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β-Aminobutyric acid-induced resistance in plants

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Abstract

The broad spectrum protective effect of the non-protein amino acid β-aminobutyric acid (BABA) against numerous plant diseases has been well-documented in the literature. Here, we present an overview of BABA-induced protection in various pathosystems. Contradictory reports concerning the mechanism of action underlying this type of protection incited us to take advantage of Arabidopsis/pathogen interactions as model systems to investigate the action of BABA at the genetic and molecular level. We present evidence that the protective effect of BABA is due to a potentiation of natural defense mechanisms against biotic and abiotic stresses. In order to dissect the pathways involved in potentiation by BABA we describe the use of a mutational approach based on BABA-induced female sterility in Arabidopsis.

Abbreviations: AABA – α-aminobutyric acid; BABA – β-aminobutyric acid; GABA – γ-aminobutyric acid; SA – salicylic acid; DDG – 2-deoxy-D-glucose; BTH – benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; INA – 2,6-dichloroisonicotinic acid; ppm – parts per million; PR – pathogenesis related; ROS – reactive oxygen species; HR – hypersensitive response; TLC – thin layer chromatography; PGPR – plant growth promoting rhizobacteria.

Introduction

Various synthetic and biological compounds have been described which are capable of controlling a large variety of plant diseases without displaying a direct antibiotic effect themselves. These substances are called inducers based on their ability to induce resistance in the treated plants. Both biologically as well as chemically induced resistance against pathogen attack have been described for many plant species against a wide variety of pathogens ranging from oomycetes, fungi, bacteria to viruses (Sticher et al., 1997). Both types of induction share similarities at the phenotypic level, such as a hypersensitive response (HR), trailing necroses, wall strengthening in form of papillae and lignification, and at the molecular level, where a similar set of genes has been observed to be induced. These genes are termed SAR (systemic acquired resistance) genes and their expression depends on salicylic acid (SA). In contrast, systemic resistance induced by plant growth promoting rhizobacteria (PGPR) is characterized by its dependency on the two plant hormones jasmonic acid (JA) and ethylene (Pieterse and Van Loon, 1999), although the phenotype of the observed protection is the same as for SAR.

Chemical inducers of resistance seem to enter at different points in defense pathways. The most thoroughly investigated chemical inducers are those interfering with the SA pathway, such as INA (2,6-dichloroisonicotinic acid) or BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester), commercialized under the tradename of BION (Friedrich et al., 1996; Görlach et al., 1996). Protection by ORYZEMATE (Watanabe et al., 1977; Sekizawa

and Mase, 1980), a commercial inducer used in rice against blast and bacterial leaf blight functions via an unknown mechanism.

In addition, numerous other inorganic (Gottstein and Kuć, 1989; Walters and Murray, 1992; Chérif et al., 1993; Reuveni et al., 1994; Schneider and Ullrich, 1994) and organic substances (Hadwiger and Beckman, 1980; Ricci and Pernollet, 1989; Cohen et al., 1991; Benhamou and Theriault, 1992; Namai et al., 1993; Benhamou et al., 1994; Coquoz et al., 1995; Yu, 1995), as well as extracts from plants (Daaf et al., 1995) and microorganisms (Strobel et al., 1996), have been described to induce disease resistance in plants.

BABA as chemical inducer of resistance

The present review concentrates on induced resistance based on the action of a simple chemical compound, β-aminobutyric acid (BABA) (Figure 1). BABA is a non-protein amino acid which occurs rarely in nature. The only report in connection to plants describes its presence in root exudates of tomato plants grown in solarized soil (Gamliel and Katan, 1992). What makes this substance interesting though is its close relation to a highly bioactive substance, the neurotransmitter γ-aminobutyric acid (GABA) (Figure 1). GABA, besides glycine, is the major inhibitory neurotransmitter in the central nervous system of animals. Both GABA and glycine lead to hyperpolarization of the neural membrane through the stimulation of Cl^- influx due to the binds to specific receptors in the membrane. The $GABA_A$ receptor binds GABA and the glycine receptor (GlyR), a ligand-gated ion channel, binds glycine (Betz, 1992). Interestingly, in the latter case, BABA acts as a partial agonist of glycine. At low concentrations, it competitively inhibits glycine responses. At higher concentrations, it leads to the build-up of a significant membrane current (Schmieden and Betz, 1995). In contrast to BABA, the natural occurrence of GABA is well-documented in plants (Shelp et al., 1999).

Although BABA is only rarely found naturally in plants, it has proved to be a potent inducer of acquired resistance (Table 1). Almost 40 years ago, Papavizas and Davey (1963) and Papavizas (1964) reported the role of BABA in the protection of pea plants against the oomycete pathogen *Aphanomyces euteiches*. They demonstrated that from 10 amino compounds and related substances tested in the greenhouse against root rot of peas, only DL-β-aminobutyric acid and DL-methyl-β-aspartic acid effectively reduced the root rot severity. The substances showed the highest activity when applied as a soil drench three days prior to inoculation with *Aphanomyces* at a concentration of 100 ppm in the soil. It was claimed, that the two amino acids did not act directly on the pathogen, but prevented expression of disease symptoms.

BABA has a broad sprectrum of activity against many disease-causing organisms such as virus, bacteria, oomycetes, fungi and nematodes (Table 1). This wide range of activity supports the notion of BABA as an inducer of resistance and not simply a biocidal substance.

The possibility of a direct toxicity of BABA has been tested repeatedly *in vitro* on many plant pathogens by different research groups and can be ruled out since no toxic effects have ever been observed (Cohen, 1994; Cohen et al., 1994; 1999; Li et al., 1996; Sunwoo et al., 1996; Hong et al., 1999; Tosi et al., 1999). An *in vivo* toxicity based on the action of metabolites of BABA is also not probable since experiments using ^{14}C-labeled BABA clearly demonstrated that the substance does not undergo any changes while in the plant, as shown for tomato (Cohen and Gisi, 1994) and Arabidopsis in our group (Figure 2). The fractionation of protoplasts of ^{14}C-labeled BABA-treated Arabidopsis showed that the label was found almost exclusively inside the protoplast and that it was neither

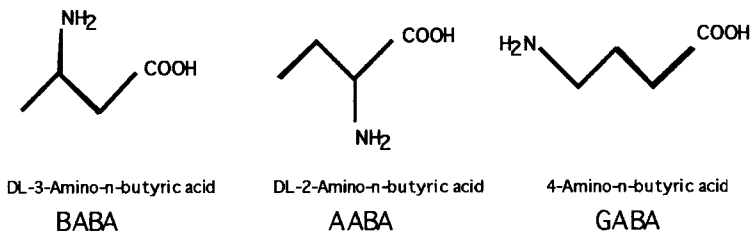

Figure 1. Chemical structure of α-, β-, and γ-aminobutyric acid.

Table 1. Overview of plants exibiting induced resistance to different pathogens after BABA-treatment

Plant	Protection against	Reference
Pea (*Pisum sativum*)	*Aphanomyces euteiches*	Papavizas (1964)
Cucumber (*Cucumis sativus*)	*Sphaerotheca fuliginea*	Vogt and Buchenauer (1997)
	Pseudoperonospora cubensis	Cohen (2000)
	Botrytis cinerea	Cohen (2000)
Cotton (*Gossypium hirsutum*)	*Verticillium dahliae*	Li et al. (1996)
		Kalix et al. (1996)
Tobacco (*Nicotiana tabacum*)	*Peronospora tabacina*	Cohen et al. (1994)
	Tobacco mosaic virus	Siegrist et al. (2000)
Pepper (*Capsicum annuum*)	*Phytophthora capsici*	Sunwoo et al. (1996)
	Colletotrichum coccodes	Hong et al. (1999)
Tomato (*Lycopersicon esculentum*)	*Phytophthora infestans*	Cohen et al. (1994)
	Meloidogyne javanica	Oka et al. (1999)
	Fusarium oxysporum fsp. *lycopersici*	Cohen (2000)
	Botrytis cinerea	Cohen (2000)
Grape (*Vitis vinifera*)	*Plasmopara viticola*	Cohen et al. (1999)
Melon (*Cucumis melo*)	*Pseudoperonospora cubensis*	Cohen (2000)
Sunflower (*Helianthus annuum*)	*Plasmopara helianthi*	Tosi et al. (1999)
	P. halstedii	Cohen (2000)
Broccoli (*Brassica oleracea* var *italica*)	*Peronospora parasitica*	Cohen (2000)
	Alternaria brassicicola	Cohen (2000)
Kohlrabi (*B. oleracea* var *gongylodes*)	*Peronospora parasitica*	Cohen (2000)
	Alternaria brassicicola	Cohen (2000)
Corn (*Zea mays*)	*Fusarium moniliforme*	Cohen (2000)
Pearl millet (*Pennisetum typhoides*)	*Sclerospora graminicola*	Vasanthi (2000)
Cauliflower (*B. oleracea* var *botrytis*)	*Peronospora parasitica*	Cohen (2000)
	Alternaria brassicicola	Cohen (2000)
Potato (*Solanum tuberosum*)	*Phytophthora infestans*	Cohen (2000)
Arabidopsis thaliana	*Peronospora parasitica*	Zimmerli et al. (2000)
	Pseudomonas syringae pv *tomato*	Zimmerli et al. (2000)
	Botrytis cinerea	Zimmerli (unpublished result)

associated with the organellar/membrane nor with the cell wall fraction. In plants treated with radio-labeled GABA, no radioactivity was detected in any fraction (Figure 2). Experiments performed with radio-labeled BABA also helped to show that it is taken up and transported through the plants. Cohen and Gisi (1994) tested different application methods to determine whether and how BABA is transported in tomato plants. When applied as droplets on the leaves, BABA penetrated and was transported mainly acropetally, with a preferred accumulation in the youngest leaves which are known to act as sinks. Very little accumulation in the leaves placed directly above or below the treated ones on the stem was observed. This pattern of distribution reflecting the amount of BABA reaching the respective leaves correlated with the observed expression of resistance against *Phytophthora infestans*. The transport was not totally unidirectional since some label (2%) could be recovered in the roots. Interestingly, BABA can also be fed to plants as a soil drench and is taken up by the roots and translocated through the tomato plantlets (Cohen and Gisi, 1994) and Arabidopsis (Mauch-Mani, unpublished data) (Figure 3). In our hands, using Arabidopsis as the test plant, BABA was much better tolerated when applied to roots, without deleterious effects in the concentration range used to induce resistance. Spraying BABA at higher concentrations on the leaves induced necrosis in tobacco (Cohen and Gisi, 1994; Siegrist et al., 2000). Cohen and Gisi (1994) comment on the fact that only 36% of the applied substance was taken up by the plants, in contrast to a 99% uptake through cut ends of petioles. They thus proposed that roots are partially impermeable to BABA. This partial uptake could also be the consequence of a limitation due to the transport capacity of a transporter or to competition between amino acids for the same transporter. Such transporter systems have been described in Arabidopsis (Rentsch et al., 1995) and it remains to be tested whether BABA is taken up by such transporters. Such a transport would help to explain the observation that BABA action is

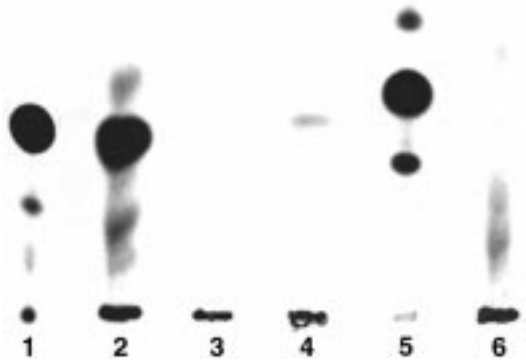

Figure 2. Autoradiogram of thin layer chromatogram of ^{14}C-labeled BABA and ^{14}C-labeled GABA treated Arabidopsis. Lane 1: ^{14}C-labeled BABA; lane 2: ground whole protoplasts of a ^{14}C-labeled BABA treated plant; lane 3: cell wall fraction of a ^{14}C-labeled BABA treated plant; lane 4: membrane and organelle fraction of a ^{14}C-labeled BABA treated plant; lane 5: ^{14}C-labeled GABA; lane 6: ground whole protoplasts of a ^{14}C-labeled GABA treated plant. Protoplast were isolated and ground directly in 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 in the protoplasting enzyme solution after the protoplasts had been released and washing them four times in MCW. TLC plates were run in a solution of n-butanol : HAC : water 60 : 20 : 20 (v/v).

stereospecific (Cohen, 1994). He showed that in the control of blue mold of tobacco, only the R enantiomer had protective activity: treatment with the S enantiomer did not differ from treatment with water.

Different methods of application and a wide range of concentrations have been used to treat plants with BABA and there seems to be a correlation between the method of application and an observed phytotoxic effect. BABA has been sprayed on the leaves, injected into stems of plants, supplied via petiole dip or applied as a soil drench to the root system. Cohen (1994) reports that when applied as a foliar spray to tobacco plants, BABA and to a lesser extent AABA (α-aminobutyric acid), but not GABA, were phytotoxic at a concentration of 100 μg mL^{-1} (ca. 1 mM). This toxicity was expressed in the form of small necrotic lesions on the treated leaves starting two days after spraying. A rapid induction of necrotic lesions in tobacco after foliar treatment with 10 mM BABA, a concentration 10 times higher than in the previously described experiment, was also observed by Siegrist et al. (2000). This pronounced phytotoxicity was acompanied by the formation of reactive oxygen species (ROS), lipid peroxidation, induction of callose around the lesion

Figure 3. Autoradiogram of Arabidopsis accession Wassilewskija after two days feeding with ^{14}C-labeled BABA via the roots. The preferential accumulation of radioactivity in younger plant parts is visualized by the darker color. Plants were grown *in vitro* for six weeks and then transferred to sterile containers containing 35 ml liquid 1/2MS medium and 10 μl ^{14}C-BABA (= 1μ Ci = 2.2×10^6 DPM). The plantlets were put in a plastic support rack to avoid direct contact of the leaves with the radioactive solution. After two days of incubation, the plants were exposed to an X-ray film.

and an increase in SA content of the leaves (Siegrist et al., 2000). No toxic effects were observed in plants treated with GABA, even at concentrations as high as 2000 μg mL^{-1} (Cohen, 1994; Siegrist et al., 2000). This might be due to the fact, that GABA is very rapidly metabolized in plants (Figure 2; Cohen and Gisi, 1994).

Mode of action of BABA

In view of these observations it is clear that it might not always be easy to separate the mechanism of action of BABA responsible for inducing resistance from this described toxic effect. Since the reaction of the plants expressing resistance often resembles the physiological reaction observed due to toxicity, the latter might even mask the former! This fact might also play a role in the decision making process on the involvement of pathogenesis-related proteins (PR) in BABA-induced resistance. It is still a matter of debate whether resistance induced by BABA is, at least partly, based on the direct induction of PRs that would bring the plant in a defense-ready state before even having been in contact with the pathogen, as has been described for INA or BTH (Ward et al., 1991; Görlach et al., 1996).

In tomato sprayed with BABA at a concentration of 19.4 mM the tomato cultivar Florida Basket showed no symptoms, whereas cv Baby displayed about 10 necrotic microlesions (0.5 mm in diameter) per leaflet on lower leaves and sometimes chlorotic microlesions on the upper leaves (Cohen et al., 1994). When BABA was sprayed at a concentration of 9.7 mM, both cultivars showed no visible lesions although it cannot be ruled out that necroses might have been detected at the microscopical level. Both cultivars were protected against infection by *P. infestans*. Although the authors observed an accumulation of the PR proteins P14a and β-1,3-glucanase in the plants, it is unfortunately not possible to draw conclusions on the possibility of a link between the appearance of lesions and the accumulation of the PRs because it remained unclear which tomato cultivar was used to perform this experiment. Hae-Keun et al. (1999) studied the induction of PR1 in tobacco following BABA treatment (the substance was brushed or sprayed onto the leaves) and came to the conclusion that the induction of PR1 mRNA was concentration-dependent, showing the highest accumulation at 40 μmol L^{-1}. Almost no signal was visible at 350 μmol L^{-1}. They compared the temporal expression pattern of PR1 mRNA after BABA- and SA-treatment and deduced that BABA might act through a SA-independent pathway. Using tobacco, Cohen (1994) demonstrated that there was a difference in the induction of PRs depending on the method of BABA application to the plants. When the substance was sprayed on leaves (1 mM), immunoblots revealed accumulation of chitinase as well as PR1. Injection of 1 mL of a solution containing 10 mg of BABA into the stems led to an accumulation of chitinase but not of glucanase or PR1. As reported above, spraying, but not stem injection, under these conditions lead to micronecrosis, suggesting a correlation between the appearance of necrotic tissue and the accumulation of some PRs. Our own experience using Arabidopsis showed that BABA applied as a foliar spray even at low concentrations induced the accumulation of PR1, 2 and 5 mRNA, although the same concentrations applied as soil drench did not. Interestingly, both application methods led to an induction of resistance in the plants (unpublished results) suggesting that in this case expression of resistance is independent of PRs. We also observed that PR1 mRNA induction in Arabidopsis was highly dependent on environmental factors. BABA-treated plants acumulated PR1 mRNA faster and to a higher extent than untreated controls after heat-treatment (Figure 4a) or salt stress

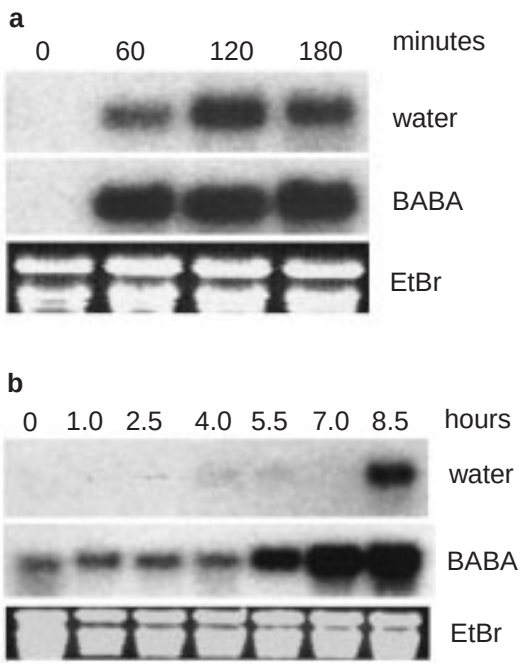

Figure 4. PR1 expression after abiotic stress. Effect of BABA treatment on the expression of PR1 during a mild heatshock (a) and a strong salt stress (b). (a) Plants were soil drenched with water or BABA (32 ppm) one day before their shift from 23 °C to 29 °C. Time points after heat-treatment are indicated on the top of the panel. (b) Plants were soil drenched with water or BABA (32 ppm) 2 days before their treatment with 250 mM NaCl in the soil. Time points after salt-treatment are indicated on the top of the panel. Total RNA was prepared and analyzed by RNA blot analysis with ^{32}P-radiolabeled probe. Ethidium bromide staining of the RNA gel (EtBr) was used to show equal loading.

(Figure 4b). Since sudden small changes in temperature or other stresses could pass unnoticed, they could account for the observed induction of PR genes in some BABA-treated plants, especially in not tightly controllable experiments performed under greenhouse conditions or in the field.

Whether induction of resistance by BABA is dependent on SA is also a topic which remains to be clarified. Using NahG tobacco plants, engineered to rapidly degrade SA to catechol (Gaffney et al., 1993), Siegrist et al. (2000) showed that it was no longer possible to induce resistance against TMV by BABA in these plants. Interestingly, the opposite is the case in NahG tobacco challenged with downy mildew, since there was no difference in protection by BABA between the NahG and wild type plants (Cohen et al., 2000). Our own investigations revealed that, in accordance with the above-mentioned observation, the requirement for SA in protection by BABA depended not on the plant species alone but also on the challenging pathogen. NahG Arabidopsis plants were very well be protected against *Peronospora parasitica* by BABA, whereas this protection totally failed when the plants were challenged with a virulent isolate of *Pseudomonas syringae* (Zimmerli et al., 2000). The independence of SA for the protection against *Peronospora* is probably due to the rapid papilla formation observed in BABA-treated plants (Zimmerli et al., 2000).

Dissection of the BABA pathway using Arabidopsis mutants

The striking ability of this simple amino acid to induce resistance in many plant–pathogen interactions incited us to take advantage of the model plant Arabidopsis and its numerous available mutants to try to elucidate the defense pathway(s) involved in BABA-induced resistance. In Arabidopsis, BABA has a distinct effect as a conditioner of plant defense mechanisms leading to a faster response to pathogen attack and resulting in a phenocopy of an incompatible interaction, i.e., HR, trailing necrosis and papilla formation (Zimmerli et al., 2000). To identify genes involved in this type of resistance, a screening system was set up in Arabidopsis. Two screening approaches were used: a classical system consisted of treating plants with BABA, infecting them with *P. parasitica* and screening for putative mutants that did not show induced resistance. This approach turned out to be laborious, very time-consuming and prone to external influences. A parallel screening system was based on the observation that higher doses of BABA induce sterility in Arabidopsis (Figure 5).

The observation that BABA-treated plants reacted with increased papilla formation after challenge with *P. parasitica* led to the hypothesis that it might somehow affect callose production in Arabidopsis, since callose is one of the main components of papillae (Aist, 1976). In addition, callose is also known to be involved in pollen/ovule interactions (De Martinis and Mariani, 1999) and in some cases there was a correlation between the degree of self-sterility and the amount of callose deposition (Kerhoas et al., 1983). In Arabidopsis, a higher and more rapid accumulation of callose in the ovules after BABA-treatment (data not shown) seems to be the reason for the observed (female) sterility of the flowers. The pollen itself is not affected since pollen from BABA-treated plants was successfully used to fertilize ovules of untreated plants; pollen from water-treated plants though was not able to pollinate BABA-treated plants (Mauch-Mani, unpublished results). The role of callose in BABA-induced sterility was also confirmed in experiments using 2-deoxy-D-glucose (DDG), an inhibitor of callose synthesis which had been used before in relation to sterility in *Brassica* (Singh and Paollillo, 1990). Treatment with DDG reversed the observed sterility in a dose-dependent manner as visualized by measuring the length of siliques and the number of seeds produced (Figure 5).

The involvement of callose in both sterility and resistance due to papilla formation was the basis for the second screen set up aimed at the identification of mutants which do not become sterile due to BABA-treatment. T-DNA tagged mutant seed lines (Feldman, 1992) were treated with 36 ppm (final concentration in the soil) of BABA starting four weeks after sowing. The plants were cultivated all the time under continuous light to accelerate flowering. One week after the onset of flowering the plants were screened for silique formation. Sterile plants were continuously torn out. The seeds of the remaining fertile plants were collected. Of the approx. 90,000 plants which were screened, a total of 15 putative BABA insensitive mutants (*bai* mutants) were rescued. They all showed formation of siliques after repeated treatments with BABA, whereas the wild type displayed sterility, visible as non-development of the siliques. Fifteen *bai* mutants have been tested for reaction to infection with *P. parasitica* after BABA-treatment and seven of them allowed growth of the pathogen. We are currently determining the DNA

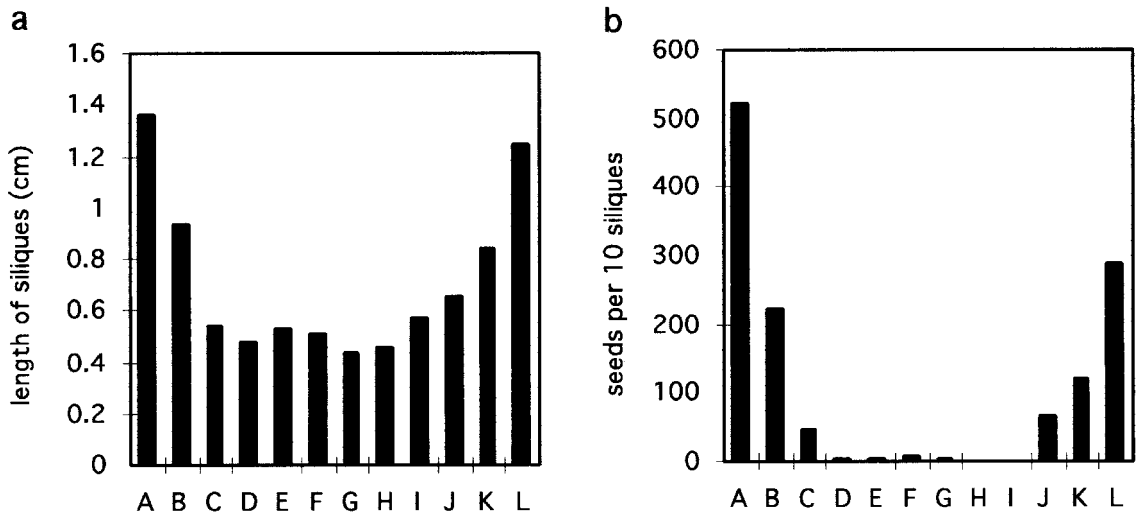

Figure 5. (a) Influence of BABA on silique length and its reversibility by DDG. (b) Influence of BABA on seed production and its reversibility by DDG. Both substances were applied as soil drench in the following concentrations: (A) H_2O, (B) BABA 24 ppm, (C) BABA 28 ppm, (D) BABA 32 ppm, (E) BABA 35 ppm, (F) BABA 40 ppm, (G) BABA 44 ppm, (H) BABA 48 ppm, (I) BABA 35 ppm + 0.5 mM DDG, (J) BABA 35 ppm + 1 mM DDG, (K) BABA 35 ppm + 2 mM DDG, (L) BABA 35 ppm + 5 mM DDG. Unfertilized pistils are about 4 mm long.

sequences flanking the insertion sites in order to be able to identify the mutated genes.

There is no doubt that BABA is a potent inducer of resistance in many plants species against a wide range of pathogens. Knowledge about the genes and mechanisms involved in BABA-mediated protection will allow development of new types of crop protectants which mimick the plants' own ways of defending themselves against pathogen attack.

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