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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Bacillus thuringiensis protein production, signal transduction, and insect control in chemically inducible *PR-1a/cry1Ab* broccoli plants

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Abstract In an effort to develop a chemically inducible system for insect management, we studied production of Cry1Ab *Bacillus thuringiensis* (Bt) protein and control of the diamondback moth (DBM), *Plutella xylostella* L., in inducer-treated and untreated tissues of a broccoli line transformed with a *PR-1a*/*cry1Ab* expression cassette. Spraying leaves of these plants with the inducer acibenzolar-S-methyl (= 1,2,3 benzothiadiazole-7-thiocarboxylic acid-S-methyl-ester) (ASM) triggered expression of the *cry1Ab* gene and produced a high level of Cry1Ab protein within 2–3 days. Cry1Ab protein persisted in leaves for at least 8 weeks, providing prolonged protection from *P. xylostella* attack. Signals generated in inducer-treated leaves were transferred to untreated newly emerged leaves or heads, as seen by production of Cry1Ab protein and/or protection from insect damage in these plant parts. Signal transduction proceeded in an attenuated manner up to the sixth newly emerged leaf. No Cry1Ab protein was detectable by ELISA in uninduced young leaves, but small amounts of the protein were present in uninduced leaves older than 3 weeks and caused some insect mortality. Such basal expression of Bt genes without induction may favor the evolution of resistant insect populations and therefore limits the application of the PR-1a/cry1Ab system for insect management. However, the rapid production and steady maintenance of a high level of transgenic protein upon induction, the signal transduction observed, and the fact that the chemical inducer can be used in field conditions make the PR-1a promoter attractive for chemical regulation of other agriculturally or pharmaceutically important genes for which low expression in the absence of induction is not a concern.

Keywords *Bacillus thuringiensis* · *Brassica oleracea* · Chemically inducible · Cry1Ab protein · Diamondback moth · *Plutella xylostella* Linnaeus · *PR-1a* promoter · Signal transduction

Abbreviations ASM: Acibenzolar-S-methyl (=1,2,3 benzothiadiazole-7-thiocarboxylic acid-S-methyl-ester) · Bt: *Bacillus thuringiensis* · DBM: Diamondback moth · ELISA: Enzyme-linked immunosorbant assay · PR: Pathogenesis-related · SA: Salicylic acid · SAR: Systemic acquired resistance · TSP: Total soluble protein

Introduction

Development of Bt crops has revolutionized agricultural practices for insect control and provided significant and multiple benefits (Shelton et al. 2002). Bt genes in currently marketed crops are under the control of a constitutive promoter, which results in constant selective pressure on insects feeding on the Bt plants. Selective pressure might be reduced by controlling expression of the Bt genes via a chemically inducible promoter. A chemically inducible system may make it possible to limit expression of Bt genes to the periods when the crop is most sensitive to damage and/or when pest populations exceed a damage threshold. This approach could provide an alternative or additional resistance management strategy to the current use of refuges of non-transgenic plants (Bates et al. 2005a, b).

A number of chemically inducible systems with components derived from either plant or non-plant sources have

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been developed in plants (Reynolds 1999; Zuo and Chua 2000; Padidam 2003); however, these have not yet been utilized for insect resistance management. Furthermore, most reports of chemically inducible systems in plants have focused on characterization of transgene expression and biological functions immediately following induction (Deveaux et al. 2003). Information about systemic responses to chemical induction of transgenes is limited (Padidam et al. 2003; Guo et al. 2003; Koo et al. 2004). To our knowledge, there are no reports on systemic responses in plant tissues that develop after induction.

A few systemically inducible systems have been reported in plants (Farmer and Ryan 1992; Malamy and Klessig 1992). Systemic acquired resistance (SAR) has been most extensively studied. During pathogen infection, an unknown signal is perceived and released in pathogen-infected target cells and travels to healthy cells to trigger SAR (Friedrich et al. 1996). The signal transduction pathway is dependent on the accumulation of salicylic acid (SA) (Rasmussen et al. 1991; Vernooij et al. 1994). Following SA accumulation, SAR genes, including the *PR-1a* gene, are induced, resulting in the establishment of the resistant state (Vernooij et al. 1994; Ward et al. 1991). Synthetic compounds can also activate expression of *PR* genes and establish SAR (Friedrich et al. 1996). ASM (acibenzolar-S-methyl = 1,2,3 benzothiadiazole-7-thiocarboxylic acid-S-methyl -ester), the preferred inducer for the PR-1a promoter, has recently been registered as an antifungal chemical for field application (Syngenta Crop Protection 2005). Thus, it would be possible to use the PR-1a/ASM system to express and test a protein of interest under field conditions.

If inducible expression of insect resistance genes controlled insects in both inducer-treated existing leaves and in untreated leaves that emerged after the induction, it could be an effective tool for insect management. To test the workability of such an approach, we produced transgenic broccoli plants carrying a *cry1Ab* gene under the control of the pathogenesis responsive PR-1a promoter from tobacco (Williams et al. 1992; Cao et al. 2001). Our previous study (Cao et al. 2001) found that young leaves of some progeny lines showed inducible production of Cry1Ab protein and inducible control of the diamondback moth, *Plutella xylostella* L. These lines were of interest for further greenhouse and field tests of chemically inducible systems for insect management. Prior to such tests, it was important to learn more about the responses of both inducer-treated and untreated transgenic materials over a longer growth period than previously examined (Cao et al. 2001). We therefore investigated the timing, level, and duration of the production of Cry1Ab protein and control of the diamondback moth (DBM) in inducer-treated plant tissues. We also determined the extent to which the inducible response was systemic by examining the production of Cry1Ab protein and control of DBM in untreated plant tissues, particularly in the distal parts of a plant that emerged at different times after induction.

Materials and methods

Plant material

A progeny of a transgenic broccoli (*Brassica oleracea* L., cv. "Green Comet") line, T73-3, was used in this study. Line T73-3 carried one insertion of the *PR-1a*/*cry1Ab* expression cassette and was inducible by ASM (Cao et al. 2001). Homozygous T2 progeny of T73-3, identified by failure to segregate on callus and shoot induction medium containing kanamycin (Cao et al. 2002), were used in the experiments. Plants were grown in a greenhouse at 20°C in the daytime and 15.5°C at night with a 16:8 h light/dark regime.

Induction of plants

Plants were induced by spraying with Actigard 50 WG (provided by Syngenta Crop Protection, Research Triangle Park, NC), a 50% wettable powder formulation of ASM at the rate of 62.5 mg/100 ml. (In our previous paper (Cao et al. 2001), we referred to the inducer as BTH, rather than ASM.) To facilitate contact between the solution and the leaf surface, 0.1% Tween 20 (Sigma, St. Louis, MO) was added to the Actigard solution. Whole plants were sprayed with ca. 25 ml of the Actigard/Tween 20 solution using a hand held mister with care to ensure that all existing leaves were thoroughly covered.

Evaluation of Cry1Ab protein production and signal transduction

To determine the time course of inducible production of Cry1Ab protein, plants with ten leaves were sprayed with either ASM/0.1% Tween 20 or water/0.1% Tween 20. The second leaf from the top was used for the assays. To measure production of Cry1Ab protein in untreated leaves adjacent to induced ones, plants with ten leaves were covered with plastic cling wrap except for the two leaves below the top leaf of the plant. The two uncovered leaves were sprayed with either ASM or water. To assess Cry1Ab protein production in newly emerged untreated leaves, plants with 8–10 leaves were sprayed with either ASM or water except for the apical portion of the plant, which was covered with plastic wrap. To measure production of Cry1Ab protein in newly emerged heads, the apical portion of plants and the surrounding two smallest leaves were covered with plastic wrap 2 days before head development. All exposed leaves were then sprayed with either ASM or water.

Proteins were isolated as described by Cao and Earle (2003) from ASM-treated and untreated (existing or newly emerged) leaves and heads at different times after the induction. The amount of Cry1Ab protein present in samples was measured by enzyme-linked immunosorbant assay (ELISA) using Cry1A plate kits from Envirologix Inc. (Portland, ME). Proteins from water-treated *PR-1a*/*cry1Ab*

plants were used as the control. Protein concentrations were determined according to the BioRad (Hercules, CA) protein assay (Bio-Rad Laboratories 2005). Protein extracts were diluted at 1:11 with extraction buffer from Envirologix Inc. before ELISA was carried out. The optical density (OD) values of samples were measured using a microplate reader set at 450 nm. The amount of Cry1Ab protein was expressed as nanograms of Cry1Ab protein per milligram total soluble protein (TSP).

Insect bioassays

Susceptible *P. xylostella* larvae were obtained from the previously described Geneva 88 colony (Zhao et al. 2002). The colony was reared on wheat-casein diet (Shelton et al. 1991) and maintained in an environmental chamber at 27 ± 1°C, 50 ± 2% RH, and a photoperiod of 16:8 h (L:D).

To determine effects of DBM on leaf defoliation, plants with eight leaves were sprayed with ASM or water. The bottom leaf (3rd leaf formed) and the top (8th) leaf were used for the bioassay. Individual whole leaves were placed in a baby food jar containing 1% water agar, and five 2nd instar larvae were placed on the surface of each leaf. Defoliation of each leaf was visually observed and percentage of defoliation estimated 3 days later. Percentages of defoliation from three to four individual leaves used in each treatment were then averaged.

Mortality of larvae was measured on leaves formed after seven-week old plants were sprayed with ASM. After 4 weeks, all new leaves formed subsequent to treatment with ASM (seven or eight leaves per plant) were removed, and half of each leaf was placed individually in a 30 ml plastic cup with five 2nd instar larvae. Percent mortality was recorded after 3 days, and corrected for control mortality using Abbott's formula (Abbott 1925). Each leaf position was replicated 6–10 times.

Statistical analysis

Cry1Ab concentration was analyzed as a completely randomized design of analysis of variance (ANOVA) with two to four repeated measures, and means were compared with standard deviation (SD).

Results

Time course of Cry1Ab protein production in ASM treated leaves

To understand the timing, level, and maintenance of the production of Cry1Ab protein after induction, we measured Cry1Ab protein in individual leaves over an eight-week period after plants with ten leaves were sprayed with ASM. Cry1Ab protein was readily detectable one day after the induction and reached a high level (815 ng/mg TSP) by 3 days (Fig. 1). The protein was maintained at a high level up to 7 weeks, with some reduction in later weeks. The level of Cry1Ab protein was still over 200 ng/mg TSP at the end of the 8th week. By this time the sprayed leaves had begun to senesce, and they wilted shortly afterward. In water-treated leaves, Cry1Ab protein was not detected initially; however, small amounts of Cry1Ab protein were marginally detectable about 3–4 weeks after the spray.

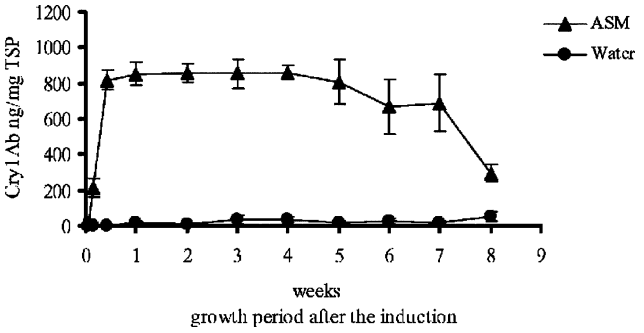

Fig. 1 Production of Cry1Ab protein in broccoli leaves over an extended growth period following induction with ASM. The production of Cry1Ab protein is expressed as ng/mg total soluble protein (TSP). Bars represent SD based on four plant samples

Production of Cry1Ab protein and insect control in induced and uninduced leaves of different ages

As the leaves treated only with water produced some Cry1Ab protein after several weeks, we also studied the production of Cry1Ab protein and the control of larval defoliation in older (3rd leaf formed) and younger (8th leaf formed) leaves of the same *PR-1a/cry1Ab* plants. The younger leaves had emerged about a week before sampling, while the older ones were about 3 weeks old. The results are shown in Fig. 2a and b. Old and young leaves of non-transgenic plants treated with either ASM or water produced no Cry1Ab protein and suffered a mean of 55% defoliation by DBM. Young transgenic leaves treated with ASM produced some Cry1Ab protein within 2 days and reached a high level (778 ng/mg TSP) afterwards. These leaves showed little defoliation by DBM larvae. Older leaves produced high levels (861 ng/mg TSP) of Cry1Ab protein soon after the spray with ASM and were completely protected from larval damage. Young transgenic leaves treated with water showed no production of Cry1Ab protein and suffered a mean of 47% defoliation by DBM larvae, which was not statistically different from the defoliation suffered by the non-transgenic plants. However, water-treated older leaves produced a low level (275 ng/mg TSP) of Cry1Ab protein and inhibited leaf damage by the larvae. We also examined the production Cry1Ab protein and insect control in older leaves without any treatment. The results were similar to those seen after water treatment (data not shown). These results indicate that the low levels of Cry1Ab protein present in older leaves even without chemical induction were sufficient to control DBM larvae.

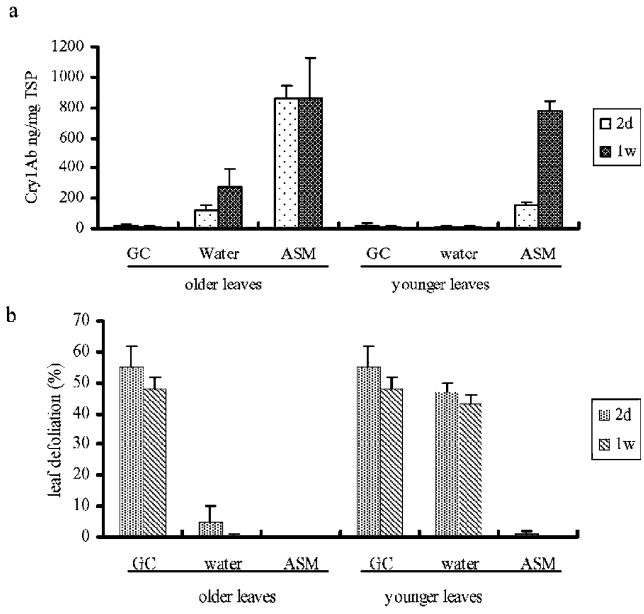

Fig. 2 Production of Cry1Ab protein (**a**), and insect defoliation (**b**) in younger (8th) and older (3rd) leaves of the same *PR-1a*/*cry1Ab* broccoli plants treated either with ASM or water. The older leaves were over 3 weeks old while the younger leaves were 1 week old at the time of sampling. Leaf defoliation was visually evaluated. Cry1Ab protein is expressed as ng/mg of total soluble protein (TSP). Bars represent SD with two samples of each treatment. GC: non-transgenic Green Comet control. d: day; w: week

Production of Cry1Ab protein and insect control in leaves newly emerged after ASM treatment

To understand the extent to which induction is systemic in our *PR-1a*/*cry1Ab* broccoli plants, Cry1Ab protein was first quantified in untreated existing young leaves above and below two leaves that had been sprayed with ASM. Figure 3 shows that 3 days after induction the ASM-treated leaves had produced high levels of Cry1Ab protein (about 760 ng/mg TSP). Cry1Ab protein was also present in untreated existing leaves immediately above and below the ASM-treated leaves 3 days and 1 week after induction, but

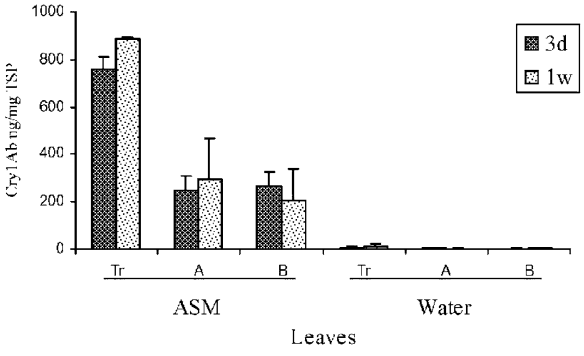

Fig. 3 Production of Cry1Ab protein in untreated existing leaves. Protein was isolated from ASM- or water-treated leaves (Tr) as well as one leaf above (A), or below (B) the treated leaves. Cry1Ab protein is expressed as ng/mg of total soluble protein (TSP). Bars represent SD ($n = 2$). d: day; w: week

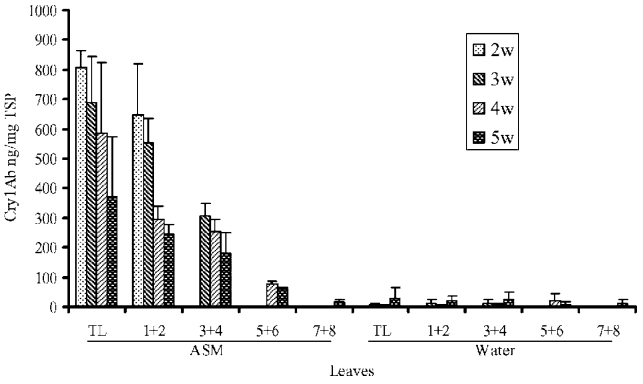

Fig. 4 Production of Cry1Ab protein in new leaves after treatment of existing leaves with ASM. Protein was isolated from top leaves (TL) treated either with ASM or water as well as untreated leaves that emerged after induction. The numbers 1 + 2, 3 + 4, 5 + 6, and 7 + 8 represent the 1st and 2nd, 3rd and 4th, 5th and 6th, and 7th and 8th newly emerged leaves. Higher numbers are the younger leaves, which are more distal from the inducer-treated parts. Cry1Ab protein is expressed as ng/mg total soluble protein (TSP). Bars represent SD ($n = 4$). w: week

at a lower level. Little or no Cry1Ab protein was detected in water-treated leaves or in nearby existing ones. These results suggest that signal transduction occurred from the ASM-treated leaves to existing untreated leaves, resulting in production of Cry1Ab protein.

To determine whether the signal transduction extended from ASM-treated leaves to newly emerged leaves, we measured production of Cry1Ab protein in new leaves that appeared after the whole plants were sprayed with ASM. The first two newly emerged leaves from ASM-treated plants contained a high level of the Cry1Ab protein (649 ng/mg TSP) 2 weeks after induction, although this value was slightly lower than that seen in the leaves that had been sprayed with ASM (Fig. 4). The amount of Cry1Ab protein in newly emerged leaves decreased at increasing distances from the ASM-treated parts of the plant. The 3rd and 4th newly emerged leaves produced a somewhat lower level of Cry1Ab protein (308 ng/mg TSP) while the 5th and 6th new leaves had a very low level (76 ng/mg TSP). New leaves that emerged later had almost no detectable Cry1Ab protein. As expected, newly emerged leaves on plants sprayed with water instead of ASM produced little or no Cry1Ab protein.

To determine how insects were affected by the Bt protein produced in newly emerged leaves via signal transduction from ASM-treated existing leaves, we evaluated mortality of larvae on leaves formed after induction. The first two leaves newly emerged from plants treated with ASM produced high levels of Cry1Ab protein and caused complete larval mortality (Fig. 5). Mortality of larvae feeding on the 3rd and 4th leaves formed after induction, which produced a moderate level of Cry1Ab protein, was 80–100%, and larval growth was severely inhibited. As the distance between ASM-treated and newly formed leaves increased, larval mortality gradually decreased. The mortality of larvae feeding on the 6th newly formed leaves was only 60%. On the 8th new leaf, which contained little or

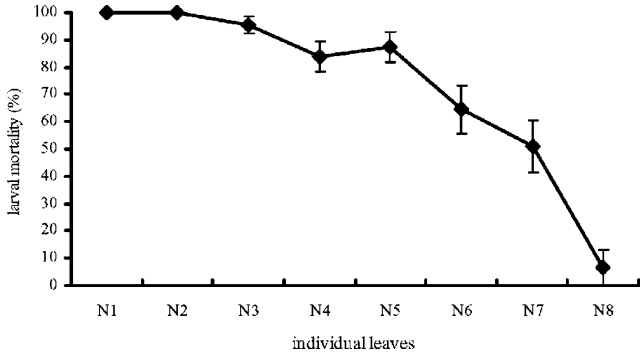

Fig. 5 Mortality of 2nd instar diamondback moth larvae on new foliage formed after induction. All leaves used for the bioassay were collected 1 month after induction. Each leaf was infested with five 2nd instar DBM larvae. Larval mortality was evaluated 3 days later and given as an average value from six same numbered newly emerged leaves. Bars represent SD

no detectable Cry1Ab protein, the larval mortality dropped to 20%. Overall, the mortality of larvae on leaves newly formed after induction was correlated with the production of Cry1Ab protein in the leaves.

Production of Cry1Ab protein in untreated heads through signal transduction

To ascertain whether there was signal transduction from vegetative leaf tissues to floral tissues, Cry1Ab protein was measured both in the uppermost ASM-treated leaves and in heads that emerged after induction. Figure 6 shows that Cry1Ab protein was at a high level in ASM-treated leaves 4 days after induction and remained at this level for at least 5 weeks. At the fourth day, heads of ASM-treated plants contained a low level of Cry1Ab protein, which later increased. Cry1Ab protein was initially undetectable in heads from water treated *PR-1a/cry1Ab* plants; however, a marginal low level of Cry1Ab protein was detected after 4–5 weeks.

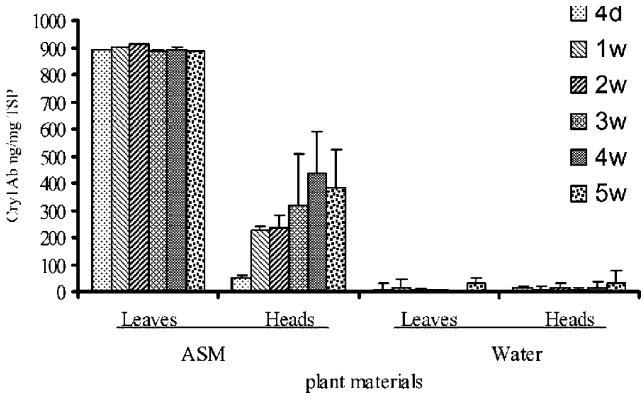

Fig. 6 Production of Cry1Ab protein in broccoli heads that emerged after the induction of leaves. Protein was isolated from the top leaves treated either with ASM or water and from untreated heads at different times after induction. Cry1Ab protein is expressed as ng/mg total soluble protein (TSP). Bars represent SD ($n = 2$). d: day; w: week

Discussion

Our study with *PR-1a/cry1Ab* broccoli plants shows that many factors can affect the successful implementation of chemically inducible insect management. Major factors include timing, level, and duration of inducible production of insect resistance proteins, systemic signal transduction, and tight control of the expression of the resistance genes.

An effective chemically inducible system must quickly turn on expression of insect resistance genes and produce sufficient insecticidal proteins soon after the induction. We found that within 2 to 3 days after treatment with ASM, *PR-1a/cry1Ab* broccoli plants produced a high level of Cry1Ab protein, similar to that achieved with the constitutive 35S promoter (Cao et al. 2002, 2005). Moreover, the high level was maintained for at least 8 weeks, when leaves began to senesce, providing an extended period of protection from insect attack.

The chemically inducible systems developed in plants have generally focused on regulation of various genes in research experiments (Gatz et al. 1992; Caddick et al. 1998; Aoyama and Chua 1997; Martinez et al. 1999). The importance of systemic inducibility for biotechnology applications has usually received little attention. In a useful inducible system for actively growing plants, gene expression must be quickly turned on not only in the treated parts but also in distal parts as soon as they appear. In the case of broccoli, a new leaf emerges about every 3 to 4 days. Thus, soon after induction the plant is composed of a mixture of treated and untreated parts. In our system, signal transduction occurred from inducer-treated to untreated tissues, but the signal attenuated with time and/or distance. As a result, varying levels of Cry1Ab protein were present within the induced plants. This is counter-productive to insect management, because sub-lethal doses can cause resistance to develop faster than high doses (Roush 1997). Consequently, repeated applications of the inducer may be required to maintain high levels of Bt protein in all parts of actively growing plants.

Successful application of a chemically inducible system for insect management also requires tight regulation of the expression of the resistance genes. Although newly emerged *PR-1a/cry1Ab* leaves produced little or no Cry1Ab protein without induction, the protein became marginally detectable in these leaves after further growth. The gradual increase in Cry1Ab protein levels during development of untreated leaves reflects loss of the tight control of *cry1Ab* gene expression due to unknown factors. The result is a basal level of the Cry1Ab protein ranging from undetectable to low, which can result in negative effects on insect fitness and a wide range of mortality from 0–100%, as seen in additional bioassays in our project (Bates et al. 2005b). The low level of Bt protein in the absence of the inducer may favor the evolution of insect resistance (Roush 1997).

When the basal level of transgene expression must be close to zero in the absence of induction, plant-derived promoters whose function is triggered by endogenous

chemicals are probably not the best choice. Plant promoter sequences usually contain various *cis* elements that are likely to respond to a number of physiological signals and environmental stimulants, in addition to the chemical in question (Gatz 1997; Zuo and Chua 2000). The observed leakiness of *PR-1a*/*cry1Ab* system in broccoli is probably due to the response of the promoter to endogenous signaling compounds or exogenous stimuli in addition to ASM sprays. Alternative promoters of non-plant origin may provide tighter control (Jepson et al. 1998; Padidam 2003) because they are less likely to respond to endogenous plant chemicals. However, lack of signal transduction (Sweetman et al. 2002) may limit the effectiveness of most non-plant derived, chemically inducible promoters in actively growing plants, without further improvement of the promoters. Recent development of the ecdysone agonist-inducible system is encouraging (Padidam 2003) because the inducer, methoxyfenozide, is a registered agrochemical that moves systemically in plants (Martinez and Jepson 1999). However, systemic inducibility in untreated and newly emerged tissues in actively growing plants remains to be studied. Furthermore, foliar uptake of the inducer must be assessed before the system can be effectively applied in field conditions.

Conclusions

Although the basal level of expression seen in older leaves limits the value of the *PR-1a*/*cry1Ab* system for insect management, the fact that the preferred inducer ASM is registered as an antifungal chemical for field application makes it attractive for agricultural use. High gene expression can be achieved with the PR-1a promoter within a few days after induction and maintained for many weeks, and signal transduction to untreated tissues occurs. Thus, this promoter may be attractive for spatial or temporal control of expression of agronomically or pharmaceutically important genes whose basal expression in the absence of induction has no negative effects on plant growth and development.

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