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Ethylene Responsiveness of Soybean Cultivars Characterized by Leaf Senescence, Chitinase Induction and Nodulation

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Summary

A total of 161 cultivars of soybean (*Glycine max* L. Merr.) were tested for their responsiveness to ethylene treatments, using senescence of the primary leaves and the induction of chitinase activity in the roots as response markers. Cultivar «Gong jiao 6308-1» showed rapid chlorosis and the highest chitinase induction upon treatments with ethylene. The inducibility of chitinase by ethylene increased with increasing age of the plant. In addition, it was found that upon repeated ethylene treatments nodule formation of cultivar «Gong jiao 6308-1» was completely blocked in the lower part of the root system when inoculated with *Bradyrhizobium japonicum*. In several other cultivars, e.g. in «Bai tie jia qing», ethylene treatments did not induce leaf senescence or induction of chitinase activity. Cultivar «Bai tie jia qing» also showed completely normal nodulation even after repeated ethylene treatments. These results demonstrate that different soybean cultivars show a wide variation and strongly differ in their ethylene responsiveness.

Key words: Bradyrhizobium – Chitinase – Ethylene – Glycine max (cultivars) – Root nodules.

Introduction

Ethylene is a plant hormone that is often produced in response to abiotic stress factors and pathogen attack (Boller, 1991). Application of ethylene causes developmental and biochemical alterations, e.g. leaf chlorosis, senescence and abscission, and fruit ripening or the induction of hydrolases such as β-1,3-glucanase and chitinase, which are believed to play a role in plant defense (Boller, 1988; Osborne, 1991; Picton et al., 1993; Lanahan et al., 1994; Ecker, 1995).

Plant chitinases cleave chitin, a major component of most higher fungi and arthropods, and often possess lysozyme activity (Boller, 1988; Meins et al., 1992; Collinge et al., 1993). In addition, chitinases are able to cleave nodulation factors (Nod factors) of rhizobia (Staehelin et al., 1994 a, b), the lipo-chito oligosaccharide signals of symbiotic bacteria that fix atmospheric nitrogen in root or stem nodules of legumes (Caetano-Anollés and Gresshoff, 1991; Hirsch, 1992; Schultze et al., 1994). Chitinases often exist in multiple isoforms, and only specific chitinases are accumulated in response to ethylene. The DNA sequences responsible for ethylene regulation of chitinase have been analyzed for bean chitinase in transgenic tobacco and in bean protoplasts (Broglie et al., 1989; Roby et al., 1991).

Another effect of exogenous ethylene is the inhibition of the establishment of the *Rhizobium*-legume symbiosis (Goodlass and Smith, 1979; Lee and La Rue, 1992 a, b). Conversely, aminoethoxyvinylglycine, an inhibitor of the ethylene biosynthesis in plants, stimulates nodulation in some cases, indicating a possible role of ethylene in formation and autoregulation of nodules (Peters and Crist-Estes, 1989; Zaat et al., 1989; Guinel and La Rue, 1992).

In the present study we screened 161 soybean cultivars for their responsiveness to exogenous ethylene, using the induction of leaf senescence and the increase of chitinase activity in the roots as response parameters. We selected cultivars that clearly differed in these responses. In order to investigate the impact of exogenous ethylene and ethylene-induced chitinase activity on the *Bradyrhizobium*-legume interaction, two

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selected soybean cultivars differing in ethylene responsiveness were tested for their capacity to form nodules.

Material and Methods

Origin of plant material and surface sterilization of seeds

Seeds of 161 different cultivars of soybean (*Glycine max* L. Merr.) were kindly provided by Mr. Fei-jing Wang from the Chinese Academy of Agricultural Sciences (Beijing, People's Republic of China). Seeds were surface sterilized by treatment with H_2O_2 (purum, containing 30–31 % (w/v) H_2O_2, Fluka, Buchs, Switzerland) for 20 min, followed by repeated washing with sterilized tap water (3×1 min).

Plant growth conditions

In initial experiments, seeds of 11 soybean cultivars were planted in plastic pots (8 cm diameter, 150 mL volume) containing vermiculite (one seed per pot). Plants were grown in the greenhouse and watered daily with nutrient solution (Werner et al., 1975) containing 20 mmol/L nitrate for 3 weeks. After ethylene treatment (see below), roots were harvested and their fresh weight was determined and used for the chitinase assay. For a large screen, 150 cultivars were grown in trays containing vermiculite in the greenhouse. After 3 weeks, plants were treated with ethylene (see below). Cultivars exhibiting chlorosis or abscission of the primary leaves were selected and chitinase was measured in their root extracts. For the time course experiment, seeds of the cultivars «Bai tie jia qing» and «Gong jiao 6308-1» were grown in pots in the greenhouse as described above. Every week new seeds were planted. After 5 weeks, all plants were treated with ethylene, and chitinase activity was determined in the harvested roots.

Ethylene treatment

Two sets of plants raised in the same way were incubated for 48 h in two airtight transparent plastic chambers in the greenhouse. One of them received 100 nL · mL^{-1} ethylene, the other without ethylene served as a control. The addition of an ethylene-absorbing agent in the control chamber was omitted because neither leaf chlorosis nor chitinase induction were observed on control plants. For the nodulation experiment, the 48-h ethylene treatment was repeated weekly.

Nodulation assay

Surface sterilized seeds of the cultivars «Bai tie jia qing» and «Gong jiao 6308-1» were germinated on agar plates and grown in sterilized Leonard jars (one plant per jar) filled with perlite and nutrient solution in a phytotron as described previously (Leonard, 1943; Staehelin et al., 1992). Two weeks later plants were treated with ethylene as described above. Thereafter, plants were inoculated with 5 mL of a suspension of *Bradyrhizobium japonicum* 61-A-101 (Stripf and Werner, 1978) grown to stationary phase in 20E-medium (Werner et al., 1975) at 27 °C on a rotary shaker at 140 rpm. Plants were treated weekly with ethylene for 48 h and harvested 4 weeks after inoculation. The dry weight of stem, root and nodules was determined after lyophilisation.

Extraction of root material and chitinase assay

Roots were extracted with mortar and pestle in cold 0.1 mol/L phosphate (Na^+) buffer, pH 7 (4 mL per g fresh weight). The extracts were centrifuged at 15,000 g_n for 15 min, and the supernatants assayed for chitinase activity at pH 5 using [^{3}H]chitin as substrate (Boller et al., 1983). One nkat was defined as the activity that catalyzes the release of soluble chito-oligosaccharides corresponding to 1 nmol *N*-acetylglucosamine in 1 s at infinite dilution (Boller et al., 1983). Where indicated, various amounts of an antiserum against bean chitinase (Vögeli et al., 1988) were added until maximum inhibition was reached; preimmune serum served as a control.

Results

Chitinase activities were compared in root samples of 11 different soybean cultivars, incubated in airtight containers with and without ethylene for 48 h. Chitinase activity in roots of plants incubated without ethylene was lower than 2 nkat per g fresh weight for all tested cultivars. The pattern of chitinase activity of ethylene-treated soybeans differed depending on the cultivar used in this experiment: Some cultivars clearly showed an induction of chitinase, while ethylene had no effect in other cultivars, for example «Bai tie jia qing» (Table 1). This cultivar was selected as a potentially ethylene-insensitive soybean cultivar and characterized in further studies.

In order to screen soybean cultivars with strong responses to ethylene, we made use of the observation that ethylene-sensitive cultivars responded to the exogenously applied hormone not only with chitinase induction but also with strong chlorosis of the primary leaves. This alteration, indicative of senescence of the leaves, was visible after incubation in the ethylene-containing chambers and was usually followed by leaf abscission. From 150 different cultivars treated with exogenous ethylene, 56 cultivars responding with leaf chlorosis or abscission were selected, and chitinase activity was determined in extracts of harvested roots (Table 2). Cultivar «Gong jiao 6308-1» showed highest chitinase activity and was selected as an ethylene-sensitive cultivar for further studies. Similarly, chitinase activities of more than 10 nkat per g fresh weight were measured for the cultivars «Dong liao xiao jin

Table 1: Chitinase activity in roots of 3-week-old soybean cultivars after treatment in chambers with or without ethylene.

Cultivar	Chitinase activity[a] nkat (g FW)$^{-1}$		Factor of induction by ethylene
	no ethylene[b]	ethylene[b]	
Bai tie jia qing	1.73±0.7	1.68±0.5	1.0
Da zi hua	1.25±0.5	1.39±0.3	1.1
Ai hui ben di zhong	1.11±0.5	1.68±1.1	1.5
Bai tie jia	1.07±0.2	1.78±0.8	1.7
Bei feng 2 hao	0.84±0.2	1.70±0.1	2.0
Bai qi kuai dou	1.90±0.5	3.98±1.3	2.1
Bai pi dou	1.21±0.1	2.79±1.9	2.3
Dong da li	1.65±0.5	4.69±1.0	2.8
Bai pi zi	1.58±0.4	4.98±1.1	3.2
An da 37-1	0.87±0.3	3.32±1.6	3.8
An tu bai hua lu da dou	1.07±0.4	4.07±1.2	3.8

[a] Means and SD for three independent crude homogenates.
[b] Plants were treated in airtight chambers with or without 100 nL · mL^{-1} ethylene for 48 h.

Table 2: Chitinase activity in roots of ethylene-treated soybean cultivars that showed leaf senescence. One hundred and fifty different cultivars were grown in trays in the greenhouse for 3 weeks. A total of 56 cultivars were selected that responded to ethylene treatment with chlorosis of the primary leaves. Chitinase activity was measured in roots of these selected cultivars.

Chitinase activity <4 nkat·g (FW)$^{-1}$	Chitinase activity 4–10 nkat·g (FW)$^{-1}$	Chitinase activity >10 nkat·g (FW)$^{-1}$
9 cultivars	43 cultivars	4 cultivars: Hei qi huang da dou Dong liao xiao jin huang Fu song you xian bai hua Gong jiao 6308-1[a]

[a] Cultivar Gong jiao 6380-1 showed highest chitinase activity and was selected for further studies.

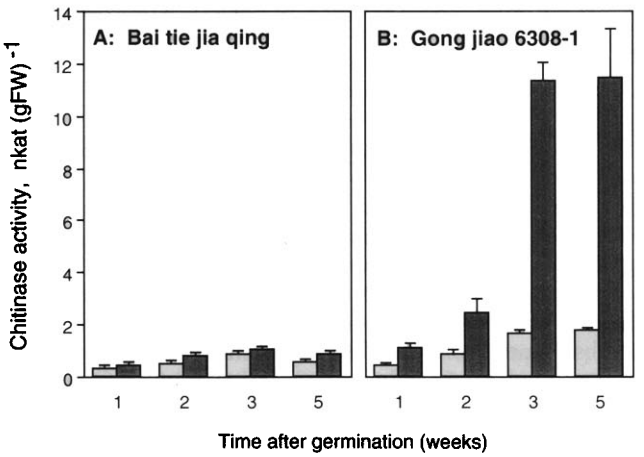

Fig. 1: Chitinase activity in roots of soybean cultivars «Bai tie jia qing» (A) and «Gong jiao 6308-1» (B) grown for different times in the greenhouse. Chitinase activity was determined in crude root homogenates of plants kept for 48 h in airtight chambers without ethylene (open columns) or with 100 nL · mL^{-1} ethylene (shaded columns). Data represent mean and SD of three independent extracts.

huang», «Fu song you xian bai hua» and «Hei qi huang da dou» (Table 2).

In order to characterize the ethylene-responsiveness in roots in more detail, chitinase activity was measured during root development for the cultivar «Bai tie jia qing» and for the cultivar «Gong jiao 6308-1» (Fig. 1). Chitinase activity in roots of mock-treated plants was lower than 2 nkat per g fresh weight for both cultivars and only increased slightly during the first weeks. Chitinase induction by ethylene depended on the age of the plants. Induction was low in young roots of cv. «Gong jiao 6308-1» but strongly increased in plants that were at least 3 weeks old. Cultivar «Bai tie jia qing» showed no differences between the chitinase activity of ethylene-treated and mock-treated plants during all tested stages of root growth.

The influence of ethylene on soybean chitinase was further studied using an antiserum against ethylene-induced bean chitinase (Fig. 2). Chitinase activity of mock-treated roots was only weakly inhibited by the antiserum. However, when ethylene-treated root extracts of the cultivar «Gong jiao 6308-1» were incubated in the presence of various amounts of antiserum, the chitinase activity decreased with increasing amounts of added antiserum and reached a plateau (data not shown). Preimmune serum showed no inhibitory effect. These data indicate that roots of «Gong jiao 6308-1» contain two antigenically different chitinase isoenzymes that are differentially stimulated by ethylene.

The effect of an ethylene treatment on nodulation was tested for the selected cultivars «Bai tie jia qing» and «Gong jiao 6308-1». Plants were inoculated with *Bradyrhizobium japonicum* 61-A-101 3 weeks after germination, the age of highest ethylene-responsiveness with respect to chitinase induction. In order to prevent the formation of roots that do not show ethylene-induced alterations, ethylene treatment was repeated weekly for 4 weeks. As shown in Table 3, the selected cultivars differ in their symbiosis capacity. Nodule formation of the ethylene sensitive cultivar «Gong jiao 6308-1» was not affected in the roots that developed first in the upper part of the Leonard jar but it was completely blocked in the younger parts of the root system that developed in the lower part of the Leonard jar. In contrast, nodulation in cultivar «Bai tie jia qing» was not influenced by ethylene. Both the

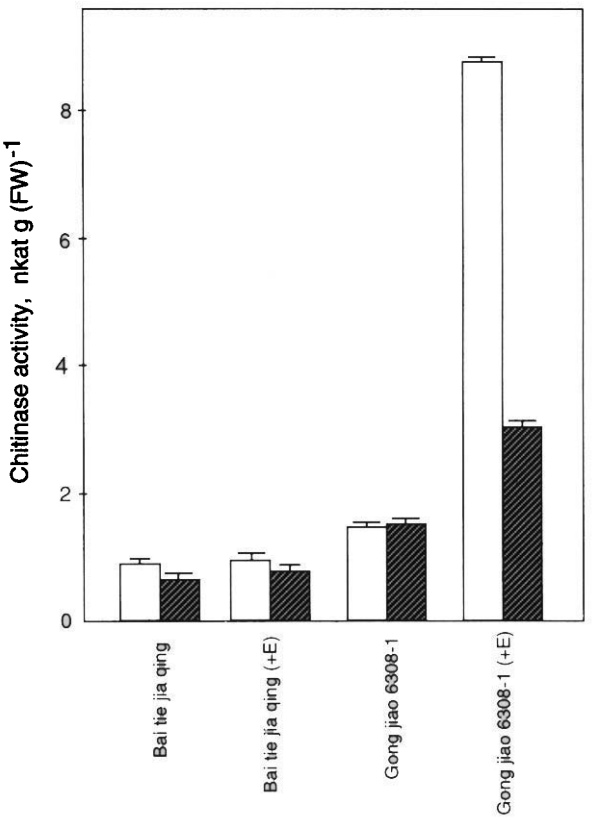

Fig. 2: Effect of an antiserum raised against ethylene-induced bean chitinase on chitinase activity in soybean roots. Chitinase activity was assayed in crude root homogenates of 3-week-old soybean plants kept for 48 h in airtight chambers without ethylene (−E) or with 100 nL · mL^{-1} ethylene (+E). Activities of extracts incubated without antiserum (open columns) or with an amount of antiserum yielding maximal inhibition (shaded columns) are indicated.

Table 3: Effect of ethylene on nodulation of two selected soybean cultivars. Plants were harvested 4 weeks after inoculation with *Bradyrhizobium japonicum*. Values indicate average and standard deviation of nodues from three plants each grown separately in a Leonard jar.

	Number of nodules	
	– Ethylene[a]	+ Ethylene[a]
Bai tie jia qing		
upper root system[b]	106±51 NS[d]	140±48 NS[d]
lower root system[c]	11± 9 NS[d]	11±18 NS[d]
Gong jiao 6308-1		
upper root system[b]	73±26 NS[d]	99±87 NS[d]
lower root system[c]	129±87 S[d]	0± 0 S[d]

[a] Two-week-old plants were incubated for 48 h in airtight chambers without or with 100 $nL \cdot mL^{-1}$ ethylene. Thereafter, plants were infected with *Bradyrhizobium japonicum*. The same ethylene treatment was repeated weekly until plants were harvested.

[b] Nodules were harvested from the root system above the plug of the Leonard jar.

[c] Nodules were harvested from the root system below the plug of the Leonard jar.

[d] NS, not significantly different; S, significantly different between control and ethylene-treatment (t-test, $p<0.0025$).

upper and lower root systems showed normal nodulation after ethylene treatment. The data indicate that inhibiton of nodulation by ethylene in soybean is cultivar specific.

Discussion

Leaf senescence and chitinase induction by ethylene were found to be a convenient tool to characterize the diversity of soybean cultivars from different origin and to select soybean cultivars that differ in their responsiveness to ethylene. Cultivar «Gong jiao 6308-1» was selected as the one with the strongest chitinase induction in the roots, comparable with the induction of chitinase in leaves and roots of other legumes after ethylene treatment (Boller et al., 1983; Ishige et al., 1991; Vad et al., 1991; Staehelin et al., 1994 a, b). However, the time course experiment demonstrated that ethylene-dependent induction of chitinase is developmentally regulated. According to the immunological characterization, two chitinase isoenzymes are strongly induced by ethylene in older roots, and one of them resembles the ethylene-induced chitinase of bean leaves (Boller et al., 1983; Vögeli et al., 1988) and the main chitinase present in soybean nodules (Staehelin et al., 1992). Moreover, cultivar «Gong jiao 6308-1» inoculated with *B. japonicum* was also characterized by complete supression of nodulation in the lower root part of the root system after repeated ethylene treatments (Table 3). Conversely, cultivar «Bai tie jia qing» treated with ethylene showed neither leaf chlorosis nor chitinase induction in the roots during the observed period. In addition, nodule formation of cultivar «Bai tie jia qing» was not affected by ethylene. We therefore conclude that this cultivar is insensitive to ethylene at least with respect to the parameters investigated, similar to certain mutants of *Arabidopsis* that did not show leaf chlorosis after mutagenesis in the presence of exogenous ethylene (Bleecker et al., 1988; Guzmàn and Ecker, 1990). Recently it has been shown that protein phosphorylation and protein kinases are involved in perception or transduction of the ethylene signal (Kieber et al., 1993; Raz and Fluhr, 1993; Ecker, 1995). It remains to be investigated whether ethylene perception or a step in the signal transduction pathway is supressed in the ethylene-insensitive soybean cultivar «Bai tie jia qing».

When compared with other legumes (Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and La Rue, 1992 a, b), inhibition of nodule formation in soybean by exogenous ethylene is less strong (Lee and La Rue, 1992 b; Hunter, 1993) and cultivar-specific (Table 3). It is not known yet how ethylene can block nodulation, but it is tempting to speculate that ethylene-induced chitinases may play a role in nodule inhibition. Specific chitinases are able to cleave differently substituted Nod factors of rhizobia, and they may thereby determine their biological activities on the host plant (Hunter, 1993; Staehelin et al., 1994 a, b). However in pea roots, exogenous ethylene appears to inhibit cell division and hence the formation of a nodule primordium but not earlier events, such as the formation of infection threads in the epidermis (Lee and La Rue, 1992 b). This indicates that in these plants, ethylene affects the later stages of nodule develoment, during which a regulatory role of Nod factors has not yet been established. It remains to be seen whether ethylene blocks nodulation in sensitive plants through alterations of specific hydrolase activities or through other mechanisms.

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