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Molecular genetic analysis of beta-aminobutyric acid-induced stress resistance in Arabidopsis

THESE

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RESUME

Au cours de l'évolution, les plantes ont développé des mécanismes de défense très variés afin de survivre aux attaques menées par les agents pathogènes. Généralement, un premier contact avec des agents pathogènes qui provoquent une nécrose suffit à induire une réaction de résistance dans les zones non infectées de la plante. La résistance systémique acquise (RSA) se caractérise par une accumulation d'acide salicylique (SA) accompagnée par une expression de gènes liés à la pathogénicité. D'autre part, les hormones végétales tel que l'acide jasmonique (JA) et l'éthylène sont impliquées dans une voie de transduction divergente qui protège contre des agents infectieux différents. Les acides aminés non protéiques tel que les acides γ- et β-aminobutyriques (ABAB) ont certains effets sur les animaux et les plantes. Ainsi, les plantes stressées induisent une accumulation d'acide γ-aminobutyrique et des traitements avec ABAB apportent une protection contre différents pathogènes, et ce, chez de nombreuses espèces distinctes. Comme la compréhension des mécanismes d'action d'ABAB n'est pas résolue; nous avons, lors de ce travail, recherché à comprendre le mode d'action de ce produit chimique à l'aide de la plante modèle *Arabidopsis thaliana*.

En premier lieu, nous avons testé ABAB contre le champignon virulent *Peronospora parasitica*. En fait, ABAB protège Arabidopsis par une activation des méchanismes naturels de défense tel que l'accumulation de callose, la réponse hypersensible et la formation de nécroses le long des hyphes. ABAB est aussi efficace contre *P. parasitica* dans des plantes transgéniques ou des mutants déficient dans les voies de signalisation dépendantes de l'SA, du JA et de l'éthylène. Des traitements avec ABAB n'induisent pas l'accumulation d'ARNm des gènes *PR-1* ou *PDF1.2*, respectivement associé à la RSA ou dépendant de l'JA et de l'éthylène. Par contre, ABAB conditionne l'accumulation d'ARNm du gène *PR-1* après attaque par des bactéries virulentes. Dans ce cas, les plantes traitées avec ABAB présentent moins de symptômes que les contrôles non traités. En outre, ABAB protège les mutants insensibles à l'JA et à l'éthylène, mais n'est pas efficace dans des plantes qui présentent une RSA déficiente. ABAB protège donc Arabidopsis par une potentialisation des mécanismes de résistance spécifiques aux différents agents pathogènes rencontrés. De plus, nous apportons certains éléments qui démontrent une indépendance par rapport à la RSA du mécanisme de formation des papilles.

D'autres agents pathogènes peuvent induire des mécanismes de défense dépendant de l'JA et de l'éthylène. Afin d'étendre l'étude du mode d'action d'ABAB à ce type d'agent infectieux, nous avons évalué l'effet de ce produit chimique sur des plantes d'Arabidopsis infectées par l'agent de la pourriture grise *Botrytis cinerea*. Les plantes traitées avec ABAB ont montré une moindre sensibilité à deux différentes souches de cet agent pathogène. En outre, ABAB protège les mutants insensibles à l'JA et à l'éthylène, mais est inactif dans des plantes avec une RSA déficiente. Des traitements avec le benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, un analogue fonctionnel de l'SA réduit de manière similaire l'infection. De plus, ABAB potentialise l'accumulation d'ARNm du gène *PR-1*. L'expression des gènes dépendants de la voie de transduction de l'JA et de l'éthylène n'est par contre pas potentialisé. Ainsi chez Arabidopsis, en plus d'une dépendance à l'JA et à l'éthylène, la protection contre *B. cinerea* est dépendante de l'SA. Nos résultats suggèrent que cette voie de signalisation est réprimée lors de l'infection par *B. cinerea*. Finalement, la surexpression du gène *PDF1.2* observée lors de l'infection par *B. cinerea* de plantes avec une RSA déficiente révèle l'existence d'une connexion entre voies de transduction distinctes.

Certaines rhizobacteries sont capables de provoquer l'induction d'une résistance contre des agents infectieux qui attaquent la plante au niveau des feuilles. Ce type de résistance induite produit des changement dans l'expression de gènes qui sont impliqués lors de stress

biotiques et abiotiques. Nous avons donc testé l'action de ABAB sur la résistance au froid. Le traitement avec ABAB augmente la résistance d'Arabidopsis à la congélation. En effet, après un traitement au froid de 2 jours, la majorité des Arabidopsis traitées avec ABAB ont survécu, alors que tous les contrôles non traités sont morts. Chez Arabidopsis, la stimulation de certains gènes lors de basses températures a pu être corrélée avec une résistance accrue à la congélation. La potentiation de l'expression de ces gènes par un traitement au ABAB n'a toutefois pas été observée. Néanmoins, un traitement avec ABAB conditionne l'expression du gène " heat shock protein 83" lors d'un choc au chaud. Ainsi, ABAB module également les réponses des plantes à des stress tels que le froid ou le chaud, révélant une probable connection entre réponses aux stress biotique et abiotique.

Lors de cette thèse, l'analyse du mode d'action d'ABAB chez Arabidopsis a permis d'atteindre un point de départ pour aborder la compréhension de la modulation de la réponse des plantes aux stress multiples. Dans le futur, la découverte du site d'action de ce produit chimique devrait permettre l'acquisition de nouvelles connaissances concernant la résistance induite ou plus largement la perception de stress par les plantes. Cela devrait apporter de nouveaux outils à l'amélioration de la résistance des cultures par une augmentation du potentiel de défense naturel des plantes.

ABSTRACT

Plants have developed numerous, complex defense mechanisms to escape infection by pathogens. In many cases, resistance is expressed locally and systemically in response to either necrotizing pathogens or root-colonizing soil bacteria. Systemic acquired resistance (SAR) is characterized by a systemic accumulation of salicylic acid (SA) and the concomitant activation of pathogenesis-related (PR) genes. Besides SA, the plant hormones jasmonic acid (JA) and ethylene have been shown to be involved in a separate signal transduction pathway mediating resistance against distinct pathogens. Non-protein amino acids such as γ-aminobutyric acid (GABA) and β-aminobutyric acid (BABA) have known biological effects in animals and plants. In plants, stress provokes an accumulation of GABA and treatment with BABA protects numerous plant species against various pathogens. However, the BABA-mediated protective mechanisms in plants are still poorly understood. To clarify this point, we analyzed the mode of action of BABA in the plant model system *Arabidopsis thaliana*.

We first tested BABA against the virulent pathogen *Peronospora parasitica* and demonstrated that this chemical protected Arabidopsis through activation of natural defense mechanisms of the plant such as callose deposition, the hypersensitive response (HR), and the formation of trailing necroses. BABA was still fully protective against *P. parasitica* in transgenic plants or mutants impaired in the SA, JA, and ethylene signaling pathways. Treatment with BABA did not induce the accumulation of mRNA of the systemic SAR-associated *PR-1* and the ethylene- and jasmonic acid-dependent *PDF1.2* genes. However, BABA potentiated the accumulation of *PR-1* mRNA after attack by virulent pathogenic bacteria. As a result, BABA-treated Arabidopsis plants were less diseased compared to the untreated control. In the case of bacteria, BABA protected mutants insensitive to JA and ethylene, but was not active in plants impaired in the SAR transduction pathway. Thus, BABA protects Arabidopsis against different virulent pathogens by potentiating pathogen-specific plant resistance mechanisms. In addition, this work provides evidence that BABA-mediated papilla formation after *P. parasitica* infection is independent of the SAR signaling pathway.

Other pathogens, such as necrotrophs can induce plant defense responses through JA and ethylene-dependent signal transduction pathway. To extend the analysis of the mode of action of BABA to necrotrophs, we evaluated the effect of this chemical on Arabidopsis plants infected with the gray mold fungus *Botrytis cinerea*. BABA-treated Arabidopsis were found to be less sensitive to two different strains of this pathogen. BABA protected mutants defective in the JA and ethylene pathways, but was inactive in plants impaired in the systemic acquired resistance transduction pathway. Treatments with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, a functional analogue of SA, also markedly reduced the level of infection. Moreover, BABA potentiated mRNA accumulation of the SA-associated *PR-1*, but not the JA/ethylene-dependent *PDF1.2* defense gene. Thus, besides JA/ethylene-dependent defense responses, SA-dependent signaling also contributes to restrict *B. cinerea* infection in Arabidopsis. Our results also suggest that SA-dependent signaling is downregulated after infection by *B. cinerea*. Furthermore, the observed upregulation of the *PDF1.2* gene in mutants defective in the SA-dependent signaling pathway points to a cross-talk between SA- and JA/ethylene-dependent signaling pathways during pathogen ingress.

Plant resistance against pathogens can also be induced by plant-growth-promoting rhizobacteria. Interestingly, this type of induced resistance provokes changes in plant gene expression linked with both biotic and abiotic stresses. We therefore tested the effect of BABA on the resistance to cold stress. BABA treatment increased the tolerance of Arabidopsis plants to freezing: the majority of the BABA-treated Arabidopsis survived, whereas all the water controls died. In Arabidopsis, cold regulated (COR) genes has been shown to be coordinately stimulated during low temperature treatment with a concomitant enhanced freezing tolerance. The possible increased resistance through potentiation of COR genes expression after BABA treatment was monitored. In this case, conditioning effect was not observed. However, BABA treatment leads to potentiation of the expression of the heat shock protein 83 gene during heat stress. Hence, BABA also modulates plant responses to abiotic stresses such as cold or heat shock, revealing possible connection between biotic and abiotic stress responses.

In this thesis, analyses of the mode of action of BABA in Arabidopsis have contributed to reach a starting point for further elucidation of the modulation of multiple stress responses by plants. In the future, the discovery of the site of action of this chemical would give us tremendous knowledge's of the basic mechanisms involved during induced resistance or eventually, in general stress perception. This might provide new tools to enhance crop protection through enhancement of the natural defense potential of plants.

Contents

CHAPTER 1

General introduction

In general, plants are resistant to the majority of pathogenic microorganisms they encounter and disease is the exception rather than the rule. The yield of diseased plants can be reduced to various degrees depending on the nature of the pathogen and on the environmental conditions. When weather conditions are particularly favorable for the development of the pathogen, infection can develop quickly and extensively over a broad geographic region and may lead to an epidemic with all its economic and social consequences. For example, the destruction of potato plants caused by *Phytophthora infestans* in Ireland around 1840 (Bourkes, 1991) has been responsible for the death as well as the emigration of thousands of Irish people to the United States of America, Australia and New Zealand. Hence, the control of plant disease development is crucial in agriculture. As a consequence, resistant varieties, chemical treatments and phytosanitary measures have been widely used to limit the spread of disease. Inbreeding consists in selecting cultivars more resistant to disease and crossing them with other varieties to breed in more characters of agronomical interests. While this approach is of common use, resistant varieties have often a short life-span and the appearance of new

destructive pathogen races is quicker than the time needed for the development of new resistant cultivars. A more recent approach is the use of genetic engineering to introduce single genes. This approach is more rapid than classical inbreeding. Furthermore, it is possible to introduce specific characters for improving plant resistance to pathogen infection without changing the agronomical interest of a particular cultivar. Understanding the molecular and genetic basis of natural plant defense mechanisms becomes therefore a crucial element of this approach.

Plant resistance to pathogen infection

Resistance of plants to pathogenic microorganisms ingress is based on both constitutive and inducible resistance defense mechanisms.

Constitutive resistance mechanisms

The first line of defense a pathogen will encounter during plant infection consist of preformed barriers such as the cell wall, the cuticle and/or inhibitory secondary metabolites. For pathogens penetrating directly through the cell wall, the cuticle layer represents a first barrier to penetration (Kerstiens, 1996). Indeed, most pathogens seem to be equipped with cutinases. Lignin and/or suberin are extremely resilient cell wall components and their occurrence can greatly improve plant resistance to fungal penetration (Sticher et al., 1997). On the other hand, inhibitory secondary metabolites may represent another preformed barrier. These compounds, also called phytoanticipins, generally accumulate in the vacuole as inactive glycosides. The active form of these metabolites is released by hydrolytic enzymes of the cytoplasm when cell compartmentation is lost (Osbourn, 1996).

Inducible resistance mechanisms

Upon recognition of the pathogen, the plant activates various inducible defense mechanisms that counteract growth and spread of the pathogenic microorganisms.

R-gene mediated resistance

The ability of plants to resist pathogen invasion is often dependent upon the recognition of the product of a matching avirulence (*avr*) gene expressed by the pathogen. These *avr* genes are recognized by the products of corresponding resistance (*R*) genes of the host leading to race-specific pathogen resistance (Crute and Pink, 1996). *R* gene action triggers a signal transduction cascade leading to the elicitation of defense responses such as the rapid death of host cells called the hypersensitive response (HR) (Hammond-kosack and Jones, 1996). Concomitantly, in cells adjacent to the lesion, many defense-related genes are activated. The products of these defense genes play a role in limiting the development of the pathogen through reinforcement of the host cells wall or by producing antimicrobial compounds such as phytoalexins as well as pathogenesis-related (PR) proteins. Pathogens that trigger gene-for-gene resistance are called avirulent pathogens. When pathogens do not carry an *avr* gene recognized by the plant, they are called virulent. During a compatible interaction, the plant does not recognize a virulent pathogen and defense responses are triggered too slowly and too weakly, resulting in disease development (Van Loon, 1997).

Systemic acquired resistance

Necrotic lesions such as those provoked by a hypersensitive reaction often lead to the expression of a signal that activates systemic acquired resistance (SAR) in distant tissue of the plant. As a consequence, plants can acquired resistance to normally virulent pathogens and they decrease the size of hypersensitive lesions arising from the interaction with avirulent

pathogens. This resistance state is characterized by an early increase in synthesized salicylic acid (SA) in both primary infected and distally non-infected leaves (Malamy et al., 1990; Metraux et al., 1990). Moreover, SAR is typified by systemic enhanced expression of so-called SAR genes (Ward et al., 1991; Ryals et al., 1996). These genes encode for PR proteins (Van Loon, 1985). In Arabidopsis, PR-1, BGL2 (PR-2) and PR-5 are systemically induced during the onset of SAR (Uknes et al., 1992). Since some of these PR proteins have demonstrated antifungal activity (Kombrick and Somssich, 1997), it is thought that they contribute to protection and are therefore commonly used as markers for SAR.

SA is a key component for the activation of SAR (Hammond-kosack and Jones, 1996; Durner et al., 1998). Exogenous application of SA is sufficient to mimic pathogen-induced SAR with concomitant expression of *PR* genes (Ryals et al., 1996). Construction of transgenic tobacco and Arabidospis expressing a bacterial salicylate hydroxylase gene (*NahG*), the product of which converts SA into inactive catechol (Gaffney et al., 1993; Delaney et al., 1994), demonstrates the requirement for SA as a signal in SAR. Indeed, these plants fail to develop SAR when infected with pathogen or after exogenous application of SA.

While SA is required in the systemic tissue for the expression of *PR* genes, it was proposed that SA is not the (only) primary long-distance signal for the induction of SAR (Willits and Ryals, 1998). Indeed, wild type scions grafted onto NahG rootstocks were still able to express SAR, indicating that a SAR-inducing signal different from SA is probably responsible for resistance (Vernooij et al., 1994). Recently, a lipid transfer protein has been implicated in the modulation of the SAR mobile signal (Cameron et al., 2000). However, SA seems to be necessary in non-infected tissue for SAR expression (Vernooij et al., 1994; Willits and Ryals, 1998). It is possible that SA is produced *de novo* after the accumulation of a mobile signal in systemic leaves (Smith-Becker et al., 1998).

Genes controlling SA-dependent signalling

To understand the mechanisms underlying SAR signalling, many research groups have used a genetic approach in Arabidopsis. A number of mutants with defects in the SA signalling have been characterized during the past few years. A first group of gain-of-function mutants include the *lsd* (lesion simulating disease) (Dietrich et al., 1994), the *acd* (accelerated cell death) (Greenberg et al., 1994) and the *cpr* (constitutive PR) mutants (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998). These plants are characterized by constitutively elevated levels of SA, increased expression of PR-1, BGL2 and PR-5 as well as high resistance to *Pseudomonas syringae* and *Peronospora parasitica.* Plants carrying both the *cpr* mutations and the NahG transgene do not exhibit elevated defense gene expression or elevated resistance to *P. syringae* suggesting that the *CPR* genes act upstream from SA. Mutants such as the allelic mutants *npr1/nim1/sai1* (no PR/no immunity/salicylate insensitive) (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997) do not express *PR* genes in response to SA treatments. *NPR1* encodes a novel protein that contains ankyrin repeats (Cao et al., 1997; Ryals et al., 1997). NPR1 operates down-stream of SA and interacts with b-zip transcription factors that act on the *PR-1* promoter. Hence, NPR1 may contribute to the expression of defense genes by modulating the action of transcription factors (Zhang et al., 1999). Other mutants with defects in genes required for the activation of the SA signalling pathway have been characterized. The allelic *eds5* (enhanced disease susceptibility) (Rogers and Ausubel, 1997) and *sid1* or *sid2* (salicylic acid induction deficient) (Nawrath and Metraux, 1999) mutants accumulate very little SA and *PR-1* transcript after infection with *P. syringae*. Interestingly, *eds5* and *sid2* do not have defects in the accumulation of the antimicrobial metabolite camalexin (Nawrath and Metraux, 1999). On the other hand, plants carrying the *pad4* (phytoalexin deficient) mutation show reduced

SA accumulation and *PR-1* expression accompanied by a low level of camalexin after infection with *P. syringae* (Zhou et al., 1998).
Analysis of double mutants with mutations in both *CPR* and *NPR1* genes confirms that CPR acts upstream of NPR1 and SA (Bowling et al., 1997; Clarke et al., 1998; Dong, 1998). However, CPR6 seems to activates an SA-dependent, but NPR1-independent mechanism for the activation of *PR* genes (Clarke et al., 1998), revealing still unknown aspects of the SAR signalling network.

JA/ethylene-dependent signalling
When a plant is attacked by a pathogen, production of JA and ethylene generally occurrs (Penninckx et al., 1996; 1998). In Arabidopsis, *Alternaria brassicicola* infection induces the production of these two chemicals concomitantly with the accumulation of a particular set of defense-related genes. Among those are the plant defensin PDF1.2, a basic PR-3-type chitinase and an acidic hevein-like protein (PR-4) (Penninckx et al., 1996; 1998). Thionin (Thi2.1) is induced in Arabidopsis in response to JA (Epple et al., 1995) and basic PR genes are inducible by ethylene in tobacco plants (Linthorst, 1991). It has been postulated that these genes might be involved in a separate signalling pathway to systemic induced resistance. Indeed, inoculation of Arabidopsis with the avirulent fungal pathogen *A. brassicicola* induces the expression of the above mentioned JA/ethylene-dependent PR genes, but not the SA-dependent *PR-1* gene (Penninckx et al., 1996; Thomma et al., 1998). Induction does not require SA or NPR1, but it does require JA and ethylene signalling. Resistance to isolates of *A. brassicicola* and *Botrytis cinerea* is compromised by *coi1* (coronatine insensitive), a mutant defective in the JA signalling (Feys et al., 1994), but is unaffected by NahG or *npr1* plants (Thomma et al., 1998). In contrast, the same study shows that resistance to *P. parasitica* is unaffected by *coi1*. Furthermore, *jar1* (jasmonate response) mutants (Staswick et al., 1992) which exhibit reduced sensitivity to MeJA, have been shown to be more susceptible to the soil fungus *Pythium irregulare*. Clearly, SA-dependent defense responses are not the only mechanism by which a plant can counteract pathogen infection and JA/ethylene-dependent signalling pathway is also involved in plant-pathogen interactions.

Rhizobacteria-mediated induced systemic resistance
Selected nonpathogenic, root-colonizing *Pseudomonas* bacteria have been shown to induce resistance in all parts of the plant. This type of induced resistance is called rhizobacteria-mediated induced systemic resistance (ISR) (Van Loon et al., 1998). This phenomenon has been observed in several plant species (Van Peer et al., 1991; Duijff et al., 1998). In Arabidopsis, *Pseudomonas fluorescens* strain WCS417r has been used to induce ISR against fungal and bacterial pathogens, demonstrating, as for SAR, a broad-spectrum of activity (Pieterse et al., 1996; Vanwees et al., 1997).

Interestingly, WCS417r-mediated ISR functions independently of SA and *PR* gene activation (Pieterse et al., 1996; Vanwees et al., 1997), but requires components of the JA and ethylene responses. Indeed, *jar1* and *etr1* (ethylene response) (Bleecker and Kende, 1988) mutants do not express ISR upon treatment with WCS417r (Pieterse et al., 1998; Knoester et al., 1999). Thus, SAR and ISR are regulated by distinct signalling pathways. However, SAR and ISR are both blocked in the regulatory mutant *npr1* (Cao et al., 1994; Pieterse et al., 1998). Therefore, NPR1 is not only acting on SA-dependent defense-responses, but also regulates JA/ethylene-dependent signalling of rhizobacteria-mediated ISR.

Cross-talk between defense signalling pathways
As mentioned above, plant defense responses are controlled by multiple signal transduction pathways where SA, JA and ethylene are involved. These regulators control and potentiate

the activity of distinct defense pathways helping the plant to prioritize a specific activity in response to single or multiple stresses (Reymond and Farmer, 1998; Genoud and Metraux, 1999; Glazebrook, 1999; Pieterse and vanLoon, 1999). For example, Penninckx *et al.* (1998) showed that treatment with ethylene or MeJA of *coi1* and *ein2* (ethylene insensitive) (Guzman and Ecker, 1990) mutants respectively, did not provoke the accumulation of PDF1.2 mRNA as it did in wild type Col-0. It indicates that JA and ethylene signalling has to be triggered concomitantly and not sequentially to activate PDF1.2 expression. Moreover, exogenous application of MeJA and ethylene had a synergistic effect on the expression level of PDF1.2 (Penninckx et al., 1998), demonstrating a concerted action of both JA and ethylene.

Negative interactions have also been reported, where SA-dependent and JA/ethylene-dependent signalling can be mutually inhibitory. Genes activated by JA/ethylene are hyper-inducible in the SAR defective plants NahG and *npr1* (Penninckx et al., 1996; Clarke et al., 1998). Similarly, two mutations that cause reduced SA level, *eds4* and *pad4*, displayed a higher PDF1.2 mRNA accumulation after treatments with either rose bengal or MeJA or rose bengal alone (Vaijayanti et al., 2000), supporting the idea that SA interferes with JA-dependent signalling. In other plants, it has been demonstrated that SA, INA and BTH suppress JA-dependent defense gene expression (Doherty and Bowles, 1988; PenaCortes et al., 1993; Niki et al., 1998) through a possible inhibition of JA synthesis and action (Doares et al., 1995). Conversely, JA and ethylene have also effects on the level of SA-induced gene expression. The ethylene Arabidopsis mutant *etr1* showed less mRNA accumulation of the SA-dependent *PR-1* gene after pathogen infection (Lawton et al., 1995). Furthermore, pretreatment of wild type Arabidopsis plants with ethylene resulted in an enhanced *PR-1* expression when induced by SA (Lawton et al., 1995). Taken together, ethylene response are likely to modulate SA action. All these examples of cross-talk provide evidence that a complex interconnecting network of activation and/or inhibition between defense pathways should help the plant to fine-tune defense-responses to different aggressors.

Potentiation of defense responses

Some defense responses are not directly activated during induced resistance but are induced more rapidly and efficiently after challenge inoculation, a phenomenon referred to as potentiation. For example, cucumber leaves exhibiting induced SAR showed a more rapid and up-regulated lignification of host cell walls in response to inoculation with *Colletotrichum lagenarium* (Hammerschmidt and Kuc, 1995). On the other hand, SAR-induced tobacco plants overexpressed PR-10 and PAL genes when infected with TMV (Mur et al., 1996). Tissue priming or conditioning and the resulting potentiation of local defense responses was also demonstrated in parsley cells treated with SA, INA or BTH. Indeed, these primed cells show enhanced elicitation of the oxydative burst (Kauss and Jeblick, 1995), the secretion of cell wall phenolics (Kauss et al., 1993) or phytoalexins (Kauss et al., 1992) and activation of some defense genes (Thulke and Conrath, 1998). In this later case, a dual mechanism was observed: some genes such as the PR genes are directly induced whereas some local defense genes are only up-regulated upon challenge with a pathogen. Recently, it has been proposed that a ubiquitin-proteasome system may play a role in the potentiation process (Becker et al., 2000).

Only a few studies of the potentiation of the JA/ethylene-dependent signalling pathway have been conducted in the last years. JA has been shown to potentiate elicitor-induced accumulation of active oxygen species in cultured parsley cells (Kauss and Jeblick, 1995). In addition, ethylene potentiates the SA-dependent *PR-1* gene expression in SAR-expressing Arabidopsis (Lawton et al., 1995). Hence, induced resistance is not only acting directly on

the expression of defense responses but also sensitizes the tissue to react faster and stronger to pathogen ingress.

Chemical SAR activators

To be considered as a chemical SAR activator, neither the chemical nor its metabolites must demonstrate direct antibiotic activity *in vitro* or *in planta*. Additionally, the compound has to be efficient against the same broad-spectrum of pathogens as biological SAR, with similar protection at phenotypic and genetic levels (Kessmann et al., 1994).

INA (2,6-dichloroisonicotinic acid) and BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester) have been shown to activate the same spectrum of resistance as SAR does with concomitant activation of SA-dependent *PR* genes (Uknes et al., 1992; Lawton et al., 1996). Consequently, they are considered as synthetic chemical activators of SAR (Sticher et al., 1997). Indeed, INA and its methyl ester are efficient agents against a wide spectrum of pathogens, ranging from viruses, bacteria to fungi (Uknes et al., 1992; Kogel et al., 1994; Nielsen et al., 1994). INA directly induces the expression of SAR genes and demonstrates a low *in vitro* antifungal activity (Ward et al., 1991). In tobacco and in Arabidopsis, INA is still fully active in NahG-expressing plants, demonstrating that this compound can replace or operates downstream of SA (Vernooij et al., 1994).

BTH shows also SAR-like activities in a number of plants such as wheat, rice, tobacco and Arabidopsis (reviewed in Sticher et al., 1997). Like INA, BTH has almost no direct antifungal activity and leads to the activation of the same SAR genes as SA (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). Since neither INA nor BTH protect *nim1/npr1* mutants, it has been postulated that both compounds act through a common signalling cascade downstream of SA perception (Delaney et al., 1995; Lawton et al., 1996).

ß-aminobutyric acid

Non-protein amino acids such as γ-aminobutyric acid (GABA) and BABA demonstrate biological activities in both animals and plants. In animal, GABA as glycine, are major inhibitory neurotransmitters (Waagepetersen et al., 1999), whereas BABA can be a partial agonist of the glycine receptor (Schmieden and Betz, 1995). In plants, GABA is produced in response to stress (Shelp et al., 1999) and local treatments with BABA have been shown to protect plants against various pathogens. First observations were reported some 40 years ago, when protection of pea was demonstrated against the oomycete *Aphanomyces euteiches* (Papavizas and Davey, 1963; Papavizas, 1964). It was described that soil drench application of BABA at a concentration of 100 ppm 3 days before inoculation was sufficient to reduce the root rot severity in unsterilized soil. Since then, systemic protection of tomato, potato and tobacco plants against the oomycetes *Phytophthora infestans* and *Peronospora tabacina* have been reported (Cohen, 1994; Cohen and Gisi, 1994). Moreover, recent reports show that BABA also protects plants against a nematode and a virus (Oka et al., 1999; Siegrist et al., 2000), demonstrating the broad range of activity of this chemical. The possible direct toxicity of BABA on many plant pathogens was repeatedly tested *in vitro* and *in vivo* by different research groups. A direct antimicrobial activity of this chemical was never observed (Cohen, 1994; Cohen et al., 1994; Li et al., 1996; Sunwoo et al., 1996; Cohen et al., 1999; Hong et al., 1999; Tosi et al., 1999). Experiments with ^{14}C-labelled BABA clearly shows that this chemical is not metabolized in tomato (Cohen and Gisi, 1994) or in Arabidopsis (Jakab et al., 2000), ruling out the involvement of a BABA-metabolite acting as an antimicrobial compound in the plant. Thus, BABA-mediated resistance is most likely based on the activation of host resistance mechanisms. Nevertheless, the mode of action of this chemical is still a matter of controversy : some studies report an induction of *PR* genes after BABA treatment (Cohen et al., 1994), while others state the contrary (Cohen, 1994). The objective

of the following thesis is to gain more insight into the mechanisms underlying BABA-induced resistance.

Outline of this thesis

As mentionned above, the protective effect of BABA has been studied extensively in many different plant-pathogen interactions. But the use of crop plants make the study of fundamental mechanisms involved by this chemical difficult. This might perhaps be the reason why the mode of action of BABA is still a matter of controversy. With this observations in mind, I decided to analyzed the effect of BABA on the model plant *Arabidopsis thaliana*. Arabidopsis is frequently used in fundamental research on plant-microbe interactions, since it is a well known genetic system. Most conveniently, a growing collection of well-characterized Arabidopsis mutants is available, allowing us an examination of particular genetic requirements for BABA-induced resistance. Moreover, Arabidopsis are very small plants with advantages such as a short generation time, a small nuclear genome and well described pathosystems.

In Chapter 2, I first analyzed the effect of BABA on the resistance of Arabidopsis against the oomycete pathogen *P. parasitica*. BABA activated the natural defense mechanisms of the plant such as callose deposition, the HR and the formation of trailing necroses, leading to protection against a virulent isolate of this pathogen. In this case, BABA-mediated resistance was found to function independently of the SA, JA and ethylene defense signalling pathways. Interestingly, BABA primed the accumulation of *PR-1* mRNA after infection with a virulent bacteria. As a result, only wild type Arabidopsis or mutants with a full expression of this defense gene were protected after BABA treatment. These differences in the mode of action of BABA depending on the type of pathogen revealed that BABA potentiates pathogen-specific plant resistance mechanisms.

The mode of action of BABA was further investigated in Chapter 3, where BABA-induced resistance was tested against a necrotrophic pathogen such as *Botrytis cinerea*. In this chapter, evidence is provided that BABA has no effect on the JA/ethylene-dependent signalling pathway, but potentiates the SA-dependent defense responses. Thus, it suggests that Arabidopsis plants activate not only JA/ethylene-dependent, but also SA-dependent defense responses to restrict infection by necrotrophs. Nevertheless, this activation is probably too slow to be efficient.

The effect of BABA was also investigated on the resistance to abiotic stress such as cold treatment. The majority of BABA-treated Arabidopsis plants was able to recover after a two days cold shock treatment, whereas almost all the non-treated control died. No potentiation of the cold-regulated genes was observed. But interestingly, during heat shock treatment, the mRNA of the heat shock protein 83 gene accumulated faster in BABA-treated Arabidopsis, indicating that BABA can also potentiate genes induced during abiotic stress. These results suggest that BABA modulates a connecting point of both biotic and abiotic stresses.

Finally, a general synthesis of the results obtained during this work is presented in Chapter 5 with reference to current ideas about the signalling network regulating plant disease resistance and their relationship with abiotic stress.

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CHAPTER 2

Potentiation of pathogen-specific defense mechanisms in Arabidopsis by ß-aminobutyric acid

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Abstract

The non-protein amino acids γ-aminobutyric acid (GABA) and β-aminobutyric acid (BABA) have known biological effects in animals and plants. Their mode of action has been the object of thorough research in animals, but remains unclear in plants. Our objective was to study the mode of action of BABA in the protection of *Arabidopis* plants against virulent pathogens. BABA protected *Arabidopsis* against the oomycete pathogen *Peronospora parasitica* through activation of natural defense mechanisms of the plant such as callose deposition, the hypersensitive response (HR), and the formation of trailing necroses. BABA was still fully protective against *P. parasitica* in transgenic plants or mutants impaired in the salicylic acid (SA), jasmonic acid (JA), and ethylene signaling pathways. Treatment with BABA did not induce the accumulation of mRNA of the systemic acquired resistance (SAR)-associated *PR-1* and the ethylene- and jasmonic acid-dependent *PDF1.2* genes. However, BABA potentiated the accumulation of *PR-1* mRNA after attack by virulent pathogenic bacteria. As a result, BABA-treated *Arabidopsis* plants were less diseased compared to the untreated control. In the case of bacteria, BABA protected mutants insensitive to JA and ethylene, but was not active in plants impaired in the SAR transduction pathway. Thus, BABA protects *Arabidopsis* against different virulent pathogens by potentiating pathogen-specific plant resistance mechanisms. In addition, we provide evidence that BABA-mediated papilla formation after *P. parasitica* infection is independent of the SAR signaling pathway.

Introduction

Plants have evolved numerous, complex defense mechanisms to survive attacks by fungal and microbial pathogens. Plant resistance responses are genetically determined (1) and , in the case of gene-for-gene resistance, they are manifested through the development of a HR (2). The cloning of resistance genes from several plant species has given exciting clues to a better understanding of race-specific resistance (3). In addition to the gene-for-gene resistance, plants have developed inducible defense mechanisms. In this publication we show that the non-protein amino acid BABA can induce disease resistance in *Arabidopsis* independently of known resistance markers. Typically, after attack by a necrotizing pathogen, the plant reacts by developing a long-lasting defense response (SAR) against a broad spectrum of pathogens. SAR is characterized by an early increase in newly synthesized SA (4) followed by the activation of genes encoding *PR* proteins (5). Application of SA and functional analogs of SA, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), correlates with the induction of both *PR* gene expression and resistance (5, 6). One possible mode of action of SA in pathogen defense is to condition defense reactions leading to a faster response of the plant after pathogen attack (7). *Arabidopsis* mutants impaired in SAR have helped to understand the signal transduction pathway leading to resistance. *Npr1* mutants do not accumulate *PR-1* mRNA in response to SA or its functional analogs and are highly susceptible to infection by virulent pathogens (8). *Arabidopsis* overexpressing a salicylate hydroxylase gene (*NahG*) have low levels of SA and are unable to undergo SAR (9). Besides SA, the plant hormones JA and ethylene have been shown to be involved in a separate signal transduction pathway providing resistance against distinct pathogens (10, 11, 12, 13). The JA- and ethylene-dependent signaling events could be analyzed with mutants such as *jar1* and *etr1*, which exhibit reduced sensitivity to MeJA and altered perception of ethylene, respectively (14, 15), and have been shown to be more susceptible to certain soil-borne pathogens (16, 17).

Non-protein amino acids such as GABA and BABA have known biological effects in animals and plants. In animals, GABA and glycine are major inhibitory neurotransmitters (18), whereas BABA is a partial agonist of the glycine receptor (19). In plants, GABA is produced as a response to stress (20) and treatments with BABA were shown to provide protection against various pathogens (21, 22, 23). However, little is known concerning the mode of action of BABA: some studies report an induction of *PR* after BABA treatment (24), whereas others state the contrary (25). Thus the mode of action of BABA remains a matter of controversy. In the present article, we analyze the effect and the mode of action of BABA in *Arabidopsis*. We show that BABA mediates the conditioning of induced plant defense mechanisms leading to a phenocopy of genetic resistance after infection with a normally virulent pathogen.

Material And Methods

Biological Material

The transgenic *Arabidopsis thaliana* line harbouring the *NahG* gene (9) was obtained from J. Ryals (Novartis, Research Triangle Park, NC). The Columbia (Col-0) ecotype mutants *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. *Arabidopsis* accessions Columbia (Col-0) and Wassilewskija (WS) were obtained from Lehle Seeds (Round Rock, TX). Plants were grown on a steam sterilized soil mix of commercial potting soil/perlite (3:1) at 22°C day/18°C night temperature with 12 h light per 24 h. Conservation procedures of *Peronospora parasitica)* have been described previously (26). Strain DC 3000 of *Pseudomonas syringae* pv *tomato* (*Pst* DC 3000) and the isogenic strain carrying the avirulence gene *avrRpt2* (27) were cultivated at 28°C/220 rpm in King's medium B (28) containing rifampicin for selection.

Plant Inoculation and Treatment

P. parasitica was inoculated by spraying until shortly before droplet run-off occurred with a suspension of 10^5 conidia per mL of water. Plants inoculated with *P. parasitica* were kept at 20°C in a 12/12 h light/dark cycle. Hundred percent relative humidity was necessary during the first and the last day of the growth cycle to insure infection and sporulation.

For bacterial inoculation, cells were collected by centrifugation, resuspended in 10 mM $MgCl_2$ at A_{600} = 0.2, corresponding to a concentration of 10^8 cfu mL^{-1}. Bacteria were then diluted to 10^5 cfu mL^{-1} in 10 mM $MgCl_2$. Titers were determined as follows. Three leaves per plant were infiltrated using a 1 mL syringe without a needle. Each time-point represents 24 leaf discs (0.5 cm diameter) from 8 different plants. One disc from each plant was pooled, resulting in 3 groups containing 8 leaf discs each. Leaf discs were washed twice with sterile water and homogenized in 10 mM $MgCl_2$. Quantification was done by plating appropriate dilution's on King's B agar containing rifampicin (50 mg L^{-1}). Tissue samples were harvested from inoculated leaves at 0, 1, 2 and 3 days after infiltration.

For *P. parasitica* infections, pots containing about 30 2 to 3-week-old *Arabidopsis* plants were soil drenched with indicated chemicals dissolved in water. Five-week-old *Arabidopsis* plants treated with 16 mg L^{-1} BABA were used for bacterial infiltrations. Treatments were performed one day before inoculation with the pathogen, when not otherwise indicated. Only soil drench treatments were used to avoid formation of necroses observed after spraying, because such necroses induce the SAR pathway and mask the primary effect of BABA.

***In Vitro* Assay**

In vitro assays for antimicrobial BABA activity against pathogenic fungi were evaluated as radial growth of mycelia discs placed onto the middle of agar plates. Growth was determined after several days on PDA (Difco) medium containing BABA at a final concentration of 1000 mg L^{-1}. For bacterial assays, *Pst* DC 3000 and *Pseudomonas syringae* pv *maculicola* were cultivated in the minimal medium M9 (29). At A_{600} = 0.4, the culture was divided into 2 volumes, one containing a final concentration of 1000 mg L^{-1} BABA and the other not. Bacterial growth was determined every hour up to the stationary phase. Each experiment was performed with 6 replicates.

***In Vivo* Assay**

To analyze the germination rate of the obligate biotroph *P. parasitica*, leaves from untreated control and 16 mg L^{-1} BABA-soil drenched Col-0 plants were harvested 60 h after inoculation with *P. parasitica* isolate NOCO. Plant tissue was destained overnight in ethanol 95% and stained with aniline blue (30). The germination rate was evaluated by determining the number of germinated conidia on 10 leaves per treatment. Experiments were repeated twice with

similar results. Observations were performed with a fluorescence microscope with UV filter (BP 340-380 nm, LP 425 nm).

BABA Metabolism Analysis

Arabidopsis seeds were sterilized and subsequently grown for 6 weeks on half strength MS (1/2 MS) medium (31). Plantlets were then transferred to sterile containers with 35 mL liquid 1/2 MS medium and 1μCi ^{14}C-BABA (1.03 Ci mol^{-1}; Novartis, Basel, Switzerland) or 1μCi ^{14}C-GABA (15.5 Ci mol^{-1}, Novartis, Basel, Switzerland), respectively. Plastic support racks were used to avoid direct contact of the leaves with the radioactive solution. After 2 days of incubation, protoplasts were prepared (32) to determine the presence of radioactivity inside the cells. After harvesting, the protoplasts were subjected to a viability test with fluorescein diacetate (FDA) (33) and counted to make sure that at least 80 % of the protoplasts were viable. A further purification step, consisting of centrifuging (30 s, 15,800 *g*, in a microfuge) the protoplasts through a hydrophobic layer (381 μL di-butyl phthalate, 119 μL phthalic acid bis(2-ethyl-hexylester) was introduced. Protoplasts were then ground directly in the microtubes, the debris spun down, and the supernatant (cell contents) applied to TLC plates. The pellet was washed four times with MCW (MeOH:$CHCl_3$:water 12:5:3, v/v) to yield the membrane fraction. The cell wall fraction was obtained by spinning down the remains after protoplasts had been released and washing them four times in MCW. TLC plates were run in a solution of n-butanol:acetic acid:water (60:20:20, v/v). Amino acids were visualized by spraying with 0.2% ninhydrin in ethanol before heating for 5 min at 140°C. The radioactive amino acids were detected by autoradiography using a Kodac Xomat film.

RNA Gel Blot Analysis

RNA was extracted from frozen pulverized plant material in a buffer containig 2 M Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, and 20% SDS in a ratio of 1:2:1 (v/v) (34) with an equal volume of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1, v/v). After centrifugation to separate the phases, the RNA from the aqueous phase was precipitated with 1 volume of 6 M LiCl overnight at 4°C. The pellet was then washed with 70% ethanol and resuspended in H_2O. Five μg of total RNA were separated on a formaldehyde-agarose gel and transferred to a Nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was probed with ^{32}P-labeled cDNA (RadPrime DNA Labeling System, Life technologies, Merelbeke, B) encoding pathogenesis-related proteins *PR-1* (35) and *PDF1.2* (11).

RESULTS

Protection against *P. parasitica*

To test whether the observed protective effect of BABA on crop plants (36) can be extended to *Arabidopsis*, the plants were treated with BABA or its isomers, α-aminobutyric acid (AABA) and GABA, one day prior to inoculation with the oomycete *Peronospora parasitica*, isolate NOCO. This isolate is virulent on *Arabidopsis thaliana* accession Col-0 with conidiophores emerging from the leaf seven days after inoculation (37). *Arabidopsis* displayed a remarkable selectivity towards aminobutyric acid isomers; only BABA protected against *P. parasitica* (Table 1). The protection became first apparent at a concentration as low as 8 mg L^{-1} applied in the soil one day prior to inoculation (Table 1). Similar levels of

protection were obtained with the virulent isolate EMWA on *Arabidopsis* accession WS (data not shown).

Table 1. Sporulation of *P. parasitica* isolate NOCO on *Arabidopsis thaliana* (Col-0) treated with isomers of aminobutyric acid

		Sporulation intensity*		
Treatment [#]	Concentration (mg L^{-1})	6 dbi [†]	1 dbi [†]	6 dpi [‡]
BABA	0	++++	++++	++++
BABA	8	+	-	+++
BABA	16	-	-	+++
BABA	32	nd	-	nd
AABA	32	nd	++++	nd
GABA	32	nd	+++	nd

*Sporulation was scored 7 days after inoculation. -, no sporulation, + to ++++, increasing degrees of sporulation. Experiments were repeated four times with similar results.
[#]Plants were soil drenched with chemicals before or after inoculation as indicated.
[†]days before inoculation
[‡]days post inoculation
nd, not determined

BABA does not Act as an Antimicrobial Compound

The protection due to BABA could be the result of a direct antibiotic activity. *In vitro* tests on various fungi and bacteria showed that this is not the case, even at 1000 mg L^{-1}, a concentration more than 50 times higher than the one used for treating plants (data not shown). The antifungal activity of BABA against the obligate biotroph *P. parasitica* was also analyzed in an *in vivo* assay by determining the germination rate of conidia on leaf surfaces of treated *Arabidopsis* plants. No difference was observed on BABA-treated plants compared to controls (data not shown). In addition, *P. parasitica* spores were directly incubated in 12 mg L^{-1} BABA and sprayed in this solution on susceptible *Arabidopsis* plants. Seven days later, sporulation on these plants was similar to control plants sprayed with spores suspended in water only (data not shown). In addition, BABA protected *Arabidopsis* only when applied before inoculation (Table 1), demonstrating that this chemical has no curative effect once *P. parasitica* is established in the leaf. Moreover, a study of the metabolism of BABA with labeled molecules showed that BABA is not metabolized whereas GABA rapidly breaks down. Most of the BABA label was found in the soluble fraction (cell content) and only very little was detected in the cell wall fraction on autoradiograms (data not shown). Therefore, it is very unlikely that protection due to BABA is based on a direct antibiotic activity of BABA or its metabolites. Such protection results very likely from an activation of disease resistance mechanisms in the host plant.

Host Defense Reactions after Infection of BABA-treated *Arabidopsis*

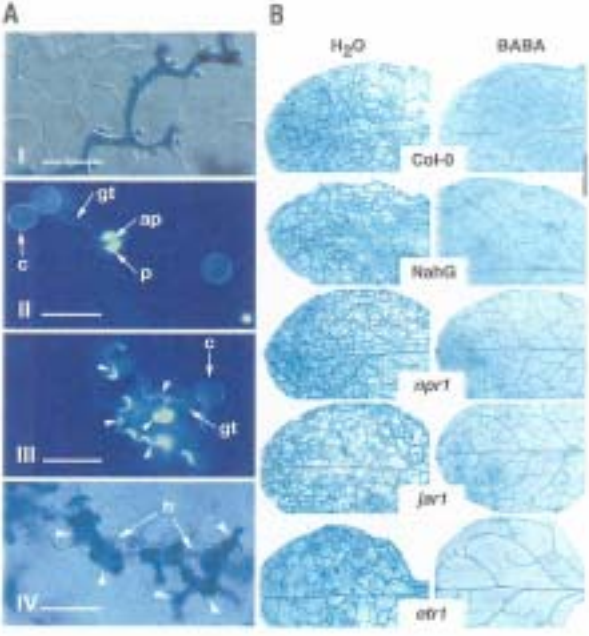
To understand the nature of resistance induced by BABA, cytological observations were performed at infection sites of a virulent *P. parasitica* isolate in *Arabidopsis* leaves (Fig. 1A,

I). At concentrations of 12 mg L^{-1} or higher, hyphal penetration in the host was completely suppressed. Callose deposits, termed papillae (38), were observed at the site of attempted penetration (Fig 1A, II). At lower concentrations, cells underwent a phenocopy-HR at the site of attack (Fig 1A, III) and, in some cases, hyphae were able to grow between cells into the leaf tissue. In this situation, the plant often reacted by developing trailing necroses along the growing hyphae (Fig 1A, IV). These observations all indicate that BABA stimulates the natural defense of the plant by converting phenotypically a compatible into an incompatible host-pathogen interaction.

Analysis of the Mode of Action of BABA

The mode of action of BABA against *P. parasitica* on *Arabidopsis* was investigated using transgenic plants or mutants impaired in the signal transduction pathways activated by pathogen infection. We first analyzed whether BABA acts through the SAR transduction pathway. NahG-expressing *Arabidopsis* plants and *npr1* mutants were treated with 12 mg L^{-1} BABA one day prior to inoculation with a virulent isolate of *P. parasitica.* These plants, like wild-type Col-0, were fully resistant, showing no fungal colonization in the leaf up to 7 days after inoculation. By comparison, the water controls showed an extensive ramification of hyphae in the leaf (Fig. 1B), as well as development of conidiophores and oospores (data not shown). The contribution of JA and ethylene to BABA-induced resistance was analyzed with *jar1* and *etr1* mutants, respectively. Like the lines deficient in the SAR signaling pathway, these two mutants were completely resistant to *P. parasitica* infection (Fig 1B). Thus, despite phenotypical similarities with SAR, BABA-induced resistance against *P. parasitica* in *Arabidopsis* is neither dependent on SA accumulation, nor on accumulation of *PR* genes and in addition, it is independent of JA and ethylene perception. Consistent with these observations and in contrast to treatments with the SAR activator BTH (39), no *PR-1* transcript accumulation after BABA treatment was observed (Fig 2A). Furthermore, mRNAs for the plant antifungal proteins defensin and thionin, respectively responsive to both JA and ethylene or JA alone (11, 40), were not induced after BABA treatment (data not shown).

Fig. 1. BABA-induced resistance in *Arabidopsis* against *P. parasitica*
(A) Microscopic aspects of the protective effect of BABA.
(I) Growing hyphae in untreated control plant. Bar = 50 μm.
(II) Callose (yellow) deposition (papilla=p) below the appressoria (ap) at the end of the germ tube (gt) on leaf treated with 12 mg L^{-1} BABA. (c) conidium. Bar = 20 μm.
(III) Phenocopy-HR reaction in plants treated with 8 mg L^{-1} BABA. Callose deposition (arrowheads) around the appressoria and cells undergoing necrosis appear in yellow. (c) conidium; (gt) germ tube. Bar = 20 μm.
(IV) Trailing necrosis (arrowheads) along a growing hypha (h) in a plant treated with 4 mg L^{-1} BABA. Bar = 50 μm. Plants were treated with BABA one day before inoculation and stained 3 days later with aniline blue for callose observation (Wasserblau Standard Fluka) (30) and Calcofluor White M2 R. S. New (Cyanamid) (44) (II and III) or with lactophenol-trypan blue (I and IV) (45) for fungal structure coloration. Picture IV was taken 6 days after inoculation.
(B) Effect of BABA in *Arabidopsis* lines altered in their response to *P. parasitica.* Wild-type (Col-0) control, NahG, *npr1, jar1*, and *etr1* plants were treated with water or 12 mg L^{-1} BABA and inoculated with the virulent *P. parasitica* isolate NOCO. Pictures show leaves stained with lactophenol-trypan blue (45) 7 days after inoculation. Fungal structures and damaged cells are stained in blue. Genotypes and treatments are indicated in the middle and top of the figure, respectively. A representative example for each genotype is shown.


A
B
H2O
BABA
I
gt
ap
c
p
II
c
gt
III
IV
Col-0
NahG
npr1
jar1
etr1

Conditioning Effect

The BABA-induced conversion of a compatible to a phenocopy of an incompatible interaction observed after *P. parasitica* infection, was further explored by analyzing the expression of *PR* genes after pathogen infection. To induce a strong localized reaction of the plant tissue, we used the virulent bacterium *Pst* DC 3000 to inoculate plants, and the time-course of the expression of *PR-1* mRNA was monitored. BABA treatment conditioned the plant to produce *PR-1* mRNA more rapidly. Typically, *PR-1* mRNA expression in plants inoculated with *Pst* DC 3000 was induced 12 h earlier in BABA-treated plants compared to the untreated control. Indeed, the plant reacted as fast as after an infection with avirulent bacteria (*Pst* DC 3000 *avrRpt2*) (Fig. 2B). Thus, as observed in the interaction with *P. parasitica*, BABA treatment mimicked some aspects of genetic resistance through conditioning of plant defense responses.

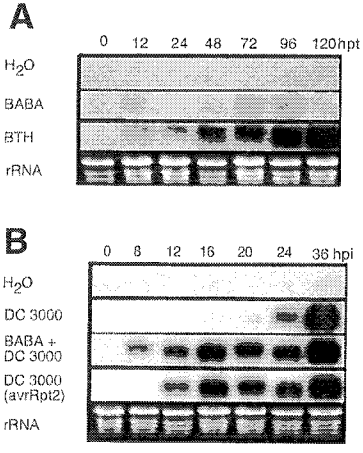


Fig. 2. Time-course of the expression of *PR-1* mRNA in *Arabidopsis* .
(A) Effect of chemical treatments. Total RNA was extracted at various times after soil drench with water, 16 mg L^{-1} BABA or 300 µM BTH. (hpt) hours post treatment.
(B) Conditioning effect of BABA. Plants were soil drenched with water or 16 mg L^{-1} BABA one day before infiltration with bacteria (time zero). Each time point represents 9 infected leaves harvested from 3 different plants. Total RNA was prepared and analyzed by RNA gel blot analysis. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. (hpi) hours post inoculation.

Protection against Bacteria

To verify whether this conditioning is effective to protect *Arabidopsis* against pathogenic bacteria, we infected Col-0 plants with the virulent bacterium *Pst* DC 3000. As shown in Figure 3A, BABA treatment resulted in a ten-fold reduction of the bacterial titer and strongly decreased symptoms. *Arabidopsis* plants pretreated with BABA did not exhibit the typical chlorotic leaf spotting associated with *Pst* DC 3000 infection (Fig 3B). Thus, the conditioning effect observed after BABA treatment could be linked to resistance. To further analyze the mode of action of BABA on *Arabidopsis* against *Pst* DC 3000, NahG-expressing *Arabidopsis* plants and *npr1* mutants were treated with 16 mg L^{-1} BABA one day prior to inoculation with *Pst* DC 3000. These plants were not protected, but mutants deficient in the JA or ethylene signaling pathways were protected at a similar level as the wild-type Col-0 (Fig. 3A and B). Consequently, BABA protects *Arabidopsis* not only against an oomycete, but also against a pathogenic bacterium. Moreover, in the case of bacterial infection, the plant protection required *PR* genes activation or a functional SA signal transduction pathway.

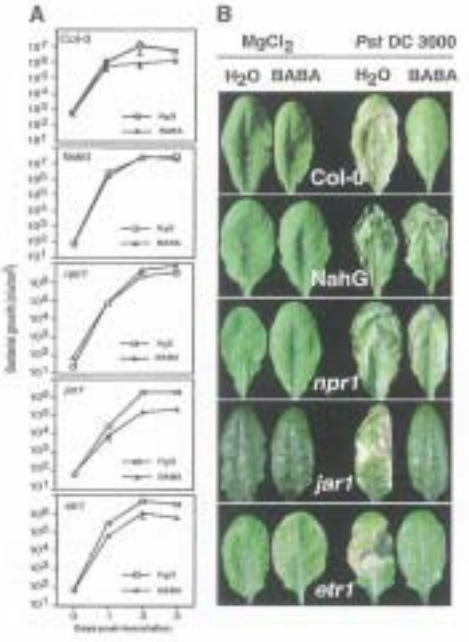


Fig. 3. Protection effect of BABA in *Arabidopsis* infected with *Pst* DC 3000.
(A) Bacterial growth. Wild-type Col-0, NahG, *npr1*, *jar1*, and *etr1* leaves were analyzed for bacterial density at different time points after infiltration. Data represent the mean ± SE of 3 pools stemming from 8 replicate samples. Experiments were repeated four times with similar results.
(B) Symptoms. Pictures show disease symptoms 3 days after infiltration in wild-type Col-0, NahG, *npr1*, *jar1*, and *etr1* plants. Treatments are indicated at the top of the figure. Representative examples are shown.

Discussion

Non-protein amino acids are secondary plant metabolites exhibiting diverse properties. GABA accumulation is observed in stressed plants (20), and BABA treatment protects different plant species against various pathogens (21, 22, 23). Since the mode of action of BABA is still largely unknown in plants (41), we have analyzed the protective effect of this chemical in *Arabidopsis*. Our data indicate that, among the isomers tested, only BABA protects *Arabidopsis* against a virulent isolate of the oomycete pathogen *P. parasitica*. It therefore shows, as in other plant species (21, 22, 23), a high degree of specificity among aminobutyric acid isomers. Soil drench treatment at a concentration as low as 8 mg L^{-1} given one day prior to inoculation was sufficient to protect the plants. These results are consistent with protection observed in other plants (21, 22, 25). We have also shown that BABA is protecting *Arabidopsis* against pathogenic bacteria at a level similar to protection due to chemically induced resistance (35, 39). Hence, BABA protects different plant species against different fungi (21), a bacterium, a nematode (22) and a virus (23), demonstrating the broad range of activity of this chemical. Importantly, *in vitro* and *in vivo* experiments with diverse fungi and bacteria or *P. parasitica* show that BABA has no direct toxic effect. Furthermore, BABA is not metabolized in *Arabidopsis* ruling out the involvement of a BABA metabolite acting as an antimicrobial compound in the plant. BABA does not show any curative effect, since it protects *Arabidopsis* against *P. parasitica* only when applied before inoculation. Taken together, BABA-mediated resistance is most likely based on the activation of host resistance mechanisms.

Microscopical analysis of the interaction between *P. parasitica* and BABA-treated *Arabidopsis* suggests that active defense mechanisms are involved in BABA-mediated resistance. *Arabidopsis* plants treated with BABA show typical responses observed in the course of induced resistance (35). Treatment with a high concentration of BABA leads to callose deposition termed papillae at almost all attempted penetration sites. At a lower concentration, a spectrum of responses from phenocopy HR to trailing necroses was observed, demonstrating a modulation of the plant response depending on the endogenous concentration of BABA. Importantly, BABA induced callose deposition only after attempted penetration of the epidermis by *P. parasitica*; spontaneous deposition without prior inoculation was never observed. Thus, BABA may induce resistance by accelerating the normal responses of the plant to infection, leading to a higher level of resistance. The same phenomenon was observed after infection with a virulent bacterium. In this case, soil drench treatments with BABA did not induce *PR-1* mRNA accumulation, but conditioned the plant to induce this defense gene more rapidly after infection. Indeed, the plants reacted as fast as after infection with avirulent bacteria. Therefore, BABA enhances defense mechanisms triggered upon sensing of the pathogen by the plant, since no changes are detected in BABA-treated plants before infection. This phenomenon is known as potentiation or conditioning (41, 42).

The observation that BABA induces resistance against *P. parasitica* in transgenic NahG plants suggest that BABA could activate the SAR pathway downstream of SA accumulation. However, the protection of *npr1* mutants and the fact that BABA does not induce accumulation of *PR-1* mRNA make this hypothesis unlikely. The plant hormones JA and ethylene have been implicated in a separate defense transduction pathway (10). Since BABA-induced resistance against *P. parasitica* is not dependent on sensitivity to JA and ethylene,

this rules out the involvement of the resistance mechanisms mediated by these signaling molecules. Furthermore, the phytoalexin camalexin is also not a key factor of the BABA-induced resistance against *P. parasitica*, since camalexin deficient mutants were fully protected (data not shown). Papillae are formed extensively after *P. parasitica* infection in BABA-treated *Arabidopsis*, a phenomenon rarely observed in non-treated controls. Therefore, this structural barrier could be sufficient to completely block *P. parasitica* penetration. After BABA treatment, even in the absence of *PR* protein accumulation, production of massive papillae is detected. Thus the observed BABA-mediated conditioning leading to an earlier and stronger papilla formation may explain resistance against a normally virulent *P. parasitica* even in mutants impaired in the SAR transduction pathway. It demonstrates the value of papillae as an early defensive barrier sufficient to block *P. parasitica* penetration, making downstream defense mechanisms, such as *PR* proteins or camalexin, no longer necessary. This also suggests that BABA acts at a very early step in plant-pathogen interactions, probably at the recognition level. Interestingly, protection against bacteria is dependent of the SAR transduction pathway, since BABA potentiates PR-1 mRNA accumulation and both transgenic NahG plants and *npr1* mutants were not protected. These results are in agreement with data obtained in tobacco where BABA protects against the tobacco mosaic virus through a SA-dependent signal transduction pathway (23). Hence, different mechanisms of protection are effective against distinct pathogens and BABA can stimulate the plant to deploy such pathogen-specific reactions much faster.

All these observations highlight a new aspect of the biological action of the non-protein amino acid BABA. Clearly, BABA enhances resistance through potentiation of pathogen–specific plant defense responses leading to a restriction of pathogen growth and spread. Furthermore, the observed early papilla formation obviously acts independently of known signaling cascades. These experiments add to our understanding of the importance of induced defense responses in plants. The site of action of BABA represents an attractive target for the development of novel crop protectants which capitalize on the natural potential of plants to ward off pathogens.

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CHAPTER 3

β-aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*

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Submitted

ABSTRACT

The non-protein amino acid β-aminobutyric acid (BABA) protects numerous plants against various pathogens. Protection of Arabidopsis plants against virulent pathogens involves the potentiation of pathogen-specific defense-responses. To extend the analysis of the mode of action of BABA to necrotrophs, we evaluated the effect of this chemical on Arabidopsis plants infected with the gray mold fungus *Botrytis cinerea*. BABA-treated Arabidopsis were found to be less sensitive to two different strains of this pathogen. BABA protected mutants defective in the jasmonate and ethylene pathways, but was inactive in plants impaired in the systemic acquired resistance transduction pathway. Treatments with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, a functional analogue of salicylic acid (SA), also markedly reduced the level of infection. Moreover, BABA potentiated mRNA accumulation of the SA-associated *PR-1*, but not the jasmonate/ethylene-dependent *PDF1.2* gene. Thus, besides jasmonate/ethylene-dependent defense responses, SA-dependent signaling also contributes to restrict *B. cinerea* infection in Arabidopsis. Our results also suggest that SA-dependent signaling is downregulated after infection by *B. cinerea*. Finally, the observed upregulation of the *PDF1.2* gene in mutants defective in the SA-dependent signaling pathway points to a cross-talk between SA- and jasmonate/ethylene-dependent signaling pathways during pathogen ingress.

INTRODUCTION

Plants have developed a battery of complex defense mechanisms to escape infection by pathogens. Besides constitutive barriers, a number of mechanisms are induced upon recognition of the pathogen by the host. Depending on the pathogen, specific signal transduction pathways are induced leading to the expression of sets of defense responses which include rapid programmed cell death (hypersensitive response, HR), strengthening of the cell wall or expression of antimicrobial genes (Hammond-Kosack and Jones, 1996). In many cases, resistance is expressed locally and systemically in response to either necrotizing pathogens or root-colonizing soil bacteria. Systemic acquired resistance (SAR) induced by pathogens is in many cases dependent on the endogenously synthesized signal salicylic acid (SA) (Sticher et al., 1997). Other pathogens can induce defense responses characterized by jasmonic acid- (JA) and ethylene-dependent signal transduction pathways (Thomma et al., 1998; 1999). Furthermore, the spectrum of resistance is different depending on the signal transduction pathway. In SA-controlled SAR, plants deploy barriers that are effective against pathogens such as *Peronospora parasitica* (Thomma et al., 1998) or *Pseudomonas syringae* (Summermatter et al., 1995), while JA or ethylene-controlled resistance leads to protection against *Alternaria brassicicola* or *Botrytis cinerea* (Thomma et al., 1998; 1999). These results made it clear that induced defense responses are mediated by multiple signal transduction pathways. In addition, these signaling pathways are not simple linear and isolated cascades but can crosstalk with each others (Reymond and Farmer, 1998; Genoud and Metraux, 1999).

Defense responses induced by a primary infection can be expressed before the contact with a secondary challenging organism. A primary infection can also lead to a faster activation of defense responses after challenge inoculation, a phenomenon known as potentiation. Tissue priming or conditioning and the resulting potentiation of local defense responses was demonstrated in parsley cells treated with SA, 2,6-dichloroisonicotinic acid (INA) or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). Indeed, these primed cells show enhanced elicitation of the oxidative burst (Kauss and Jeblick, 1995), the secretion of cell wall phenolics (Kauss et al., 1993), phytoalexin production (Kauss et al., 1992) and activation of defense genes (Mur et al., 1996; Thulke and Conrath, 1998). In the latter case, a dual mechanism was observed: some genes such as the pathogenesis-related (PR) genes are directly induced whereas some local defense genes are only potentiated. Recently, it has been proposed that an ubiquitin-proteasome system may play a role in potentiation processes (Becker et al., 2000).

Synthetic and natural compounds called inducers can effectively trigger induced resistance responses (Kessmann et al., 1994; Sticher et al., 1997). Some of the best characterized examples are INA and BTH. These compounds induce the same spectrum of resistance as pathogen-induced SAR with concomitant activation of SA-dependent PR genes (Uknes et al., 1992; Lawton et al., 1996). The non-protein amino acid β-aminobutyric acid (BABA) has been shown to protect Arabidopsis against *P. parasitica* through activation of defense mechanisms such as callose deposition, HR and the formation of trailing necroses (Jakab et al., 2000; Zimmerli et al., 2000). BABA is fully active against *P. parasitica* in transgenic plants or mutants impaired in the SA, JA, and ethylene signaling pathways. While BABA did not induce the accumulation of mRNA of the SA-associated *PR-1* or the JA- and ethylene-dependent *PDF1.2* genes, it potentiated the accumulation of *PR-1* mRNA after attack by virulent pathogenic bacteria. In the case of bacterial pathogens, BABA protected mutants insensitive to JA and ethylene, but was inactive in plants impaired in the SAR transduction pathway. Thus, BABA protects *Arabidopsis* against different virulent pathogens by potentiating pathogen-specific plant resistance mechanisms.

Here, we have evaluated the effect of BABA in Arabidopsis infected by the necrotrophic fungus *B. cinerea*. We have found that BABA-treated Arabidopsis are protected against infection by *B. cinerea*. BABA also potentiates the accumulation of *PR-1*, but not *PDF1.2* mRNA after infection. Our results indicate that the SAR signaling pathway contributes to restrict *B. cinerea* infection in Arabidopsis. Furthermore, we have shown that components of the SA-dependent signaling pathway inhibit the expression of JA/ethylene dependent defense-responses after *B. cinerea* infection.

RESULTS

BABA Enhances Arabidopsis Resistance to *B. cinerea* Infection

BABA protects Arabidopsis plants against *P. parasitica* and *P. syringae* pv *tomato* DC 3000 (Zimmerli et al., 2000). These two pathogens activate the SA-dependent signal transduction pathway in Arabidopsis. In this paper our analysis was extended to pathogens inducing defense responses via the JA/ethylene signal transduction pathway. Soil drench treatment with 30 $\mu g\ mL^{-1}$ BABA one day prior to the deposition of 3 μL droplets containing 75 conidia of *B. cinerea* each led to a significant reduction of the surface of the necroses as observed 3 days after inoculation. Furthermore, BABA was effective against both strains of the gray-mold fungus tested here (Fig. 1A). Fungal hyphae grew concentrically from the site of inoculation resulting in a visible necrosis 3 days after inoculation. Necroses were smaller in BABA-treated Arabidopsis plants compare to the untreated controls (Fig 1B). *B. cinerea* hyphae developed similarly in water- and BABA-treated Arabidopsis as shown by microscopical observations, but the surface invaded by *B. cinerea* hyphae was less important in BABA-treated Arabidopsis plants compared to untreated controls (Fig 1C). Consequently, the macroscopic symptoms reflect the level of infection in both treated and untreated plants.

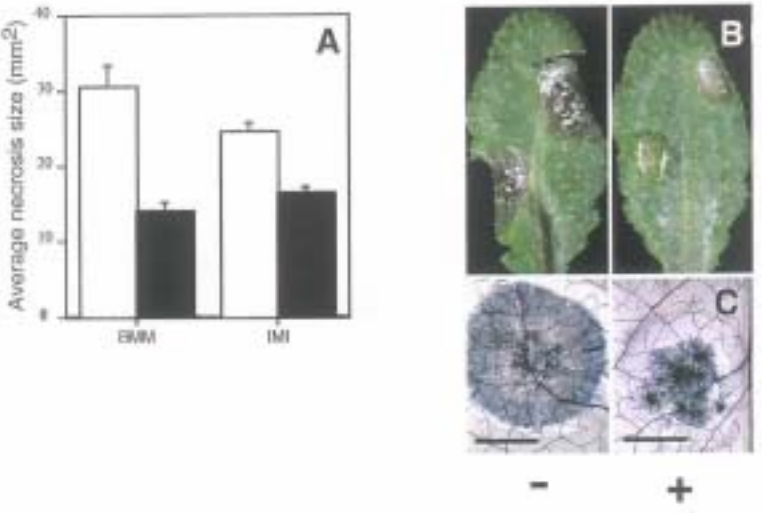

Figure 1. Protection of Arabidopsis by BABA against infection with *B. cinerea*.
A, Average size of necroses formed 3 days after inoculation on 5- to 6-week-old Arabidopsis Col-0 plants drop-inoculated with *B. cinerea*. Plants were soil-drenched with water (open bars) or 30 mg mL-1 BABA (closed bars) one day prior to inoculation with strains BMM (BMM1) and IMI (IMI169558). The experiment was repeated at least 3 times with similar results. B, Symptoms observed 3 days after inoculation on leaves of 5-week-old Arabidopsis Col-0 drop-inoculated with *B. cinerea* strain BMM1. Plants were soil-drenched with water (-) or 30 mg mL-1 BABA (+) one day prior to inoculation. C, Microscopical aspect of *B. cinerea* strain BMM1 infection. Micrographs show leaves stained with lactophenol-trypan blue (Keogh et al., 1980). Inoculation and treatment were performed as in (B). Bar = 20 mm.

BABA Protects Arabidopsis Mutants Impaired in the JA/ethylene, but not Mutants or Transgenic Arabidopsis Defective in the SA-Dependent Signal Transduction Pathway

The mode of action of BABA was analyzed using transgenic Arabidopsis or mutants impaired in the signal transduction pathway to infection. NahG-expressing Arabidopsis unable to accumulate SA (Lawton et al., 1995) and *npr1-1,* a mutant nonresponsive to inducers of SAR (Cao et al., 1994), were used to probe the SA pathway. Interestingly, BABA did not enhance the resistance against *B. cinerea* in any of these plants (Fig 2A). Analysis of the contribution of the JA/ethylene signal transduction pathway to BABA-mediated protection of Arabidopsis against *B. cinerea* was investigated with the ethylene-insensitive *etr1-1* mutant (Bleecker and Kende, 1988) and with *coi1-1*, a mutant affected in the JA response pathway (Feys et al., 1994). Two days after inoculation, both mutants were protected to a similar level as Col-0 wild type (Fig 2B). Furthermore, *etr1-1* mutants were also protected 3 days after inoculation (Fig 2C). The fact that *B. cinerea* hyphae had already completely invaded the leaves of the untreated *coi1-1* plants 3 days after inoculation did not allow us to analyze the level of infection of this highly sensitive mutant at this late time-point. Hence, BABA protects Arabidopsis against *B. cinerea* through SA-dependent defense responses.

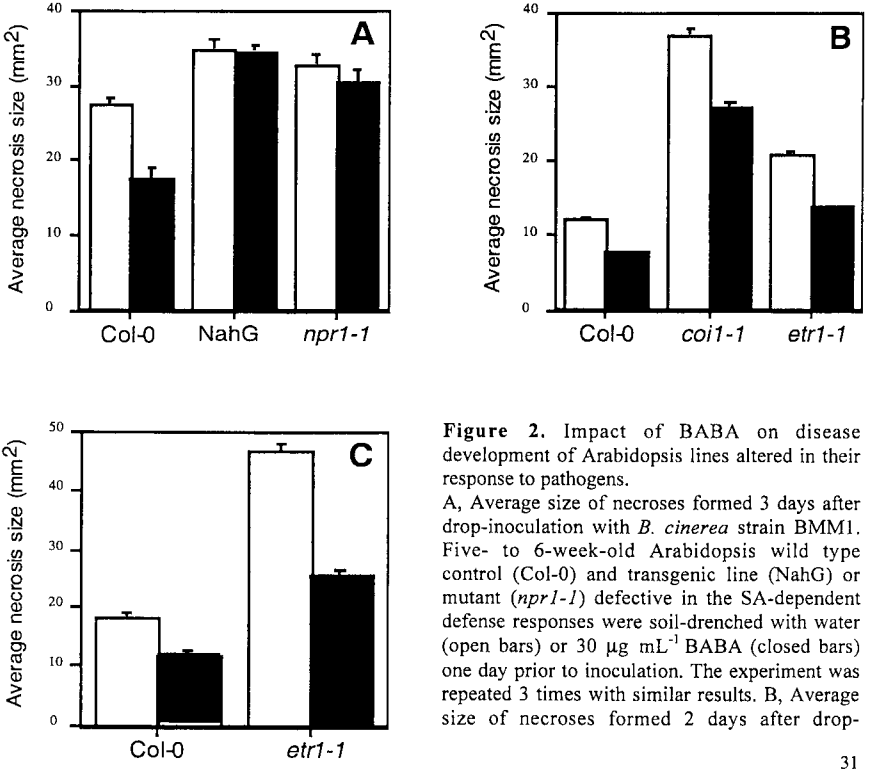

Figure 2. Impact of BABA on disease development of Arabidopsis lines altered in their response to pathogens.
A, Average size of necroses formed 3 days after drop-inoculation with *B. cinerea* strain BMM1. Five- to 6-week-old Arabidopsis wild type control (Col-0) and transgenic line (NahG) or mutant (*npr1-1*) defective in the SA-dependent defense responses were soil-drenched with water (open bars) or 30 µg mL^{-1} BABA (closed bars) one day prior to inoculation. The experiment was repeated 3 times with similar results. B, Average size of necroses formed 2 days after drop-

inoculation with *B. cinerea*. Arabidopsis wild type control (Col-0) and mutants defective in the JA (*coi1-1*)/Ethylene (*etr1-1*)-dependent signaling pathway were inoculated and treated as in (A). C, Average size of necroses formed 3 days after drop-inoculation on Arabidopsis wild type control (Col-0) and *etr1-1* mutants. Plants were inoculated and treated as in (A).

BTH Protects Arabidopsis against *B. cinerea*

Since SA-dependent defense-responses are involved in the BABA-mediated protection of Arabidopsis against *B. cinerea*, we evaluated the effect of BTH, a functional analogue of SA, on the protection of Arabidopsis to infection to *B. cinerea*. A soil drench application of 0.33 10^{-3} M BTH one day prior to inoculation with two different strains of *B. cinerea* is sufficient to drastically slow down the infection (Fig 3). This confirms the implication of SA-dependent defense responses in the protection of Arabidopsis against *B. cinerea*.

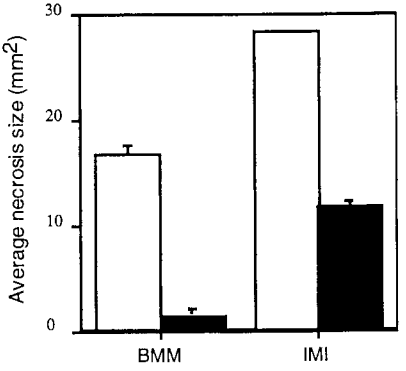

Figure 3. Effect of BTH on the infection process of *B. cinerea* in Arabidopsis.
Average size of lesions formed on Arabidopsis Col-0 leaves 3 days after drop-inoculation with *B. cinerea* strain BMM (BMM1) and IMI (IMI169558). Plants were treated with water (open bars) or 0.33 10^{-3} M BTH (closed bars) one day before inoculation. The experiments were repeated twice with similar results.

BABA Potentiates *PR-1* but not *PDF 1.2* mRNA Accumulation

BABA treatment has been shown to prime the SA-dependent defense-response pathway through potentiation of *PR-1* mRNA accumulation after infection with virulent pathogenic bacteria (Zimmerli et al., 2000). JA/ethylene-dependent defense-responses are essential for resistance against *B. cinerea* (Thomma et al., 1998; 1999). Moreover, treatment of Arabidopsis plants with these two plant hormones induces the accumulation of the plant defensin gene *PDF1.2* (Penninckx et al., 1996). However, treatment with BABA potentiated the plant to induce *PR-1* mRNA more rapidly and more intensively, but no differences were observed for *PDF 1.2* mRNA accumulation (Fig 4). Indeed, *PDF 1.2* transcripts accumulation was recorded starting 36 hours post-inoculation in both treated and untreated Arabidopsis. Thus, SA-dependent defense-responses are boosted in BABA-treated Arabidopsis plants during infection with *B. cinerea*.

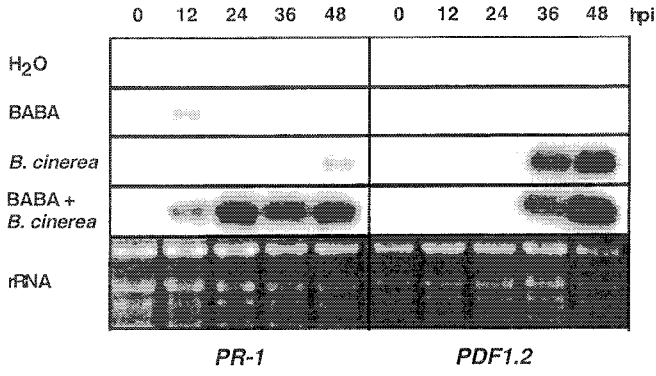

Figure 4. Effect of BABA treatment on the time-course of the expression of defense genes in Arabidopsis infected with *B. cinerea*.
Arabidopsis Col-0 plants were soil drenched with water or 30 μg mL^{-1} BABA one day prior to inoculation. Total RNA was extracted at different hours post inoculation (hpi) with *B. cinerea* strain BMM1. Each time point represents 9 infected leaves harvested from 3 different plants. RNA blots were hybridized with *PR-1* and *PDF1.2* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading.

Pathogen-Induced Expression of *PR-1* and *PDF 1.2* mRNA in NahG, *npr1-1*, *etr1-1* and *coi1-1* Plants

BABA-primed defense responses after *B. cinerea* infection were investigated by analyzing the expression of *PR-1* and *PDF 1.2* mRNA in NahG, *npr1-1*, *etr1-1* and *coi1-1* plants. As expected, potentiation of *PR-1* transcript accumulation was observed in *etr1-1* and *coi1-1* mutants, but not in NahG and *npr1-1* plants. Moreover, *PDF 1.2* mRNA accumulated in NahG and *npr1-1* plants, but not in *etr1-1* and *coi1-1* mutants (Fig 5). Therefore, potentiation of *PR-1* mRNA accumulation is dependent of a functional SAR defense pathway and expression of *PDF1.2* mRNA during *B. cinerea* infection is linked to a functional ETR1 and COI1. Furthermore, NahG and *npr1-1* plants accumulated more *PDF1.2* mRNA 2 days after inoculation with *B. cinerea* than the wild type Col-0 plants confirming that defective SAR signaling provoked altered sensitivity to JA/ethylene signaling (Fig 5).

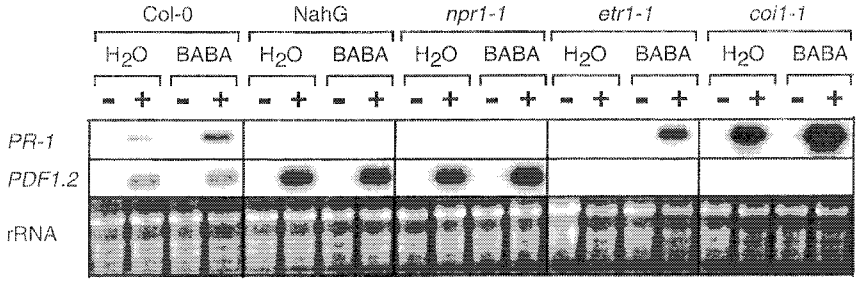

Figure 5. BABA-mediated induction of defense genes in Arabidopsis signal transduction mutants infected with *B. cinerea*.
Arabidopsis NahG, *npr1-1*, *coi1-1* and *etr1-1* plants were soil drenched with water or 30 μg mL^{-1} BABA one day prior to inoculation. For each treatment, total RNA was extracted from 9 infected leaves harvested from 3 different plants. Leaves were collected 48 hours after inoculation with PDB (-) or PDB containing spores of *B. cinerea* strain BMM1 (+). RNA blots were hybridized with *PR-1* and *PDF1.2* probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading.

DISCUSSION

The effect of BABA on necrotrophic pathogens is still largely unknown. We have shown here that this chemical protects Arabidopsis plants against two different races of the grey-mold fungus *B. cinerea*. The infection process was not completely stopped by BABA, but the disease incidence was clearly reduced, leading to smaller lesions 3 days after inoculation (Fig. 1). Both the NahG lines and the *npr1-1* mutants were not protected by BABA, indicating that SA and NPR1 are involved in the BABA-mediated protection of Arabidopsis against *B. cinerea*. This also demonstrates that a direct antibiotic effect of BABA on *B. cinerea* can be excluded. Interestingly, *coi1-1* and *etr1-1* mutants, defective in the JA and ethylene pathways respectively were protected at a level similar to the wild type control Col-0. In agreement with previous results (Thomma et al., 1998; 1999), mutants defective in the JA/ethylene-dependent defense-responses are more sensitive than wild types to *B. cinerea* infection. Thus, BABA can inhibit infection even in mutants highly sensitive to *B. cinerea*, confirming the independence of BABA-mediated defense mechanisms on JA and ethylene signaling. The dependence of BABA on the SA pathway was further evaluated in Arabidopsis plants treated with BTH, an activator of the SAR signal transduction pathway (Lawton et al., 1996). BTH-treated Arabidopsis plants showed a reduction of the size of the necroses on both strains tested (Fig 3). This is in contrast with observations on tobacco where BTH does not induce resistance against *B. cinerea* (Friedrich et al., 1996). The reasons for this species-specific protective effect of BTH are not known.

The action of BABA against *B. cinerea* is not based on a direct fungitoxic effect (see above; Zimmerli et al., 2000). Rather, it seems to act like an inducer of plant resistance mechanisms. We have therefore investigated the effect of BABA on the expression of

defense-related genes. We have given a special attention to JA/ethylene-dependent genes, since they are associated with resistance against necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1998; 1999). However, unlike the effect of BABA on the potentiation of *PR-1* gene after infection with virulent bacteria (Zimmerli et al., 2000), neither accumulation nor potentiation of *PDF1.2* gene was observed in BABA-treated plants after *B. cinerea* inoculation. By contrast, potentiation of *PR-1* transcript accumulation was observed during *B. cinerea* infection (Fig. 4). Consequently, BABA stimulates the SA-dependent but not the JA/ethylene-dependent signaling pathway in Arabidopsis infected with widely diverse pathogens.

The expression of the *PDF1.2* gene in response to *B. cinerea* infection was enhanced in plants with a defective SA-dependent signaling pathway (Fig. 5). On the other hand, potentiation of *PR1* mRNA accumulation was stronger in mutants defective in the JA/ethylene-dependent signaling pathway. However in this case, the upregulation of *PR-1* gene expression is probably due to a more extensive fungal colonization. Since the same expression level of *PDF1.2* gene was observed in water- and in the less-infected BABA-treated Col-0 plants, the level of *PDF1.2* mRNA accumulation did not reflect the rate of fungal colonization. Upregulation of *PDF1.2* gene expression has also been observed in mutants defective in the SA-dependent signaling after inoculation with *A. brassicicola* (Penninckx et al., 1996) or treatments with rose bengal or methyl JA (Gupta et al., 2000). All these data indicate that SA-dependent signaling interferes with JA/ethylene-dependent defense-responses.

BABA enhances resistance through potentiation of SA-dependent defense-responses leading to restriction of *B. cinerea* growth and spread. *A. brassicicola* and *B. cinerea* infection of water-treated Arabidopsis leads to a weak and delayed *PR-1* mRNA accumulation, whereas *PDF1.2* mRNA is strongly induced (Thomma et al., 1998; this work). Furthermore, *coi1-1* and *etr1-1* mutants defective in the JA and ethylene signaling pathway respectively, are more sensitive to these two necrotrophs and fail to express *PDF1.2* upon infection (Thomma et al., 1998; this work). Consequently, both SA and JA/ethylene-dependent defense responses are involved in protection to *B. cinerea*. The question arises why *B. cinerea* fails to induce a strong SAR response. *B. cinerea* might either downregulate the SA-dependent signaling pathway or, alternatively, fail to induce it due to a defective recognition or signal transduction leading to a delayed expression of *PR-1* gene. To overcome this, Arabidopsis plants may have evolved the JA/ethylene-dependent pathway. BABA might counteract or shortcut such inhibitory mechanisms and allow the expression of the SA-dependent signaling pathway after *B. cinerea* infection. Similarly, it was demonstrated recently that necrotrophs can exploit a host defense mechanism such as HR for their pathogenicity (Govrin and Levine, 2000). Alternatively, induction of *PR-1* gene results from tissue damage inflicted by *B. cinerea* and this is potentiated by BABA. A small necrosis would be sufficient to induce *PR-1* gene expression in BABA-treated plants whereas in water-treated controls, a larger lesion would be required for the expression of SA-dependent genes.

These observations document the action of the chemical inducer BABA against necrotrophic pathogens. BABA acts by potentiation of a normally underexpressed pathway. Our results also show how a pathogen can modulate the network of defense pathways to its advantage.

MATERIALS AND METHODS

Biological Material

The transgenic *Arabidopsis thaliana* line harbouring the *NahG* gene (Lawton et al., 1995) was obtained from J. Ryals (Novartis, Research Triangle Park, NC). The Columbia (Col-0) ecotype mutants *npr1-1*, *etr1-1* and *coi1-1* were provided by X. Dong (Duke University, Durham, NC), the Nottingham *Arabidopsis* Stock Center and J. Turner (University of East Anglia, Norwich, U.K.) respectively. *Arabidopsis* accessions Columbia (Col-0) were obtained from Lehle Seeds (Round Rock, TX). Plants were grown on a pasteurized soil mix of commercial potting soil/perlite (3:1) at 22 °C day/18 °C night temperature with 12 hours light per 24 hours. *B. cinerea* (strains BMM1, isolated from *Pelargonium*; and IMI169558, International Mycology Institute, Kew, UK) were grown on 19.5 g L^{-1} Potato Dextrose Agar (Difco, Detroit, USA) at 20 °C for 10 days. The conidia were collected and suspended in sterile Potato Dextrose Broth (12 g L^{-1}) (PDB, Difco, Detroit, USA).

Chemical Treatment and Plant Inoculation

Chemicals were all dissolved in water and applied as soil drench. Evaluation of symptoms was done on thirty 5- to 6-week-old soil-grown Arabidopsis plants. Treatments were performed one day before inoculation with *B. cinerea*. The 3 smallest leaves (numbers 5, 6 and 7 from the apex) able to support two 3 µL droplets of a suspension of 2.5 x 10^4 conidia mL^{-1} in PDB (12 g L^{-1}) were used for inoculation. Droplets were deposited on fixed positions left and right from the midvein.

For the time course experiments and analysis of defense genes expression in different genotypes, soil drench treatment of 30 µg mL^{-1} BABA was done one day before inoculation. Inoculation time corresponds to the time 0. Tissue was harvested at the times indicated for RNA extraction and analysis. Inoculation was performed by spraying a suspension of 2.5 x 10^4 conidia mL^{-1} in PDB (12 g L^{-1}). For all the experiments, each time point represents a pool of nine leaves coming from three different plants. To ensure infection, inoculated plants were kept at 100% relative humidity during all the infection process, at 19 °C/17 °C day/night temperature with 12 hours light per 24 hours.

Monitoring Susceptibility to *B. cinerea*

Susceptibility to *B. cinerea* was evaluated by macroscopic observation of the diameter of the necroses. Since *B. cinerea* hyphae developed concentrically, results were expressed as necrosis size in square millimeters.

RNA Extraction and Analysis

RNA was isolated from frozen tissue samples as described previously (Zimmerli et al., 2000). Total RNA samples (6 µg) were separated through formaldehyde-agarose gels and blotted to a Nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK). ^{32}P-labeled cDNA probes of pathogenesis-related genes *PR-1* and *PDF1.2* were synthesized by random priming of isolated insert DNA using the random primers DNA labeling system (RadPrime DNA Labeling System, Life technologies, Merelbeke, B). Equal loading of samples was shown by ethidium bromide staining of the rRNA.

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CHAPTER 4

β-aminobutyric acid-mediated tolerance to freezing reveals possible connection between biotic and abiotic stress responses

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ABSTRACT

Plant-growth-promoting rhizobacteria mediate plant resistance to pathogens. This type of induced-resistance provokes changes in plant gene expression linked with resistance to biotic and abiotic stresses. We therefore tested the effect of BABA on the resistance to cold stress. BABA treatment increased the tolerance of Arabidopsis plants to freezing. Indeed, after two days at –5°C, the majority of the BABA-treated Arabidopsis survived, whereas all the water controls died. In Arabidopsis, cold-regulated (COR) genes have been shown to be coordinately stimulated during low temperature treatment with a concomitant enhanced freezing tolerance. The possible increased resistance through potentiation of COR genes expression after BABA treatment was monitored. Potentiation of these genes was not observed. However, the expression of the heat shock protein gene 83 was primed during heat stress in BABA-treated Arabidopsis. Hence, BABA also modulates plant responses to cold or heat shock, revealing possible connections between biotic and abiotic stress responses.

INTRODUCTION

Temperature is one of the most important environmental factors limiting the geographical distribution of land plants (Lewitt, 1980). Cold acclimation is observed when a plant increases its freezing tolerance after exposure to low non-freezing temperatures (Guy, 1990). Changes in gene expression (Thomashow, 1994), membrane composition (Lynch and Steponkus, 1987) and accumulation of cryoprotectants such as proline (Chu et al., 1974) and glycinebetaine (Rhodes and Hanson, 1993), have been associated with cold acclimation. The phytohormone abscisic acid (ABA) is also known to play an important role in cold acclimation. ABA treatment at normal growth temperature elevates the freezing tolerance of plants (Chen et al., 1983; Mohapatra et al., 1988) and transient exposure to low temperature increases ABA levels in a number of plant species (Chen et al., 1983; Lang et al., 1994). In Arabidopsis, genetic analyses indicate that several cold-regulated (COR) genes are coordinately stimulated in response to low temperature (Hajela et al., 1990). Coordinate overexpression of these COR genes has been shown to enhance freezing tolerance of non-acclimated Arabidopsis, suggesting a direct implication of these genes in resistance to freezing (Jaglo-Ottosen et al., 1998).

The critical factors conferring tolerance to lethal high temperatures in plants are still poorly understood. Indirect evidences suggest that heat shock proteins (HSP) are likely to play a role. Mild heat stress-mediated induction of some HSP has been correlated with tolerance to much more severe stresses (Vierling, 1991; Howarth and Skot, 1994) and a higher basal thermotolerance was observed after overexpression of transcriptional regulators of HSP (Prändl et al., 1998). Interestingly, recent studies provided direct evidences that HSP 101 is essential for heat tolerance in Arabidopsis (Hong and Vierling, 2000; Queitsch et al., 2000).

MATERIALS AND METHODS

Four weeks-old Arabidopsis Col-0 plants were grown on a pasteurized soil mix of commercial potting soil/perlite (3 :1) at 22°C day/18°C night temperature with 12 hours light per 24 hours. Chemicals were dissolved in water at the concentration indicated and applied to the soil 2 or 1 days before cold or heat shock treatments respectively. For the cold shock treatment, plants distributed in a completely randomized design were frozen in a -5°C cold chamber in the dark. After 1 hour, the plants were covered with ice chips to nucleate the freezing. Plants were removed after 2 days and returned to normal growth conditions. Plants were considered cold resistant when the apex did not show complete tissue destruction when observed 5 days after the cold shock treatment. Heat shock was done by shifting the growth temperature from 22°C to 29°C under normal light conditions. RNA was isolated from frozen tissue samples as described previously (Zimmerli et al., 2000). Total RNA samples (6 µg) were separated through formaldehyde-agarose gels and blotted to a Nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK). ^{32}P-labeled cDNA probes of cold regulated genes COR 47 and COR 15a and heat shock protein gene HSP83 were synthesized by random priming of isolated insert DNA using the random primers DNA labeling system (RadPrime DNA Labeling System, Life technologies, Merelbeke, B). Equal loading of samples was shown by ethidium bromide staining of the rRNA.

RESULTS

BABA treatment increased the tolerance of plants to freezing as determined by the freezing survival test. Indeed, almost all the water-treated plants died after 2 days at -5°C, whereas

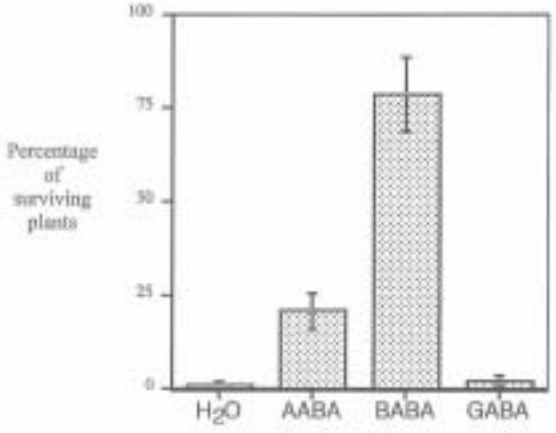

Figure 1. Freezing tolerance of Arabidopsis treated with aminobutyric acid isomers.
Col-0 plants were treated with AABA and GABA at a final concentration of 500 ppm or with BABA at 50 ppm. Surviving plants were evaluated 5 days after cold shock at -5°C for 2 days. Results are expressed as percentage of surviving plants from 8 pots per treatment containing each 35 plants. Error bars indicate standard errors. The experiment was repeated twice with similar results.

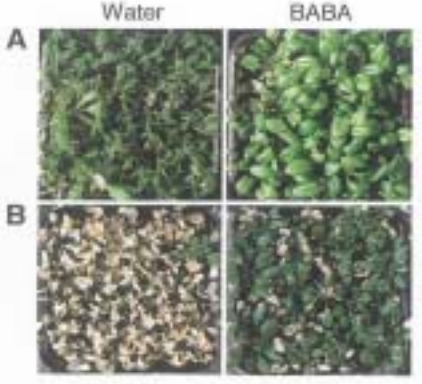

Figure 2. Effect of BABA treatment on the freezing survival.
A, Survival 5 days after freezing. Water- or BABA (50 ppm)-treated Col-0 plants were frozen at -5°C for 2 days and then returned to normal growth conditions. B, Plants growth after freezing. The experimental conditions were the same as in (A) except that plants were photographed 3 weeks after the cold shock.

more than 75 % of the BABA-treated Arabidopsis survived (Fig. 1). This resistance to freezing was isomer specific, since α-amino-butyric acid (AABA) and γ-amino-butyric acid (GABA) did not significantly enhanced freezing tolerance even at concentrations 10 times higher than those used for BABA treatment. However, AABA showed a weak, but repetitive, protective effect (Fig. 1). Five days after the cold treatment tissue of water-treated plants were completely destroyed, whereas BABA-treated plants looked normal (Fig 2A). This protective effect was long lasting, since 3 weeks after the cold shock, the majority of the BABA-treated plants developed normally (Fig 2B). Clearly, the frozen water-treated plants did not recover (Fig 2B).

Since BABA protects Arabidopsis plants against pathogen infection through potentiation of specific defense responses, we tested whether the expression of the COR 47 and COR 15a genes was potentiated after BABA treatment during cold stress. To test this, Arabidopsis plants were placed in a dark chamber at 4°C (Fig. 3A) or 8°C (Fig. 3B) and the time course of the accumulation of COR 47 and COR 15a mRNA was monitored. No potentiation effect was

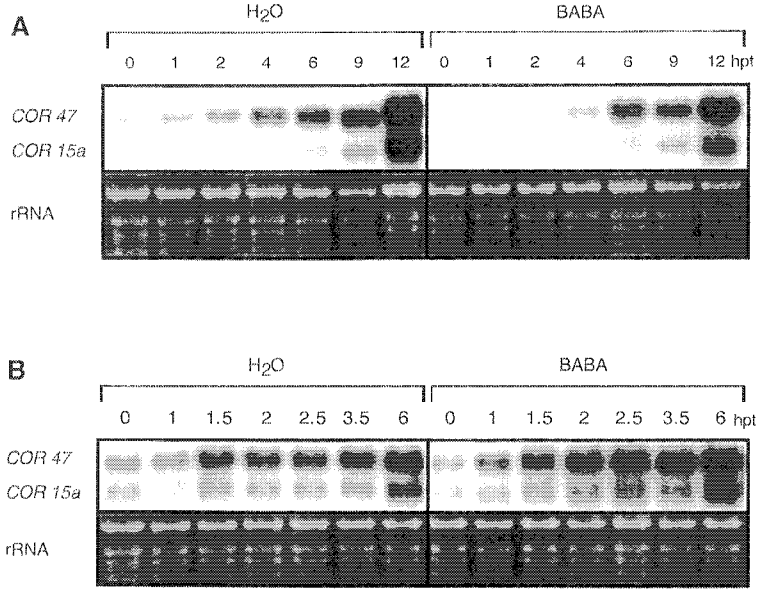

Figure 3. Impact of BABA on the time-course of the expression of COR genes in Arabidposis after cold shock.
A, Col-0 plants, grown in normal conditions (see the text) were soil drenched with water or 50 ppm BABA two days before incubation at 4°C in the dark. Total RNA was extracted at various hours post treatment (hpt). Each time point represents a pot containing 35 plants. Total RNA was prepared and analyzed by RNA blot analysis. RNA blots were hybridized with COR 47 and COR 15a probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. B, Same as in (A) except that the plants were incubated at 8°C.

observed (Fig. 3A/B). At both temperatures, accumulation of COR 47 mRNA occurred earlier than the expression of the COR 15a gene. This difference in the timing of expression is difficult to explain since both genes are regulated by Arabidopsis CBF1 (Jaglo-Ottosen et al., 1998), a transcriptional activator that binds to the C-repeat/drought-responsive element (CRT/DRE) DNA regulatory element (Yamaguchishinozaki and Shinozaki, 1994; Stockinger et al., 1997).

Although no potentiation of the COR genes was observed, HSP83 gene expression was potentiated in BABA-treated Arabidopsis. Indeed, plants treated with 32 ppm BABA accumulated a maximum of HSP83 mRNA 30 to 40 minutes after the temperature shift, whereas water-treated plants showed the highest expression 20 minutes later (Fig. 4). At a concentration of 16 ppm, the potentiation effect was less clear and showed a concentration dependency.

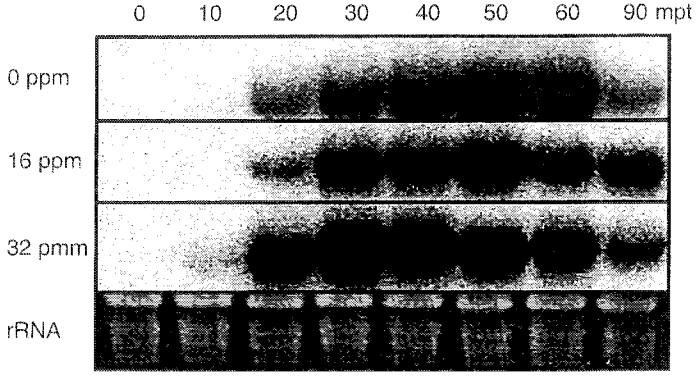

Figure 4. Conditioning effect of BABA on the expression of HSP83 gene in Arabidopsis after heat shock treatment.
Total RNA was extracted at various times after temperature shift from 22°C to 29°C. Time points were expressed in minutes post treatment (mpt). Each time point represents 35 Col-0 plants soil drenched with 0, 16 and 32 ppm BABA one day before heat treatment. Total RNA was prepared and analyzed by RNA blot analysis. RNA blots were hybridized with HSP83 probes. Ethidium bromide staining of the RNA gel (rRNA) was used to show equal loading. The experiment was repeated twice with similar results.

DISCUSSION

We have demonstrated that pre-treatment with BABA induces freezing tolerance in Arabidopsis. Only BABA enhances freezing survival, demonstrating a remarkable selectivity towards aminobutyric acid isomers. The same isomer selectivity among aminobutyric acid was observed in the protection effect against pathogens. Interestingly, the weak protective effect of AABA against pathogens (Cohen, 1994; Cohen et al., 1994; Siegrist et al., 2000) is also observed in the case of freezing tolerance, suggesting functional similarities for the regulation of both biotic and abiotic stresses.

We did not observe a potentiation effect of the expression of COR genes in BABA-treated Arabidopsis after cold shock treatment. Arabidopsis sprayed with glycinebetaine or transformed with a gene that encodes a choline oxydase which provokes an accumulation of glycinebetaine in chloroplasts also show an elevated tolerance to freezing (Sakamoto et al., 2000). Similarly, no differences in COR genes expression was observed. It is postulated that the presence of glycinebetaine in plant tissue is directly responsible for the enhancement of freezing tolerance. In our case, it might be possible to correlate concentrations of BABA treatments with levels of freezing resistance. However, other mechanisms indirectly activated by BABA could also be concentration dependent. Since glycinebetaine accumulation does not occur naturally in Arabidopsis during cold shock (Rhodes and Hanson, 1993), increase of this cryoprotectant in plant tissue after BABA treatment is very unlikely.

Eskimo1 mutants of Arabidopsis are constitutively freezing tolerant in the absence of acclimatation (Xin and Browse, 1998). This mutant accumulates high levels of proline, an other compatible osmolyte, but does not exhibit constitutive increased expression of CBF1-controlled genes. We therefore determined the proline content after BABA treatment and compared the time-course of proline accumulation in water- and BABA-treated Arabidopsis during cold shock. No potentiation or up-regulation of the accumulation of this cryoprotectant was observed during cold treatment (data not shown), demonstrating that BABA does not condition proline accumulation upon cold shock.

It has been shown that level of fatty acid unsaturation can be an important factor in chilling tolerance (Miquel and Browse, 1992; Miquel et al., 1993). Therefore, the analysis of a possible changes in the composition of polyunsaturated lipids after BABA treatment awaits further analysis.

Taken together, freezing tolerance is modulated by numerous distinct signaling pathways and activation of a single one can result in considerable freezing tolerance without activation of other pathways. Consequently, it is possible that BABA activates its own signaling pathway to increase cold tolerance in Arabidopsis, through, for example, a direct cryoprotectant effect. It is also possible that BABA potentiates other genes than the CBF1-controlled genes. Indeed, the observed potentiation of the accumulation of HSP83 mRNA shows that BABA can also condition the expression of genes involved in abiotic stresses. This observation is very interesting, since only few reports have shown connection at the gene expression level between biotic and abiotic stress responses. For example, changes in plant gene expression induced by inoculation with plant-growth-promoting rhizobacteria have been correlated with both resistance to biotic and abiotic stresses (Wagner and Timmusk, 1999). This suggests that genes and/or gene classes associated with plant defenses against abiotic and biotic stress may be co-regulated. It would be very exciting to analyze whether BABA can also protect Arabidopsis plants against heat shock damage as it did for freezing.

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CHAPTER 5

Conclusion

The non-protein amino acid β-aminobutyric acid (BABA) provides protection against various pathogens in different plant species (Jakab et al., 2000). However, its mode of action is still largely unknown (Cohen, 1994b; Cohen et al., 1994). In plants, systemic acquired resistance (SAR) (Sticher et al., 1997) and rhizobacteria-mediated induced systemic resistance (ISR) (Van Loon et al., 1998) are well-studied plant defense mechanisms. The goal of this work was to understand the mode of action of BABA in plants to provide protection against pathogens. With this idea in mind, we developed an Arabidopsis-based model system to analyze the molecular basis of BABA-induced resistance. This study provided new insights in the complexity of the regulation of induced disease resistance and its possible link to abiotic stress responses.

BABA-mediated resistance is not based on a direct antibiotic activity

It was demonstrated in Chapter 2 and 3 that soil drench treatment of Arabidopsis plants with BABA provided protection against widely diverse pathogens. BABA increased the level of protection of Arabidopsis against the oomycete *Peronospora parasitica*, the pathogenic bacteria *Pseudomonas syringae* pv. *tomato* DC 3000 and the necrotrophic fungus *Botrytis cinerea*. Protection of Arabidopsis against *P. parasitica* is illustrated by a complete lack of asexual sporulation, whereas BABA treatment resulted in a ten-fold reduction of the bacterial titer accompanied by strongly decreased symptoms (Chapter 2). BABA also protected Arabidopsis against a necrotrophic fungus: *B. cinerea* symptoms were reduced from 20 up to 50% in BABA-treated plants (Chapter 3). The observed expression of resistance correlated with the amount of BABA in the leaf, supporting the idea that BABA acts locally (Cohen and Gisi, 1994). Indeed, soil-drench treatment with BABA leaded to an accumulation of this chemical in the youngest leaves (Jakab et al., 2000), where the protection is maximal (data not shown). However, this local action does not necessarily mean that BABA acts as an antimicrobial compound. Repetitive *in vitro* test on many plant pathogens done by different research groups did not show any toxicity of this chemical (Jakab et al., 2000; Chapter 2). Furthermore, BABA is not metabolized in plants ruling out the involvement of a BABA-metabolite acting as an antimicrobial compound in the plant (Cohen and Gisi, 1994; Chapter 2). Finally, BABA-mediated protection against pathogenic bacteria and the necrotroph pathogen *B. cinerea* is blocked in the SAR-defective NahG and *npr1* plants. If a direct antimicrobial effect was involved, it is unlikely that protection would be dependent on the plant genotype (Chapter 2 and 3). Protection of Arabidopsis against *P. parasitica* is observed in all the mutants tested (Chapter 2). This independence to known defense pathways is probably based on the rapid appearance of cell-wall appositions (CWAs) such as papillae. We unsuccessfully tried to weaken these structures with 2-deoxy-D-glucose, an inhibitor of callose synthesis (Fredrikson and Larsson, 1992). The appearance of CWAs is a dynamic phenomenon involving the formation of stress fibers and direct vesicle transport. Cytochalasins (actin-microfilament inhibitors) (Kobayashi et al., 1997) abolish the CWAs appositions leading to successful haustorium differentiation in a normally *mlo* resistant barley (Schulze-Lefert and Vogel, 2000), demonstrating that resistance in *mlo* genotypes is likely to be linked with CWAs formation (Schulze-Lefert and Vogel, 2000). Therefore, it would be of great interest to test whether cytochalasins can also inhibit BABA-mediated resistance of Arabidopsis plants against *P. parasitica*.

Taken together, these results strongly suggest that the BABA-mediated protection of Arabidopsis is based on the activation of plant defense mechanisms and not on a direct antimicrobial activity of this chemical.

BABA activation of host resistance mechanisms

Microscopical observation of BABA-treated Arabidopsis plants inoculated with *P. parasitica* suggest the involvement of active defense mechanisms in BABA-mediated resistance (Chapter 2). In this case, BABA treatment converts a compatible into a phenocopy of an incompatible host-pathogen interactions (Chapter 2). During infection with pathogenic bacteria and necrotrophs, BABA treatment acts on the time-course of the expression of SA-dependent defense genes, suggesting effects on SA-dependent signaling (Chapter 2 and 3). Although BABA soil-drench treatments do not directly induce the accumulation of defense genes such as *PR-1, PR-5, BGL2, PDF1.2* and *Thi2.1* (Chapter 2), expression of the *PR-1* gene is primed

upon bacterial and necrotroph infection (Chapter 2 and 3). Potentiation was observed in both SAR (Thulke and Conrath, 1998) and ISR (vanWees et al., 1999). Hence, potentiation mechanisms are probably typical for induced resistance, suggesting that BABA acts as an inducer of plant resistance mechanisms.

BABA signaling

The accumulation of the signaling molecule SA with concomitant expression of SAR-related *PR* genes in non-infected tissue is characteristic of SAR (Sticher et al., 1997). Besides SAR, jasmonic acid (JA)- and ethylene-dependent signaling were found to be involved in signaling for resistance to pathogens such as the necrotrophic fungus *B. cinerea* (Thomma et al., 1998; 1999). Studies with transgenic Arabidopsis or mutants impaired in the signal transduction pathway for resistance, revealed that BABA activates pathogen-specific defense mechanisms in Arabidopsis (Chapter 2). However, SA-dependent signaling seems to be a key factor for the BABA-mediated protection to widely diverse pathogens (Chapter 3). In order to further elucidate BABA signaling in Arabidopsis, the expression of SA-dependent *PR-1* and JA/ethylene-dependent *PDF1.2* genes was analyzed during infection with virulent bacteria or a necrotrophic fungus. Interestingly, both pathogens potentiate the expression of the *PR-1* gene in BABA-treated plants, whereas no priming was observed with the JA/ethylene-dependent signaling, explaining the dependence of BABA on the SA pathway. Potentiation mechanisms are still poorly understood. Possibly, BABA-mediated changes in activity of transcription factors or (de)phosphorylation of particular steps in the transduction cascade could be involved in such a mechanism. However, the fact that potentiation of SA-dependent signaling is observed after infection by widely diverse pathogens, supports the notion that an alteration in cellular physiology in infected tissue rather than direct recognition of the pathogen, could be sufficient to induce such an early recognition event. The effect of BABA on to abiotic stresses also provides evidences in that direction (Chapter 4). This means that BABA may act on a general stress sensing system allowing plants to react and modulate diverse environmental stress they encountered. Only then, upregulation of appropriate transduction signaling cascade would be turned on. However, it remains to be understood why SA-dependent signaling is upregulated by necrotrophs which normally involves the JA/ethylene transduction signaling pathway (Thomma et al., 1998; 1999).

Connection between biotic and abiotic stress responses

BABA treatment enhanced freezing tolerance and provoked a potentiation of the accumulation of HSP83 gene in Arabidopsis plants (Chapter 4). Thus, BABA plays a role in the modulation of abiotic stress. The same isomer specificity among aminobutyric acids was observed during biotic and abiotic stresses (Cohen, 1994a; Chapter 4). Furthermore, BABA-mediated potentiation of specific genes was observed after pathogen infection and heat shock treatment (Chapter 4), demonstrating some striking similarities in the regulation of both types of stress. As already mentioned, BABA may act at a general stress regulator which may modulate the response of an appropriate signaling cascade after a particular aggression. However, BABA potentiates SA-dependent signaling after infection with widely diverse pathogens (Chapter 2 and 3), demonstrating a specific activity of this chemical on SA signaling. It is conceivable that particular abiotic stress can be somehow regulated by SA. Indeed, SA was shown to induce HSP70 expression in *Lycopersicon esculentum* (Cronje and Bornman, 1999). Furthermore, inoculation with plant-growth-promoting rhizobacteria

induces changes in plant gene expression suggesting a link between resistance to biotic and abiotic stresses (Timmusk and Wagner, 1999). Thus, BABA may influence both stress responses through co-regulation of separate signal transduction cascades with SA as a possible common key element.

Modulation of stress perception to restrict plant disease development

Induced resistance protects plants against a large-spectrum of pathogens (Sticher et al., 1997). Therefore, a chemical agent mimicking this defense mechanism would be of great interest. The chemical plant activator Bion is a successful example of the use of induced resistance in crop protection. Its active compound benzothiadiazole (BTH) is a functional analogue of SA and activates the SAR signaling pathway in different plants (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). The fact that BABA protects many plants against diverse pathogens (Jakab et al., 2000; chapter 2 and 3) also makes this chemical an interesting candidate as an alternative to pesticides. Its mode of action, potentiation, rather than direct upregulation of the defense signaling cascade is of great interest, since plants has to face infection before inducing defense mechanisms. This approach probably presents advantages to direct upregulation in terms of energy cost. Furthermore BABA modulates plant responses to abiotic stress such as cold or heat shock (Chapter 4). This observed broad-spectrum activity is linked with some side-effects such as sterility (Jakab et al., 2000) and probable unexpected responses to the various stresses encountered in nature. However, the discovery of the molecular site of action of BABA would give us tremendous knowledge's on the basic mechanisms involved in induced resistance or eventually, in general stress perception. This might provide new tools to control the effect of multiple stress aggressions through enhancement of the natural defense potential of plants.

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