growth was observed. As no induced protein was visible on the total protein extract from the induced culture, we attempted to purify 6 his-tag-proteins using a Ni-NTA matrix. Several proteins were observed in the eluted fraction. However, no one had a molecular mass corresponding to the expected size of CUPI1 (data not shown), indicating a possible non-specific binding of these proteins to the nickel beads. In fact, CUPI1 was probably present in the extract, but as the bacteria stopped growing within a few minutes after induction, the newly synthetized proteins were present in very small amounts and difficult to detect. Therefore, antibody detection was performed on the total extract. Unfortunately, the antibody against the synthetic peptide was not specific for CUPI1, since it detected two proteins of approximatively 38 and 25 kDa

which seemed to belong to *E.coli*, and one protein of 33 kDa in the reticulocyte lysate reaction. Indeed, these proteins were also recognized in the non-induced extracts, and their molecular mass was not in the expected range of CUPI1. Total proteins were also extracted from an induced bacterial clone not affected in its growth rate, and containing a correct sequence of CuPi1 (pQE-Did1 14). Because no induced protein was detected in the extract at the size of CUPI1, bacteria were probably affected in the induction of the RNA T7 polymerase by IPTG.
The toxic effect of CUPI1 could be avoided using the construct pGEX-CuPi1 wich encodes a fusion protein GST-CUPI1 and which contains a 6 histidines-tag at the C-terminus. The bacteria containing the plasmid grew normally after induction with IPTG. Unfortunately, I could not purify a sufficient amount of protein, neither using glutathione Sepharose, nor Ni-NTA. The further investigation of the CUPI1 function was then compromised. However, efforts to set up the purification protocol of the recombinant protein should be carried on, since another group was able to isolate GST-CUPI1 with glutathione Sepharose (Metraux, personal communication), and the induced bacteria contained a correct sequence of CuPi1 (data not shown).
Finally, I was able to obtain the expected CUPI1 protein using an *in vitro* transcription/translation system based on reticulocyte lysate. Although this system works well, it has the drawback to give a very low yield (100 ng protein/50µL reaction), and to be expensive. For these reasons, we recommend to use the *in vitro* transcription/translation system for certain purposes (e.g. 2-proteins binding, immunoprecipitation), but not to test the putative toxic effect of CUPI1.

Experimental procedures

Growth conditions for plants and treatments with chemicals

Cucumber plants (*Cucumis sativus* L, cv. Wisconsin SMR-58) were grown in a greenhouse with a 14h-photoperiod, with a night temperature of 20°C and a day temperature of 22 to 24°C. Two to three-week-old plants were soil-drenched with 1 mM solutions of SA, and 300 µM solution of BTH. Control plants were treated with water for SA and wetting powder solution for BTH. To analyze the effect of ethylene and methyl jasmonate, plants were either gassed for 24 hours in 100 L chamber with 50 ppm ethylene, or closed in 100 L chamber with a Whatman paper soaked with 50 µL MeJA.
RNA gel blot analysis

Total RNA was extracted from ground cucumber leaves, fruits, flowers and roots tissue with 0.5 mL of 2 M Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, and 20% SDS (1:2:1). The aqueous phase was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (50:49:1), precipitated with 1 volume of 6 M LiCl overnight at 4°C, and resuspended in DEPC-treated H_2O. Six micrograms of RNA were separated on a formaldehyde/agarose gel, visualized under UV light, and transferred to a Nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was probed with the cDNA of *CuPi1* and exposed to an X-OMAT AR film (Kodak, Rochester, NY). RNA loading was estimated on ethidium bromide stained gel under UV light.

Southern blot analysis

Genomic DNA was extracted from cucumber leaves and Arabidopsis plants according the method of Saghai-Maroof (1984). Ten µg of DNA were digested individually with the following restriction enzymes, EcoRI, EcoRV, HindIII, DdeI and PstI. Digested genomic DNA was separated on a 0.7% agarose gel. The gel was then depurinated in 0.25 N HCl for 15 min, denatured in 0.5 N NaOH/ 1.5 M NaCl for 30 min, neutralized in 0.5 M Tris-HCl (pH 8.0)/ 1.5 M NaCl for 30 min, and blotted to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was probed with the cDNA of *CuPi1* and exposed to an X-OMAT AR film (Kodak, Rochester, NY).

PCR amplification of CuPi1

DNA was amplified in a 35-cycles PCR reaction using 1 ng of CuPi1 cDNA, or 1 µg of genomic DNA from cucumber and the specific primers for CuPi1, 5'-GAATTCCTC-GAGATGAGTAGTGGAAAGGGA-3' and 5'-CGCGGATCCCTACGGCAAGCTGAC-ATCC-3''. The reaction product was analyzed on agarose gel stained with ethidium bromide.

Expression of CuPi1 cDNA in bacteria, E. coli

The vector pQE30 (Qiagen, Hilden, Germany) containing 6 histidine residues at the N-terminus was used as an expression vector to express a protein encoding the mature CUPI1. The *CuPi1* cDNA was amplified by PCR using Taq polymerase, one primer with a BamHI site, 5'-GGATCCCCGCAAGGAAGCTCAAGATGC-3', and one primer with a SalI site, 5'-TGTCGACTACGGCAAGCTG-3', and cloned into TOPO vector (Invitorgen, Carlsbad, CA). The cloned product was digested with BamHI and SalI, purified on agarose gel and cloned into PQE-30. Plasmid DNA was sequenced to confirm the predicted sequence, and then introduced by electroporation into *E.coli* host strain M15 (Qiagen, Hilden, Germany).
A second PCR product correspondig to the sequence encoding the mature CuPi1, was cloned into UNI vector (Invitorgen, Carlsbad, CA). and then introduced by bacterial recombination into a modified pGEX-6P-1 (Amersham Pharmacia Biotech, Little Chalfont, England) containing the LOX bacterial recombination sites and 6 histidine residues at the C terminus of the recombinant protein. CuPi11 fused to glutathione *S*-transferase (GST) was sequenced to confirm the predicted sequence, and then introduced by electroporation into the *E.coli* host strain BL21(DE3) (Novagen, Madison, WS).

The bacterial host strains containing pQE30-CuPi1, or pGEX-6P-1-CuPi1 were grown in LB medium containing 100 µg/mL ampicillin, or 50 µg/mL kanamycin respectively. The bacterial culture was grown at 37°C with vigorous aeration (220 rpm) on a shaker until the absorbance at 600 nm reached 0.5. Expression was induced by the addition of isopropyl-D-thiogalactopyranoside (IPTG), at a final concentration of 1mM. Bacteria were grown for an additional 4 h. Bacterial growth rates were measured with a spectrophotometer at 600 nm every hour before and after induction.
For protein isolation, bacteria containing pQE30-CuPi1 were precultured overnight in LB medium containing the 100 µg/mL ampicillin. Prewarm (37°C) fresh LB medium (50 mL) with ampicillin was inoculated with 1 mL of the preculture and shaken at 200rpm at 37°C. Once the OD_{600} reached 0.5, IPTG was added to a final concentration of 1mM. Three hours later, the cells were harvested by centrifugation, resuspended in 1.5 mL of 0.1 M NaH_2PO_4, pH 7.0, 300mM NaCl, 5mM ß-mercaptoethanol, lysed by freezing and thawing followed by

30 minutes incubation with 1mg/mL lyzozyme at room temperature and three rounds of sonication of 10 s at maximum power (MSE-Gallenkamp, Loughbourough, UK) and centrifuged to collect the debris (15000g, 10 min).
To purify GST-CUPI1 cells were harvested from liquid culture (50 mL) by centrifugation at 6,500g for 5 min, the liquid discarded and the cell pellet resuspended in 1-2 ml of lysis buffer PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) + 1%Triton X-100 and 5mM ß-mercaptoethanol. The cells were lysed as indicated above. The supernatant was loaded on a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech, Little Chalfont, England) following the manufacturer's instructions.
The proteins were extracted from the pellet with 1.5mL of 8M urea in 0.1 M NaH_2PO_4, pH 7.0, 300mM NaCl, 5mM ß-mercaptoethanol, incubated with NiNTA resin (Qiagen, Hilden, Germany) according the manufacturer's instructions.
All protein samples were separated by SDS-PAGE (Laemmli, 1970), and visualized by staining with Coomassie brilliant blue. Molecular mass of the protein was estimated with a broad range molecular weight marker (Bio-Rad, Hercules, USA).

Expression of CuPi1 in potato virus X

Full coding sequence of CuPi1, containing a 3'-6his-tag was amplified from CuPi1 cDNA, cloned into pP2C2S using 5'-blunt and 3'-SalI sites and the construct confirmed by sequencing. Ten µg of SpeI-linearized vector was used in transcription reaction. *In vitro* transcription and plant inoculation were previously described (Chapman *et al*., 1992).
For the RT reaction, first cDNA strand was synthetized at 37°C for 2 hours in a final volume of 20 µl containing 2 µg of total RNA (extracted as described above), 7 µM of oligo-dT primer, 400 µM of dATP, dCTP, dGTP, dTTP, and 100 units of M-MLV (Promega, Madison, USA). DNA was amplified in a 35-cylces PCR reaction using 2 µl of RT reaction and the specific primers for PVX (5'-CGATCTCAAGCCACTC-3', 5'-GACCCTATGGG-CTGTG-3').The reaction products were analyzed on agarose gels stained with ethidium bromide.
For protein extraction, 50 grams of leaf tissue were ground in 300 mL of denaturing buffer (100mM phosphate buffer pH 8.0, 8M urea, 2M NaCl, 10 mM imidazole), filtered through 4 layers of Miracloth and centrifuge at 15'000xg for 30 minutes at 4°C. The supernatant was incubated with Ni-NTA beads 14 hours at 4°C under gentle shaking. Beads washing and protein elution were performed according the manufacturer's instructions (Qiagen, Hilden, Germany). Proteins were separated on SDS-PAGE and silver stained.

In vitro transription/translation reaction

pQE30-CuPi1 4.1 was digested with EcoRI and SalI, purified from agarose gel and cloned into pCITE4a (Stratagene, La Jolla, CA, USA) for in vitro transcription/translation. TNT T7 Quick Coupled Transcription/Translation Systems (Promega, Madison, WS, USA) was used according the manufacturer's instructions with pCITE4a-CuPi1 plasmid DNA and [^{35}S]-L-methionine. The product was then separated on SDS-PAGE, the gel was dried under vacuum on Whatmann paper and exposed to autoradiography. To produce larger quantities of protein, [^{35}S]-L-methionine was replaced by D,L-methionine.

Protein gel blot analysis

Proteins were separated by SDS-PAGE, transfered at 100 V for 2 hours and 15 min on nitrocellulose Hybond ECL® membrane (Amersham Pharmacia Biotech, Little Chalfont, England), in 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) MeOH. The membrane was saturated for 30 min in TBS (0.3% Tris base pH 7.4, 0.8% NaCl, 0.02% KCl) containing 5% skim milk powder (Difco, Sparks, USA) incubated for 2h with anti-CUPI1 diluted 1:200 in TBS-5% skim milk powder, washed once 5 min with TBS, twice 10 min with TBS containing 0.05% NP40, and once 5 minutes with PBS. The membrane was then incubated 2 hours in in TBS+5% skim milk powder in the presence of the immunoglobulin G anti-rabbit conjugated to the alkaline phosphatase and then washed as described above. The development was performed by adding 6.6µl of 5% BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 12.2 µl of NTB (4-nitrotetrazoliumblue) per mL of developing buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

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CHAPTER 4

Conclusion

Are pathogen-induced glutathione *S*-transferases involved in resistance?

The oxidative burst generated during infection of wild-type Arabidopsis plants by an incompatible pathogen, generates a large production of toxic active oxygen species (AOS). Among them, H_2O_2 could trigger hypersensitive cell death, whereas lower doses of H_2O_2 might serve to activate cellular protectant genes such as glutathione peorxidase (GPOX) or GST in adjacent cells (Levine *et al*., 1994). The pathogen-induced GST1 was also shown to be a molecular marker for systemic micro hypersensitive response (HR) (Alvarez *et al*., 1998). Despite these results, we were particularly interested in the induction of *AtGSTF2*, described as ethylene-induced gene (Zhou and Goldsbrough, 1993), rather than in *AtGSTF6* (former GST1) which seemed to respond to many other stresses in plant (Wagner *et al*., 2001).
Interestingly, *AtGSTF2* was both SA- and ethylene-dependent when induced by pathogen, contrasting with reports on Arabidopsis pathogen-induced genes, which are usually classified either SA- dependent (Lawton *et al*., 1995), or SA-independent (Penninckx *et al*., 1996).

In addition to the *in vitro* GST activity, the recombinant *At*GSTF2 protein revealed higher GPOX activity with the natural substrate linoleic acid hydroperoxides (LinHPO). Because fatty acid hydroperoxides accumulate to substantial amounts in plants during pathogen attack (Rusterucci *et al*., 1999), they might represent natural substrates of the detoxifying enzyme *At*GSTF2 *in vivo*. Reactive hydroperoxides can be produced by the pathogen (von Tiedemann, 1997), or during HR, by non-enzymatic formation (Porter *et al*., 1995), or through lipoxygenase (LOX) activity (Rusterucci *et al*., 1999). Hydroperoxides derivatives can be toxic for plant membranes (Takamura and Gardner, 1996), or precursors of diverse signaling compounds (e.g. jasmonic acid) involved in plant defense response (Farmer *et al*., 1998; Reymond and Farmer, 1998).
As the pathogen-induced *At*GSTF2 is able to detoxify substantial quantities of hydroperoxides, it might be important for the balance of hydroperoxide levels, for the control of programmed cell death (Mauch and Dudler, 1993), for the regulation of the defense oxylipin signaling pathways, or the participation in antioxidant mechanisms for the reduction of symptoms observed in plants expressing SAR.
Finally, the phenotype of transgenic lines of *Arabidopsis thaliana* expressing *AtGSTF2* was analyzed during HR (Lieberherr, 2000; unpublished results). No difference was observed between the controls and the transgenic plants after infection with an avirulent strain *of Pseudomonas syringae*. Measurement of hydroperoxides content in transgenic lines will have to be carried out to test the *in vivo* function of *At*GSTF2.

Towards the antimicrobial function of CUPI1

I studied CuPi1, a novel gene isolated during SAR in cucumber and that shows no signigficant homology to known plant sequence. One of the first surprise was the homology of sequence motifs with ω-conotoxin, a toxin from a sea snail venom. Completely excited by this finding, I worked hard to express CUPI1, without being to far to get it. To know the function of CUPI1 is particularly interesting since plant small cysteine-rich proteins show antimicrobial activity (Broekaert *et al*., 1997; Garcia-Olmedo *et al*., 1992; Garcia-Olmedo *et al*., 1995; Shewry and Lucas, 1997), and CUPI1 could be involved in resistance in Cucurbitaceae.
Although CUPI1 could not be isolated during this work, experimental data indicate that it could be toxic for bacteria. In order to characterize its function, Marro (1997) initiated *Agrobacterium tumefaciens*-mediated transformants of Arabidopsis with *CuPi1* gene under the control of 35S promoter. However, the bacteria carrying the plasmid with CuPi1 could hardly grow, and one single transformed colony was used to produce transgenic Arabidopsis lines. Northern blot analysis of the transgenic plants revealed high expression of *CuPi1*, but sequencing of the amplified transgene, showed that the initial construct had been completely rearranged, coding for a truncated form of CUPI1 (Lieberherr, 2000; unpublished results). As the 35S promoter has low activity in *A. tumefaciens*, some CUPI1 was probably expressed and could possibly be toxic for the bacteria. Thus, mainly mutants of the CuPi1 sequence were probably selected. To avoid the toxic effect of CUPI1 in bacteria, a plasmid carrying an inducible promoter (e.g. glucocorticoid, ethanol) might offer a convenient alternative for future plant transformation.
The results presented here leave the possibility open that CUPI1 acts as antimicrobial peptide in cucumber. Now, since a lot of questions still remain open, we are left to dream about a trememdous antimicrobial activity and no binding activity to mamalian presynaptic calcium channels !

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CURRICULUM VITAE

Personal data
Name: Lieberherr
First name: Damien
Date and place of Birth: 01/11/70, Bellinzona, Switzerland
Nationality: Swiss
Status: Married, 2 children

Education

1995	Master of Science, Plant Physiology, University of Neuchâtel, Switzerland

Awards

1996	"Prix Louis Paris", Award for original research in biology, Master of Science, University of Neuchâtel, Switzerland

Research Experiences

1997 – 2001	Ph.D. student, Pathogen-induced GSTs in *Arabidopsis* and SAR genes in cucumber, Plant Biology Unit, Department of Biology, University of Fribourg, Switzerland (with J.-P. Métraux)
1996 – 1997	Researcher, Effect of drought on flowering interval in Maize, Applied Molecular Genetics Laboratory, International Maize and Wheat Improvement Center, Mexico (with J.-M. Ribaut and D. Hoisington)
1994 – 1995	Graduate Student Researcher, Lipoxygenase and lipolytic acyl hydrolase activities in vitro and in potato infected with *Phytophtora infestans*, Plant Physiology Laboratory, University of Neuchâtel, Switzerland (with P.-A. Siegenthaler)

Teaching Experience

1997-2001	Plant Biology (teaching assistant) University of Fribourg, Switzerland
1993	Plant Physiology (teaching assistant) University of Neuchâtel, Switzerland

Memberships
Swiss Society for Plant Physiology

Publications

Manuscript in preparation

Lieberherr D., Wagner U., Métraux J.-P. and Mauch F. Synergistic activity of salicylic acid and ethylene in the induction of glutathione *S*-transferase *AtGSTF2* by *Pseudomonas syringae*. To be submitted

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