The Hot and the Classic

THE MONARCH BUTTERFLY CONTROVERSY

The second most widely grown transgenic crop in the United States are maize (*Zea mays*) cultivars that have been engineered to express genes for various insecticidal protein endotoxins (*Bt* toxins) from the soil bacterium *Bacillus thuringiensis*. The principal target species for *Bt* toxin-expressing maize (*Bt* maize) is the European corn borer (*Ostrinia nubilalis*), one of the most damaging pests of maize in North America. Losses attributable to European corn borer damage exceed over $1 billion annually in the United States alone. *Bt* toxins are widely believed to be selectively toxic, only affecting those insects (e.g. lepidopteran larvae) that have a gut alkaline enough to activate the *Bt* protoxin by enzymatic proteolysis. Receptor binding by the C-terminal domain of the active toxin is the major determinant of host specificity by the different *Bt* toxins.

Given the growing agricultural importance of *Bt* maize as well as *Bt* cotton (*Gossypium hirsutum*) and *Bt* potato (*Solanum tuberosum*), it is not surprising that a storm of controversy arose following the publication in *Nature* of a preliminary study by Losey et al. (1999). This paper raised serious concerns about the ecological safety of *Bt* maize cultivation to non-target lepidopterans, in particular the larvae of monarch butterfly (*Danaus plexipus*). On the basis of laboratory assays, the authors concluded that monarch larvae reared on milkweed (*Asclepias syriaca*) leaves dusted with pollen from *Bt* maize ate less, grew more slowly, and suffered higher mortality than those reared on leaves dusted with nontransformed maize or on leaves without pollen.

The conclusions of Losey et al. (1999) were challenged on three grounds. First, the pollen doses used by Losey et al. (1999) were not quantitatively measured but were gauged by eye to match pollen dustings on milkweed leaves collected in the field. This raised concerns about subconscious biases on the part of the researchers. Second, concerns, as it turns out, valid ones, were raised as to the validity of extrapolating from the results of Losey at al. (1999), which concerned only one type of pollen, to all types of *Bt* maize pollen. Third, the soundness of extrapolating from laboratory assays to the field was uncertain, although a subsequent field study by Jesse and Obrycki (2000) did seem to confirm the fears raised by the Losey et al. (1999) study.

Regardless of the deficiencies of the study, the results of Losey et al. (1999) were widely heralded by the popular press and established the monarch butterfly, one of the more beautiful creatures on our planet, as the cause célèbre for environmentalists opposed to biotechnology. Berenbaum (2001) has written an engaging piece concerning the diametric responses that the lay and scientific presses have had as this controversy has unfolded.

This month's "The Hot and the Classic" is devoted to summarizing some new contributions that specifically address the question of the risk associated with *Bt* maize pollen to the monarch butterfly.

Field Studies

To interpret accurately the results of studies that examine the effects of *Bt* maize pollen on monarch larvae, it is necessary to know the range and distribution of naturally occurring pollen densities on milkweed leaves. This gap in our knowledge has recently been filled by Pleasants et al. (2001), who measured the density of maize pollen on milkweed plants inside and outside of maize fields in several different localities. Average pollen density was highest within maize fields (171 grains per cm^2) and was progressively lower from the field edge outward, falling to 14 grains per cm^2) at 2 m (see also Jesse and Obrycki, 2000; Wraight et al., 2000). The authors also describe complexity in the pattern of maize pollen density within the canopies of milkweed plants. Younger milkweed leaves, which typically harbor more than half of the monarch larvae, have on average only 30% to 50% of the pollen density of middle leaves due to their greater exposure to cleansing rain and to their steeper leaf angles. Rain is a principal determinant of pollen density: A single rainfall can remove 54% to 86% of the maize pollen on milkweed leaves.

Not All Transgenic Pollen Is Toxic

Transgenic maize hybrids that are currently or have been commercially available contain *cry1Ab* (events Bt11, Mon810, and 176), *cry9c* (event Cbh351), or *cry1Ac* (event Dbt418) *Bt* genes. In addition, registration was recently granted for a hybrid that expresses a *cry1F* gene (event Tc1507). Because the initial report by Losey et al. (1999) examined the effects of only one type of transgenic pollen (Cry1Ab event 176), Hellmich et al. (2001) conducted laboratory tests to establish the relative toxicity of various other *Bt* toxins to monarch larvae (see also Wraight et al., 2000). Bioassays of purified *Bt* toxins indicate that Cry9C and Cry1F proteins are relatively nontoxic to monarch first instars, whereas first instars are sensitive to Cry1Ab and Cry1Ac proteins. Older instars were 12 to 23 times less susceptible to Cry1Ab than were first instars. Pollen bioassays indicated that pollen contaminants, mostly fractured anthers, were particularly toxic to larvae. This finding suggests that caution must be exercised in interpreting the findings of Jesse and Obrycki (2000), who used pollen preparations containing 6 times more contaminants than the preparations of Hellmich et al. (2001). The only transgenic maize pollen that consistently affected monarch larvae was from Cry1Ab event 176 hybrids, currently less than 2% of the maize planted and for which reregistration has not been applied. The authors conclude that pollen from the Cry1Ab (events Bt11 and Mon810) Cry1F, and

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Cry9C hybrids will have no acute effects on monarch larvae in the field.

Risk Assessment Studies

A proper risk assessment of the impact of *Bt* maize cultivation on monarch butterfly populations requires consideration of both the expression of toxicity and the likelihood of exposure to the toxin. A collaborative research effort by scientists in Canada and the United States gathered information concerning the acute toxic effects of *Bt* maize pollen and the degree to which monarch larvae would be exposed to toxic amounts (Sears et al., 2001). The authors estimate that even if harmful *Bt* maize (event 176) cultivation were to climb to 80% of the total cultivated maize, only 6% of the monarch larval population would be at risk. If nonharmful cultivars (Mon 810 and Bt11) were to climb to 80% of total maize cultivation, the percentage of monarchs at risk would fall to 0.05%. The authors conclude that the impact of *Bt* maize pollen from current commercial hybrids on monarch butterfly populations is negligible.

Bt Maize versus Conventional Pesticides

Losey et al. (1999) concluded their report with the recommendation that "... we gather the data necessary to evaluate the risks associated with the new agrotechnology and... compare these risks with those posed by pesticides and other pest-control tactics." Toward this goal, Stanley-Horn et al. (2001) undertook to compare the effects on monarch larvae of pollen from cultivars of *Bt*-expressing maize, both harmful and harmless, with those of nontransgenic maize sprayed with a conventional chemical pesticide (λ-cyhalothrin). As expected, they found that event 176 *Bt* pollen, even at low densities (23–67 pollen grains cm^{-1}), had deleterious effects on monarch larvae, whereas the pollen of other *Bt* maize cultivars (Bt11 or Mon810) was essentially harmless. The reduction in survivorship and weight gain seen with the harmful event 176 pollen was dwarfed by the effects of the pesticide λ-cyhalothrin, which is commonly used as a chemical treatment against European corn borers. Most larvae died within hours after feeding on milkweed leaves collected from plants exposed to spray application. Survival and growth of larvae feeding on milkweed outside of the sprayed plots was also reduced because of insecticidal drift.

Effects on Other Nontarget Lepidoptera

Zangerl et al. (2001) concluded that *Bt* maize incorporating event 176 does have sublethal effects on black swallowtails (*Papilio polyxenes*) in the field. An earlier study by Wraight et al. (2000), however, failed to detect an effect of harmless *Bt* pollen (Mon810) on black swallowtails.

Conclusion

Losey et al. (1999) should be lauded for uncovering and exposing the nontarget effects of event-176 maize pollen on monarch larvae. Their publication had the salutary effect of raising both the consciousness of the public and of the biotechnology industry of the possible nontarget effects of *Bt* crops. However, those individuals and organizations who attempted to extrapolate the results of Losey et al. (1999) to other strains of *Bt* maize, and to *Bt* crops in general, have now been shown to be in error at least in regard to the monarch butterfly. The effects of *Bt* maize cultivation on monarch butterfly maize populations are negligible, and current evidence suggests that *Bt* maize is an environmentally safer insect control strategy than conventional chemical spraying.

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News from the Archives

ABSCISIC ACID: A UNIVERSAL SIGNALING FACTOR?

In 1986, two papers appeared that raised the possibility that abscisic acid (ABA) might be a universal signaling factor. In the first, Le Page-Degivry et al. (1986) established the presence of ABA in the brains of rats and pigs. In the second, Huddart et al. (1986) proposed, based on experiments with various smooth muscle preparations and a cyanobacterium that ABA may serve as a universal Ca^{2+} agonist across taxonomic kingdoms. This month's *News from the Archives* reviews how the concept of ABA being a universal signaling factor has fared in the ensuing years.

ABA Present in Brains

Le Page-Degivry et al. (1986) identified (+)-cis-ABA in the brains of pigs and rats by means of a very specific radioimmunoassay. The authors found that the final product of purification from mammalian brain had the same properties as authentic ABA: It cross-reacted in the ABA radioimmunoassay and had the same retention properties and the same gas chromatography/mass spectrometry characteristics. Moreover, like (+)-cis-ABA itself, the brain factor decreased the stomatal apertures of abaxial epidermis strips of *Setcreasea purpurea*. They also found evidence for the presence of ABA conjugates (esters and glucosides) in brain similar to those found in plants. Of course, the presence of ABA in the brain could be interpreted to be a consequence of a diet containing ABA. This would not, however, account for the especially high levels of ABA found in the pig brain compared with other organs (heart, liver, kidneys, and lungs). Moreover, rats fed a synthetic diet poor in ABA were actually found to have *higher* levels of brain ABA than rats fed a normal diet. While their discovery of ABA in mammalian brains was intriguing, the authors did not speculate as to a possible function of ABA in brain tissue. Nor has much been published about a possible role for ABA in brain function in the ensuing years.

The one exception is a report by Pidoplichko and Reymann (1994), who provided evidence that pre-exposure to trans,trans-ABA, which is *not* the ABA isomer used by plants, induced a marked increase of the fast component of *N*-methyl-D-Asp (NMDA)-gated currents in isolated rat hippocampal neurons. Since the NMDA receptor is a subtype of the ionotropic Glu receptor (*iGluR*) gene family, and members of this family have recently been discovered in Arabidopsis (Chiu et al., 1999), perhaps a closer examination of the effects of ABA and its various isomers on plant *iGluR* function might be worthwhile.

In short, the role, if any, of ABA in brain function remains as mysterious as the day its presence in brains was first reported.

ABA: A Ca^{2+} Agonist in Smooth Muscle?

Huddart et al. (1986) presented evidence concerning the effects of ABA on several mammalian smooth muscle preparations and on a cyanobacterium. Studies on smooth muscles from the vas deferens and bladder of rat showed that 10^{-6} M ABA enhanced field stimulation responses by 25%. This effect was inhibited by the Ca^{2+} channel blocker nifedipine. In K^+-depolarized bladder smooth muscle in which the fast Ca^{2+} channels were inactivated, 10^{-6} M ABA augmented contraction tension and enhanced the slow tonic phase of the response, which is known to be dependent on the activity of slow Ca^{2+} channels. Even more impressive effects were found with rat ileal smooth muscle. ABA at concentrations of 10^{-9} to 10^{-8} M caused enhancement of K^+ contracture tension by up to 400%. This response is strongly dependent upon extracellular Ca^{2+}. Preliminary data indicated that ABA enhances the influx of Ca^{2+} occurring after K^+ depolarization by about 60%. Thus, the results attained by Huddart et al. (1986) using all three smooth muscle preparations were consistent with the idea that ABA might act as a plasma membrane Ca^{2+} channel agonist.

The first follow-up study from Huddart's laboratory seemed to confirm the effects of ABA on smooth muscle function. Langton and Huddart (1988) reported that ABA was particularly effective in potentiating the tonic component of K^+-induced contractures in rat smooth muscle vas deferens, particularly in muscles isolated from the epididymis. Lynch (1991), however, found that the ABA analog SD217595 at 10^{-6} M caused an *inhibition* of K^+-induced phasic and tonic contractions of rat bladder detrusor smooth muscle strips. The inhibition of contraction induced by SD217595 was in stark contrast to the potentiation of smooth muscle contraction reported previously by Huddart's group with the parent molecule ABA. Later, Masters et al. (1994), also of Huddart's laboratory, published a near retraction of Langton and Huddart's (1988) results, and concluded that ABA was without significant Ca^{2+}-modulatory activity in their rat vas deferens smooth muscle preparation. Like Lynch (1991), however, they found that the ABA analog SD217595 possessed strong Ca^{2+} entry blocking ability.

In short, data concerning the positive effects of ABA on smooth muscle preparations seem conflicted at best.

ABA: A Ca^{2+} Agonist in Cyanobacteria?

In the case of cyanobacteria, Huddart et al. (1986) reported that 10^{-7} M ABA caused a significant ratio in the heterocyst to vegetative cell ratio of *Nostoc* 6720. Preliminary data also indicated that ABA led to an increase in Ca^{2+} uptake (see also Pandey et al., 1996). The Ca^{2+} ionophore A23187, like ABA, also caused an increase in heterocyst frequency. Subsequent studies revealed that ABA and A23187 also led to an increase in nitrogenase activity (Smith et al., 1987; see also Marsálek and Simek, 1992). The physiological significance of the above studies is heightened by the discovery that cyanobacteria produce ABA (Za-

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hardnickova et al., 1991). Current evidence, therefore, is consistent with the idea that ABA may be an important physiological factor in cyanobacteria.

ABA and Temperature Signaling in Sponges

The idea that ABA may also serve a signaling role in animal cells received a big boost from the recent findings of Zocchi et al. (2001). These authors demonstrated that ABA stimulates an ADP-ribosyl cyclase activity in sponges. This enzyme converts NAD^+ into cADP-Rib, a potent and universal intracellular Ca^{2+} mobilizer that has also been implicated in ABA signal transduction in plants (Wu et al., 1997). Pharmacological evidence indicated that ADP-ribosyl cyclase in *Axinella polypoides* was activated by temperature increases by means of an ABA-induced, protein kinase A-dependent mechanism. Sponges (phylum Porifera) are among the oldest multicellular animals, their evolution dating back to 600 million years ago. These results suggest an ancient evolutionary origin of this stress-signaling cascade.

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Corrigendum

It would appear that the events of September 11th have claimed another victim—the accuracy of my *The Hot and the Classic* column concerning the Monarch Butterfly Controversy (Plant Physiol **127:** 709–710, 2000). I was wrong in stating that Losey Rayor and Carter (Nature **399:** 214, 1999) had employed a variety of Bt11 maize in their study. In fact, those researchers used a variety of event 176 maize. I profoundly regret my introduction of this error into the literature, and thank Drs. Mark Sears, Diane Stanley-Horn, and Richard Hellmich for bringing this mistake to my attention. Their letter to me in this and other regards and my response to them will be posted on the ASPB Web site (www.aspb.org).