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Gene expression and insect resistance in transgenic broccoli containing a *Bacillus thuringiensis cry1Ab* gene with the chemically inducible PR-1a promoter

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Abstract

We produced 49 broccoli plants (*Brassica oleracea* L. ssp. *italica*) containing a *Bacillus thuringiensis cry1Ab* gene under control of the chemically inducible PR-1a promoter from tobacco. Most of them showed substantial or complete control of neonate diamondback moth larvae, regardless of whether the transgene was induced or not. Ten plants were selected for detailed study via northern and western analysis and insect bioassays. They expressed the *cry1Ab* gene and gave complete insect control when treated with the chemical inducers INA (2,6-dichloroisonicotinic acid) or BTH (1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester); however, leaves treated with water alone were also partially or completely protected from insect damage. Transgenic progeny plants showed greater inducibility than primary transformants at the molecular level. Two progeny lines produced *cry1Ab* mRNA and Cry1Ab protein and gave insect control only after induction, both when detached leaves and intact plants were tested. The relevance of these results to resistance management strategies is discussed.

Introduction

The development of transgenic crops expressing endotoxins from *Bacillus thuringiensis* (Bt) has revolutionized our ability to control some insect pests. The bacterial endotoxin in the Bt-transgenic plants is highly toxic to certain pests, yet has little or no effect on humans, most beneficial insects, and other non-target organisms (Anonymous 1990). The expression of Bt endotoxins in plants provides a remarkable level of insect control against the major lepidopteran pests of corn and cotton and against the Colorado potato beetle in potatoes. Additional advantages of Bt crops include more effective pest exposure together with reduced exposure of farm workers to insecticides, elimination of pesticide application costs, and reduced environmental impact from pesticide use (Anonymous 1990).

Although the acreage of Bt-transgenic crops has increased dramatically in recent years, there are concerns that sustained efficacy of Bt crops will be endangered by the development of resistance in populations of insect pests that they are intended to control (Roush and Shelton 1997). To date, several major pest species, including *Heliothis virescens*, *Leptinotarsa decemlineata*, and *Plodia interpunctella*, have demonstrated the ability to adapt to Bt in the laboratory (Tabashrik 1994). Moreover, *Plutella xylostella* (diamondback moth, DBM) has evolved sufficiently high resistance to Cry1A proteins in field settings to result in field control failures (Shelton et al. 1993; Perez and Shelton 1996; Tang et al. 1997). Such evidence of insect resistance to Bt proteins emphasizes the need to develop effective resistance management strategies.

Several strategies for limiting the development of resistant insect populations and prolonging the utility

of Bt have been proposed (McGaughey and Whalon 1992; Roush 1997). The only approach presently commercially available is a high dose of a single Bt gene in conjunction with refuges of non-Bt plants that can serve to maintain Bt-susceptible alleles of the insect. We have generated high expressing *cry1Ac*-transgenic broccoli plants that showed complete control of susceptible DBM (Metz et al. 1995a, b) and have used these plants in greenhouse and field studies to assess factors influencing the development of insect resistance (Shelton et al. 2000; Tang et al. 2001).

Several types of refuges from exposure to the Bt toxin can help conserve susceptible alleles. Spatial refuges can be created by planting transgenic plants near non-transgenic ones (the current refuge approach) or by expressing the toxin only in certain organs of a crop plant. Temporal refuges can be produced by triggering expression of the toxin in response to an internal developmental signal or an external environmental signal. To assess the value of temporal refuges controlled by an environmental cue, we produced transgenic broccoli carrying a *cry1Ab* Bt gene controlled by the pathogenesis-induced PR-1a promoter of tobacco. Transgenic tobacco with this promoter-gene combination accumulated *cry1Ab* mRNA and became tolerant to tobacco hornworm larvae after chemical induction (Williams et al. 1992). In this report, we describe the expression patterns and control of DBM in PR-1a/*cry1Ab*-transgenic broccoli and discuss the possible use of chemically inducible promoters for insect resistance management.

Methods and materials

Binary vector carrying the PR-1a/cry1Ab expression cassette

A synthetic truncated *cry1Ab* gene (U.S. Patent 5,625,136) with the PR-1a promoter, kindly provided by Syngenta Crop Protection (Research Triangle Park, NC), was used. The sequence of the *cry1Ab* gene was modified from the one used for study of chemical induction of Cry1Ab protein and insect control in tobacco (Williams et al. 1992). The PR-1a promoter is inducible by chemicals such as salicylic acid, 2,6-dichloro-iso-nicotinic acid (INA), and 1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester (BTH). A 3.4 kb *Not*I and *Sst*I fragment carrying the *cry1Ab* gene with the PR-1a promoter and 35S terminator was cloned between *Not*I and *Sst*I sites of a binary vector to generate the plasmid pJC12b (Figure 1). The binary vector pJC12b also contained the *nptII* gene flanked by the nopaline synthase promoter and terminator as a selectable marker for kanamycin resistance.

Broccoli transformation

Vector pJC12b was introduced into *Agrobacterium tumefaciens* strain ABI and used to transform hypocotyl and petiole explants of broccoli (*Brassica oleracea* ssp. *italica*, Green Comet hybrid), using protocols described by Metz *et al.* (1995a). Primary transformants (T_0 plants) were selfed by bud pollination to obtain T_1 progeny. T_1 seeds were germinated on MS medium (Murashige and Skoog 1962) containing kanamycin (125 mg/l) and no growth regulators to determine the segregation of kanamycin resistant (green) and susceptible (white) plants. In some cases, small leaf pieces from seedlings grown in soil were surface-sterilized and placed on MS medium containing kanamycin (50 mg/l), BA (5 mg/l), NAA (0.5 mg/l) and 5 mM arginine and scored for callus and shoot formation three weeks later.

Chemical induction

Two chemical inducers dissolved in water were used. In initial experiments, INA (provided by Dr Terry Delaney, Cornell University, as a 25% wettable powder) was used at 0.125 mg/ml. Later BTH became available from Syngenta Crop Protection as a 50% wettable powder (CGA-245,704); it was used at 0.625 mg/ml. INA and BTH gave comparable results in direct comparisons. For induction of detached leaves, two young leaves were collected from small plants transferred out of culture into soil at least five weeks before the assay. One leaf was dipped in the chemical inducer and the other in water. Leaves were brushed with a paintbrush to increase contact of the solutions with the leaves and placed individually in baby food jars containing 1% water agar. For analysis of intact plants, three leaves on each of three soil-grown T_1 plants of each line plus untransformed control plants were brushed with the chemical inducer or water. Detached leaves or whole plants with treated leaves were maintained at 25 °C under a 16/8 light/dark regime for two days before use for RNA and protein extractions or insect bioassays.

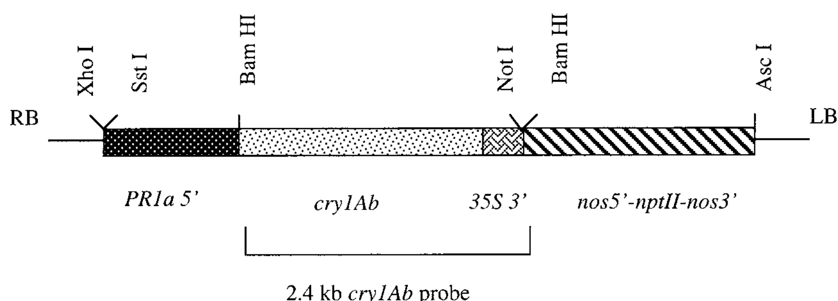

Figure 1. The binary vector pJC12b used in broccoli transformation. Expression of the *cry1Ab* gene is regulated by the PR-1a promoter and terminated by the polyadenylation sequence from the CaMV 35S RNA gene. The nopaline synthase promoter (*nos 5′*)/*nptII*/nopaline synthase terminator (*nos 3′*) expression cassette allows selection on kanamycin.

Polymerase chain reaction (PCR) and DNA dot blot analysis

Total cellular DNA was isolated from leaf tissue as previously described (Cao et al. 1999). PCR analysis of genomic DNA was carried out with *Taq* polymerase (Boehringer-Mannheim) using primers specific to the *nptII* gene. The primer sequences used were as follows: forward primer: 5′-TCGGCTATGACTGGGCACAACAGA-3′; reverse primer: 5′-AAGAAGGCGATAGAAGGCGATGCG-3′. Dot blot analysis was performed by spotting 1 μg of genomic DNA per dot onto a nylon membrane placed in a manifold (Schleicher & Schuell). Blots were probed with a ^{32}P-labeled *Bam*HI-cleaved 2.4 kb DNA fragment covering the entire coding region of the *cry1Ab* gene (Figure 1).

RNA gel blot analysis

Two days after chemical induction, total RNAs were purified from frozen leaf tissues by phenol/chloroform extraction followed by lithium chloride precipitation as previously described (Cao et al. 1997, 1999). Northern analysis of the RNA samples was carried out following the protocol of Sambrook et al. (1989). Blots were probed with a mixture of the 2.4 kb *Bam*HI fragment (carrying the entire *cry1Ab* gene plus the 35S 3′-untranslated polyadenylation sequence) and a 1.5 kb actin gene from *Brassica*.

Immunoblotting and ELISA

Proteins were extracted from leaf tissues in a buffer solution (10 mM Tris-HCl, 5 mM EDTA, 10% glycerol). Soluble protein concentrations were determined according to the BioRad protein assay (catalog No. 5000-0006). For immunoblots, proteins were electrophoretically transferred from SDS gels (10% polyacrylamide) onto nitrocellulose membranes as previously described (Cao et al. 1999). Immunoblotting was performed according to the ECL western blotting protocols (Amersham Life Science). The primary antibody was raised in goat against an *Escherichia coli*-derived truncated Cry1Ab protein carrying 645 N-terminal amino acids (GenBank accession number M15271) and diluted at 1:1000 for probing. ELISA was performed according to the manufacturers' instructions with a Cry1Ab/Cry1Ac kit (Envirologix). Sample extracts were used at a dilution of 1:11. The OD values of samples were measured by a microplate reader set at 450 nm. The amount of Cry1Ab protein produced after the induction with BTH was calculated from standard curves obtained with Cry1Ab protein (EnviroLogix). The assay detected as little as 0.25 ng Cry1Ab protein per gram fresh leaf tissue. The OD value of the untransformed Green Comet control was subtracted before determining the concentration of Cry1Ab protein.

Insects

Our colony of *Plutella xylostella* L. was collected in 1988 from cabbage at the New York State Agricultural Experiment Station, Robbins Farm, Geneva, NY, and then maintained on a wheat germ-casein artificial diet (Shelton et al. 1991). The colony was kept in an environmental chamber at 27 ± 1 °C, 35 ± 2% RH, and photoperiod of 16/8 h (light/dark). Prior to tests of second instar larvae on plants, eggs were reared on oilseed rape plants (*Brassica napus* L. subsp. *oleifera* cv. Dwarf Essex) in the greenhouse at 26–33 °C with uncontrolled RH, and photoperiod of 16/8 h (light/dark).

Insect bioassays

Two days after induction, detached leaf bioassays with neonate and second instar DBM larvae were conducted as described by Cao et al. (1999). For assays with neonates, a piece of aluminum foil containing about 20 DBM eggs was placed on the surface of the leaves. For assays with second instar larvae, four larvae were placed on each of two detached leaves from a T_1 transgenic plant of each line. In assays with intact T_1 plants, 5 second instar larvae were placed on three treated leaves from three plants of each line (total of 9 leaves per line) two days after treatment with BTH. The plants were maintained at 25 °C under a 16/8 h light/dark regime. Leaf damage (visual estimate) and larval mortality were scored after 5–6 days.

Results

Regeneration of transgenic plants

Hypocotyl and petiole explants were transformed with *A. tumefaciens* harboring a binary plasmid bearing a kanamycin resistance marker and the PR-1a/*cry1Ab* fusion gene. From about 800 explants 72 independent kanamycin-resistant plantlets were regenerated. Of these putative transformants, 49 (68%) were confirmed as transgenic by PCR with primers specific to the *nptII* gene and further by dot blot using the *cry1Ab* gene as a probe (data not shown). The overall transformation efficiency was 6.1%.

Analysis of primary transformants

To test whether T_0 plants treated with a chemical inducer were preferentially protected from insect attack, leaves of 45 transformants were treated with INA or water for two days before infestation with neonate DBM. Three transformants behaved like the controls, with 70–80% defoliation and no insect mortality, regardless of whether leaves were treated with INA or water. The others were protected from insect attack after treatment with either INA or water. Most leaves showed little or no damage (0–5%) in either case. Three plants (60, 67, 88) showed 10–20% defoliation without induction.

To confirm these initial results, 10 transgenic lines, including 60, 67, and 88, were further assayed using BTH as the chemical inducer. DBM neonates fed voraciously on untransformed leaves treated with either BTH or water (Figure 2a). These leaves suffered 70–80% damage, and the insects grew to the second instar stage by day 6 after hatching. In contrast, DBM placed on transgenic leaves treated with BTH stopped feeding and caused little or no damage (Table 1). However, transgenic leaves treated only with water also showed protection from insect damage, to various degrees (Table 1). Five plants (30, 28, 23, 24, 27) showed very little leaf damage ($\leq$ 5%) after treatment with water and caused mortality of neonates within two to three days. Four others (67, 88, 7, 72) had up to 20% defoliation without induction and caused 100% insect mortality by day six. Plant 60 showed the greatest defoliation (35%) without induction (Table 1, Figure 2a). Growth of neonates was severely inhibited on detached leaves of plant 60 although some larvae survived up to 6 days. The fact that transgenic leaves treated only with water showed partial or complete protection from insect attack indicates that the regulation of *cry1Ab* expression by the PR-1a promoter was 'leaky' in the primary transformants. These plants apparently produced sufficient Cry1Ab protein to provide insecticidal activity even without chemical induction.

Selected primary transformants were also subjected to RNA and protein analyses. The expected RNA species were detected in plants treated with BTH and to a lesser and variable extent in those treated with water (Figure 3a). Accumulation of steady-state *cry1Ab* mRNA was correlated with the production of Cry1Ab protein. Western blot analysis demonstrated the presence of the expected 79 kDa Cry1Ab protein in all BTH-treated T_0 plants although the amount of the protein varied (Figure 4a). Variable amounts of Cry1Ab protein were also detected in transgenic leaves treated with water. Plant 60, which suffered the greatest insect damage when treated with water, produced only a very low level of Cry1Ab protein without induction. No mRNA or protein was detected in untransformed control Green Comet samples treated with BTH or water.

Analysis of T_1 progeny

To identify transgenic progeny and determine the genetic behavior of the transgene, T_1 seeds were germinated on medium containing kanamycin and scored for the number of green or white seedlings. Alternatively, leaf pieces from soil-grown T_1 plants were scored for callus formation on medium containing kanamycin. Nine of the 10 lines tested exhibited a segregation ratio close to 3:1, suggesting that the *nptII*

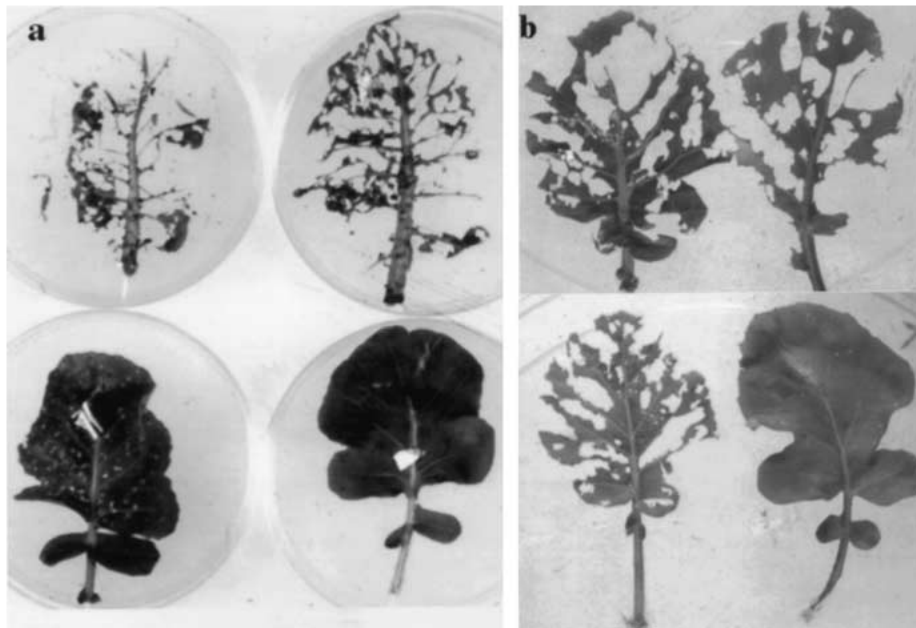

Figure 2. Insect bioassay of leaves of T_0 and T_1 *cry1Ab* broccoli lines in response to chemical induction. a. Leaves of T_0 plants. b. Leaves of T_1 plants. Top, leaves from an untransformed control plant. Bottom, leaves from transgenic line 60. Left, leaves treated with water. Right, leaves treated with BTH.

Table 1. Control of diamondback moths in T_0 and T_1 broccoli plants. Neonate DBM larvae were placed on detached leaves of T_0 or T_1 transgenic broccoli carrying a *cry1Ab* gene under control of the PR-1a promoter, with and without induction with BTH. Leaf damage and insect mortality were scored 6 days later.

Line	% defoliation caused by larvae				% mortality of larvae	
	T_0 plants		T_1 plants		T_1 plants	
	BTH	water	BTH	water	BTH	water
Green Comet (control)	70	75	80	80	15	21
60	0	35	0	75	100	29
67	0	10	0	70	100	32
88	<5	20	0	55	100	39[a]
30	0	5	0	50	100	43[a]
28	0	<5	0	35	100	75
7	0	5–10	0	20	100	100
23	0	5	0	0	100	100
24	0	0	0	5	100	100
27	0	<5	0	5–10	100	100
72	0	15	0	15	100	100

[a]The surviving larvae showed poor growth.

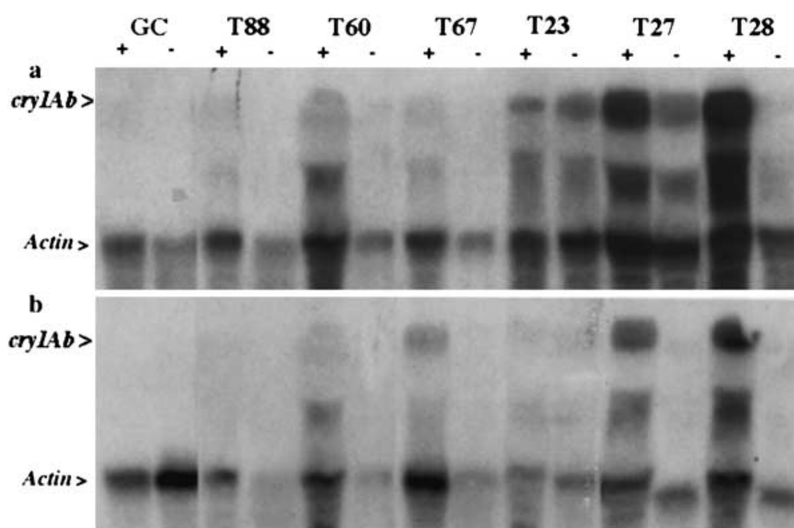

Figure 3. Accumulation of *cry1Ab* mRNA in T_0 and T_1 transgenic broccoli. a. T_0 plants. b. T_1 plants. 20 μg of total leaf RNA per sample was subjected to RNA blot analysis as described in Materials and methods with ^{32}P-labelled *cry1Ab* DNA as the probe. The transgenic line is indicated above the lanes with: +, induced with BTH, and −, treated with water. As a negative control, 20 μg of total RNA from untransformed Green Comet (GC) broccoli was loaded.

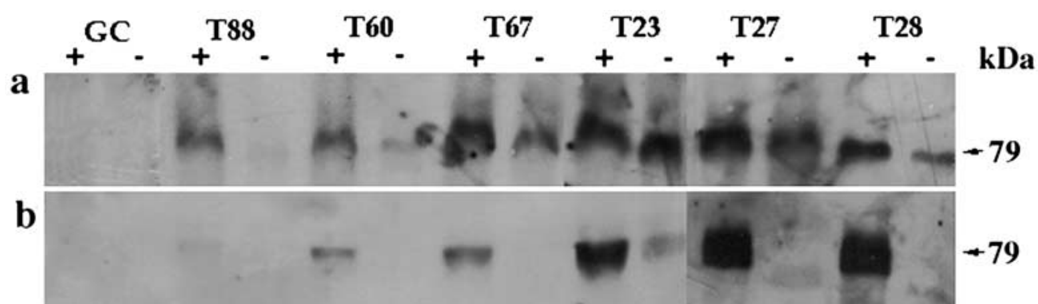

Figure 4. Production of Cry1Ab protein in T_0 and T_1 transgenic broccoli. a. T_0 plants. b. T_1 plants. 100 μg of total soluble leaf proteins per sample was subjected to western blot analysis. The transgenic line is indicated above the lanes with: +, induced with BTH and −, treated with water. As a negative control, 100 μg of soluble proteins from untransformed Green Comet (GC) broccoli was loaded.

gene was integrated into a single locus. Line 60 had a ratio of 15:1, indicating two unlinked loci. An additional line (73) tested later also segregated as a single locus. Southern dot blot analysis confirmed that kanamycin-resistant T_1 plants carried the *cry1Ab* gene as well as the *nptII* gene (data not shown).

To determine whether the leakiness of the PR-1a promoter in the absence of chemical induction was restricted to the primary transformants, T_1 transgenic plants were used in insect bioassays and northern and western analyses. Six days after infestation, extensive feeding damage was observed on untransformed leaves treated with either BTH or water. Actively feeding larvae were visible on the damaged leaves with little mortality (Figure 2b, Table 1). Transgenic leaves treated with BTH showed little or no feeding damage, and all caused 100% mortality of neonates. In comparison to the parental T_0 lines, some uninduced T_1 lines showed increased susceptibility to insect damage (Table 1). Water-treated leaves from two lines (60, 67) were similar to controls in suffering severe leaf damage and allowing survival and vigorous growth of most larvae. Others (88, 30, 28) suffered moderate leaf damage without induction. Although about 60% of the neonates survived until day six on uninduced leaves of lines 88 and 30, their growth and development were severely inhibited, and their size remained essentially unchanged. T_1 leaves from five other lines (7, 23, 24, 27, 72) showed only 0–20% leaf damage without induction and caused 100% larval mortality. The same patterns of chemical induction of different lines were observed when older leaves in later developmen-

Table 2. Control of older diamondback moth larvae by T_1 broccoli plants. Second instar larvae were placed on detached leaves of T_1 transgenic broccoli carrying a *cry1Ab* gene under control of the PR-1a promoter, with and without induction with BTH. Leaf damage and insect mortality were scored 5 days later.

Line	% defoliation		% mortality of larvae	
	BTH	water	BTH	water
Green Comet	80	80	0	0
60	0	75–80	100	0
67	0	70–80	100	12
88	0	45–50	100	87[a]
30	0	40	100	87[a]
28	0	<5	100	100
7	0	<5	100	100
23	0	0	100	100

[a]The surviving larvae showed poor growth.

tal stages were used (data not shown). Induction also occurred when leaves were sprayed with BTH rather than dipped into BTH solution and brushed (data not shown).

Similar results were seen when second instar larvae were applied to detached leaves of T_1 plants with or without induction (Table 2). Lines 60 and 67 showed insect control only when induced, while the other lines showed less leaf damage than controls and very high mortality of the larvae even when treated only with water.

To confirm that the insect control of lines 60 and 67 was inducible at the plant level, leaves on intact T_1 plants of these lines plus control plants were infested with second instar larvae. Five days later water-treated leaves of lines 60 and 67 were severely damaged; the larvae had advanced to the fourth instar, and some of them had pupated (Figure 5). In contrast, leaves of lines 60 and 67 treated with BTH showed little or no feeding damage (Figure 5) and produced 100% larval mortality.

In molecular assays, detached T_1 leaves treated with BTH showed strong induction, with increased accumulation of steady-state *cry1Ab* mRNA and production of Cry1Ab protein (Figures 3b and 4b). In contrast to their parental T_0 plants, water-treated T_1 lines showed either no accumulation or greatly reduced levels of steady-state *cry1Ab* mRNA or Cry1Ab protein (Figures 3b and 4b). This is clearly evident in the western analysis of T_1 lines (Figure 4b). Northern analysis of leaves from intact plants of lines 60 and 67 further confirmed that *cry1Ab* mRNA was induced only after treatment with BTH (data not shown).

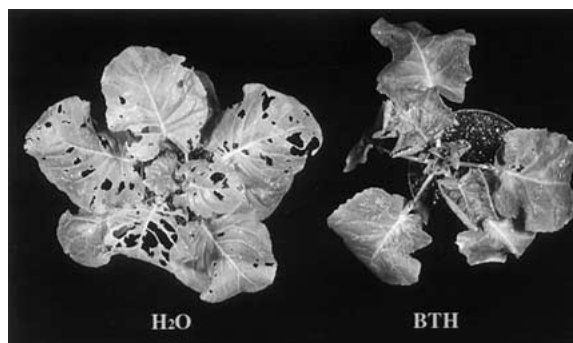

Figure 5. Bioassay of T_1 plants of broccoli line 60 treated with BTH or water and infested with second instar DBM larvae for five days.

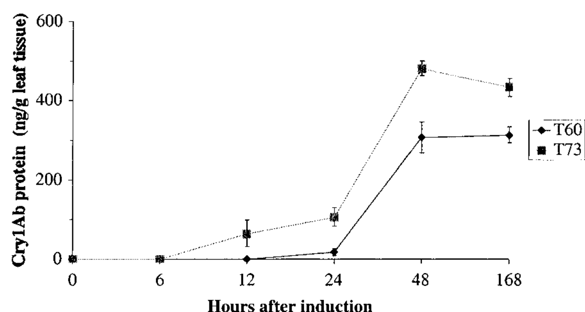

Figure 6. Time course of production of Cry1Ab protein in leaves of broccoli lines 60 and 73 after treatment with BTH, as determined by ELISA assay. Two T_1 plants of each line were used, and three assays were done on proteins from each plant at each time point.

To determine how soon after induction Cry1Ab protein appears in PR-1a/*cry1Ab* plants, ELISA was performed with proteins isolated from lines 60 and 73 at various time intervals after treatment with BTH. Two plants of each line were used, and three assays were done on leaf proteins from each plant. As shown in Figure 6, no Cry1Ab protein was detected before induction or at 6 h after BTH treatment (the assay is capable of detecting as little as 0.25 ng protein per gram leaf tissue). Cry1Ab protein was seen by 12 h in line 73 and by 24 h in line 60. The level of Cry1Ab protein reached the maximum level about 2 days after induction. In a separate experiment, western blot was performed with proteins isolated from T_1 line 60 at prolonged time intervals after treatment with BTH. Similar high levels of Cry1Ab protein were present from 2 days to 3 weeks, and the protein was present even one month after the application of BTH (Figure 7).

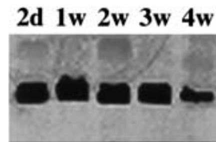

Figure 7. Presence of Cry1Ab protein at prolonged time periods after treatment with BTH. Soluble proteins were isolated from leaf tissues of a T_1 plant of line 60, 2 days and 1–4 weeks after treatment with BTH. 100 μg of soluble proteins was loaded.

Discussion

We have generated a population of transgenic broccoli plants carrying the *cry1Ab* gene under the control of the chemically inducible promoter of the tobacco *PR-1a* gene. The two chemicals we used (INA and BTH) are both known to induce systemic acquired resistance in plants (Görlach et al. 1996) and showed similar effects on induction of Cry1Ab protein in PR-1a*/cry1Ab* broccoli plants (data not shown). Untransformed broccoli plants treated with the inducers were not protected against insect damage, indicating that these chemicals did not themselves act as insecticides and did not induce production of insecticidal compounds by the plants. Induced primary transformants showed excellent control of neonate DBM; however, uninduced T_0 plants showed expression very similar to induced ones, although most suffered somewhat greater leaf damage.

The reasons for the 'leakiness' of the promoter (i.e., constitutive expression of the *cry1Ab* gene) in the primary transformants are not clear. In tobacco leaf discs transformed with a PR-1a/*gus* construct (Ohshima et al. 1990), stresses related to cutting of the leaves apparently led to induction. However, slicing of leaves of untransformed tobacco plants with a razor blade did not result in accumulation of endogenous salicylic acid or the glucoside of salicylic acid and induced little or no synthesis of PR-1 protein (Malamy et al. 1996). Mechanical wounding of our *PR-1a/cry1Ab* broccoli plants did not induce the production of Cry1Ab protein (data not shown). Production of pathogenesis-related proteins was seen in tobacco tissue culture (Antoniw et al. 1981). Our assays used leaves from T_0 plants grown in soil for 5–6 weeks prior to testing; however, Cry1Ab protein might have accumulated in the plants during the earlier tissue culture phase and persisted till the assays. This might explain the apparent constitutive expression of the T_0 plants. On the other hand, some of our T_1 plants continued to exhibit constitutive expression of the *cry1Ab* gene, even though they were grown from seed. This suggests that other unknown mechanism(s) such as position effects or rearrangements of the PR-1a promoter might also contribute to the expression of the *cry1Ab* gene in some T_1 plants. An earlier study testing the PR-1a/*cry1Ab* gene combination in tobacco (Williams et al. 1992) reported on homozygous progeny lines and did not describe the response of the primary transformants themselves. Jin et al. (2000) recently compared insect control in primary transformants of cabbage carrying a *cry1Ab3* gene under control of either the 35S promoter or the wound-inducible soybean *vspB* promoter. The *vspB/cry1Ab3* plants showed the same rapid toxicity to insects as the *35S/cry1Ab3* plants, even without prior induction of the Bt gene. This may represent constitutive expression of an inducible promoter in primary transformants, similar to our results with a chemically inducible promoter.

Uknes et al. (1993) did not detect induction of the endogenous PR-1a protein or the *gus* transgene in homozygous progeny lines of tobacco transformants treated with either water or by wounding. In our study, different T_1 progeny lines varied in their inducibility. Lines 60 and 67 showed insect control and production of *cry1Ab* mRNA and protein only when induced, both in detached leaf and whole plant assays. Their response under field conditions with possible exposure to pathogens and other stresses remains to be tested. Other lines showed partial to total control of neonate and older DBM larvae without induction, even though little or no *cry1Ab* mRNA or protein was detected. The variability of the induction of T_1 lines is probably due to position effects, since all the progeny plants were grown under similar conditions.

Inducible expression of Bt proteins in transgenic plants could contribute to resistance management strategies by limiting toxin production to situations when insect pressure would result in serious crop loss (Gould 1997). This approach would avoid the continuous selection for resistant insects that may occur when strong constitutive promoters control Bt genes. Inducible systems currently being assessed for management of insect resistance include wound-inducible proteinase inhibitors and chemically inducible PR-1a promoters. Duan et al. (1996) reported production of transgenic rice plants carrying the potato proteinase inhibitor II (PINII) gene (*pin2*) driven by its own promoter. These transgenic plants showed wound-inducible expression of the *pin2* gene and high accumulation of PINII protein, which resulted in increased resistance to pink stem borer. Because alternatives or complements to current resistance management strate-

gies for Bt transgenic plants are desirable, we have investigated use of the PR-1a promoter to direct *cry1Ab* gene expression and subsequent insect control. Our experimental results demonstrated that it is possible to obtain lines in which expression of the *cry1Ab* gene and control of DBM occur only after induction with chemicals such as INA or BTH that act on the PR-1a promoter. Production of Cry1Ab protein can be detected as early as 12 h after induction, and high levels of the protein can be maintained for insect control, once growers determine that such control is needed. Furthermore, BTH has been released commercially as a broad-spectrum plant protection compound that induces a plant's inherent disease resistance mechanisms (Görlach et al. 1986; Jepson et al. 1998). It has no deleterious effects on human health or on the insects it contacts, including non-target insects [24]. Thus, it is a more benign chemical treatment than standard insecticides and is readily available to farmers.

Release of Bt crops provoked vigorous debates. A major concern is that high dose constitutive production of Bt proteins might accelerate the evolution of insect resistance. Thus, non-Bt cultivars must be grown together with Bt crops as refuges to prevent or delay evolution of insect resistance to Bt proteins. However, the non-Bt cultivars will sustain yield losses from insect damage. Inducible lines like our PR-1a/*cry1Ab* broccoli may be valuable in several ways. First, Bt protein production can be limited to situations when insect pressure is high, thereby avoiding constant high selection for resistant insect populations and delaying resistance. The validity of this hypothesis can be tested in a greenhouse or field trial using the DBM populations and PR-*1a/cry1Ab* broccoli plants now available in our program. Such studies could provide valuable information about yield losses and delay of insect resistance in relation to timing of sprays. Second, chemically inducible lines might provide a complement to use of plants with high constitutive production of a Bt protein by limiting yield losses in the refuge. If the refuge contained a different Bt gene (or other insect control gene) under control of a chemically inducible promoter, it could be sprayed for insect control only if/when insect pressure is very high. This approach can also be tested empirically using the PR-1a/*cry1Ab* broccoli lines in combination with our constitutively expressing *cry1C* broccoli lines (Cao et al. 1999) and resistant DBM populations (Metz et al. 1995a; Zhao et al 2000).

In conclusion, transgenic broccoli lines with chemically inducible insect control were obtained through careful selection from a large number of primary transformants. Availability of these lines, in combination with constitutively expressing *cry1C* broccoli and susceptible, Cry1A- and Cry1C- resistant DBM populations (Metz et al. 1995a; Zhao et al. 2000), will allow greenhouse and field tests of alternative resistance management strategies.

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