Origin of wild beet and gene flow between *Beta vulgaris* and *B. macrocarpa* in California

D Bartsch
Biology V (Ecology), Aachen University of Technology RWTH, 52056 Aachen, Germany

J Clegg, N C Ellstrand
Botany & Plant Sciences, University of California, Riverside, CA 92521-0124, USA

ABSTRACT

We evaluated genetic variation in 52 germplasm accessions of wild and domesticated beets (genus *Beta* L) and examined the origin of wild beet accessions in California using allozyme analysis. UPGMA relationship analysis showed overall that domesticated and wild beets form genetically coherent groups. Wild beets in California have two different taxonomic origins from European *Beta vulgaris* or from *Beta macrocarpa*. Population level pattern of allozyme variation for wild California beets related to *B. vulgaris* suggest those populations evolved from naturalized populations of the cultivated *B. vulgaris* ssp. *vulgaris* which had hybridized to varying degrees with the sea beets *B. vulgaris* ssp. *maritima*. Wild California beets related to *B macrocarpa* are essentially genetically identical to European accessions. In addition, we found substantial evidence for hybridization and introgression of *B. vulgaris* alleles in Californian *B. macrocarpa*.

INTRODUCTION

The main target of genetic engineering in the genus *Beta* is sugar beet, *Beta vulgaris* ssp. *vulgaris* var. *altissima* DÖLL, which is ecologically limited due to its low competitiveness and susceptibility to several plant pathogens and phytophagous animals. Nearly all beets are wind-pollinated. Thus, gene flow between sympatric cultivated and compatible wild forms is unavoidable (Boudry et al. 1993, Raybould & Gray 1993, Bartsch & Schmidt 1997). To address biosafety concerns for beet in California three questions should be answered: 1. Where are potential areas of sympatry of wild and cultivated beet in California? 2. Can we use isozyme marker to assign wild beets of California species and subspecies? 3. Is there genetic evidence of gene flow between wild and cultivated beets in California?

MATERIAL AND METHODS

For this study, allozyme diversity was assayed from 52 wild and cultivated accessions of the genus *Beta*. Samples were obtained from seed companies, from international plant genetic resource collections or from collecting directly from wild and cultivated California populations. Accessions were selected so that most of the geographical range of wild and cultivated beets in Europe and California was represented (Table 1). Starch gel electrophoresis was performed on crude protein extracts of young leaf tissue. Approximately 100 mg of tissue from each individual was ground in 0.5 ml extraction buffer [0.1 M Tris-HCl pH 7, 4% polyvinylpyrrolidone (PVP), 0.1% dithiothreitol (DTT), and 0.1% ascorbic acid]. Gel buffers are cited below, and stain techniques are as described by Devlin & Ellstrand (1989) and Wendel & Weeden (1989).

Tab. 1: Species and accessions of beet surveyed in this study: N_i = number of individuals examined (Standard accessions recommended by WBN, World Beta Network)

No.	Species	Accession	Origin	Location	N_i
1	*B. vulgaris*	FC172	USA	(WBN standard)	15
2	- Sugar beet	KWS-2N1009	Germany		30
3		KWS-Kavetina	Germany		49
4		KWS-Rizor	Germany		48
5		KWS-246	Italy		39
6		KWS-247	Italy		41
7		Betaseed-4035	California		25
8		Betaseed-4581	California		24
9		Betaseed-4776	California		31
10		Spreckels-HH103	California		29
11		Spreckels-IV2R	California		30
12		Spreckels-NB2	California		34
13		Spreckels-SS781	California		85
14		Spreckels-VB7R	California		26
15	- Swiss chard	Dark Green	USA		70
16		Chard Fordhook	USA		21
17		Chard Lucullus	USA		21
18		Chard Rhubarb	USA		39
19	- Red beet	W300C	USA	(WBN standard)	32
20		Burpee	USA		78
21		Detroit Dark Red	USA		16
22		Red Ball	USA		25
23		Tall Top	USA		29
24	- Sea beet	PI 504266	France	Corsica, Ajaccio	26
25		PI 540575	France	Gironde County	21
26		PI 540588	France	Charante Marit. County	30
27		HHU	Germany	Oldenburg, Bot. Garden	85
28		RWTH – 1	Greece	Chalkidiki	7
29		RWTH – 2	Greece	Crete	21
30		RWTH – 3	Greece	Peleponnes	20
31		PI518398	Ireland	Kerry County, Dingle	37
32		BGRC 54228	Ireland	(WBN standard)	26
33		RWTH collection	Italy	Ravenna County, Cervia	57
34		RWTH collection	Italy	Goriza County, Grado	10
35		RWTH collection	Italy	Venice County, Venice	60
36		PI518310	UK	East Sussex County	48
37		UCR – 01	California	Contra Costa County	15
38		UCR – 02	California	Alameda County	20
39		UCR – 03	California	Santa Clara County	37
41		UCR – 05	California	San Benito County	17
42		UCR – 06	California	Los Angeles County	25
43		UCR – 07	California	Riverside County	40
44		UCR – 08	California	San Diego County	25
45		UCR – 09	California	Santa Barbara County	40
46	*B. macrocarpa*	UCR 10; UCR 13	California	Imperial County	506
47		UCR – 11	California	Ventura County	30
48		UCR – 12	California	Los Angeles County	40
49		BGRC 53034	Israel	Athlistean Plain	26
50		BGRC 57644	Cyprus	Larnaca	13
51		BGRC 57664	Spain	Cartagena	70
52		BGRC 57676	Spain	Granada	15

Our nine enzyme systems revealed a minimum of 13: aspartate amino transferase (*Aat1*, *Aat2*; E.C. 2.6.1.1), aconitase (*Aco*; E.C. 4.2.1.3), glutamate dehydrogenase (*Gdh*; E.C. 1.4.1.2), leucine aminopeptidase (*Lap*; E.C. 3.4.11.1), NAD^+ malate dehydrogenase (*Mdh1*, *Mdh2*;

E.C. 1.1.1.37), phosphoglucomutase (*Pgm1*, *Pgm2*; E.C. 5.4.2.2), shikimate dehydrogenase (*Skd*; E.C. 1.1.1.25), triose phosphate isomerase (*Tpi1*, *Tpi2*; E.C. 5.3.1.1), and uridine diphosphoglucose pyrophosphorylase (*Udp*; E.C. 2.4.1.1). To resolve these isozymes, we used three different electrophoretic buffer systems: tris-EDTA-borate pH 8.8 (Heywood, 1980) for *Gdh, Lap,* and *Udp*, lithium-borate pH 8.0 (Rieseberg & Soltis 1989) for *Aat, Pgm,* and *Tpi*, and morpholine-Citrate pH 7.0 (O'Malley et al., 1980) for *Aco, Mdh,* and *Skd*. Genetic interpretations of allozyme variation patterns were based on previously published reports for *Beta* (Abe & Tsuda, 1987, Nagamine et al., 1989, Letschert 1993, Raybould et al. 1996).

Banding patterns of polymorphic loci were congruent with typical angiosperm zymograms, and were interpreted accordingly to Weeden & Wendel (1989). Loci encoding the less anodally migrating allozyme for each enzyme system were designated "1", with additional loci numbered sequentially in order of increasing mobility. This nomenclature is inverted relative to locus/allele designations for allozymes of Letschert (1993). Data analysis was carried out with POPGENE (http://www.ualberta.ca/~fyeh/index.html)

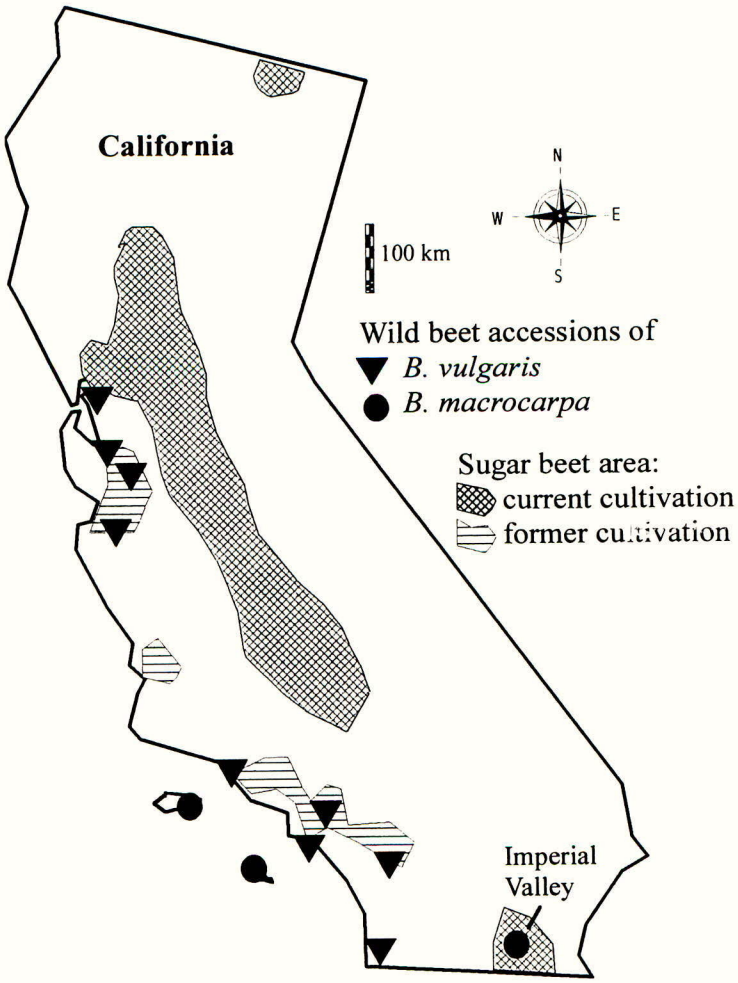

Figure 1. Sugar beet cultivation areas and wild beet accessions in California

RESULTS AND DISCUSSION

Wild beet populations in California were found in several locations (Figure 1). We compiled the different Californian wild beet accessions into groups of *B. macrocarpa* and *B. vulgaris*. The latter group clusters closely with the sea beet group (Figure 2), strongly supporting the hypothesis that at least some of the wild beets originated directly from sea beet. However, the morphologies of the collections demonstrated both sea beet and Swiss chard ancestors. Our isozyme study suggests that also some small populations of vegetable *B. vulgaris* have escaped cultivation in inland areas. The weed beet *B. macrocarpa* was found in sugar beet fields in the Imperial Valley and at coastal sites of Southwest-Californian islands.

In the Imperial Valley, McFarlane (1975) reported phenotypic observations of hybridization between wild and cultivated beets. In this area sugar beet is grown in winter culture, and vernalization of the biennial plants is a common phenomenon due to moderate cold winter temperature. An examination based on 15 sugar beet fields (representing an area of approximately 2 million m²) showed a sugar beet bolting rate of 0.6 plants/m². This rate seems to be higher than typical in this area, probably due to an extraordinary cool winter 1997/98 with periods of low freeze in some parts of the area.

The density of the annual weed *B. macrocarpa* is in the range of 2.7 plants/m² (representing an area of approximately 1 million m² sugar beet plantation examined). Although the annual *B. macrocarpa* usually flowers earlier than sugar beet bolters, a flowering time overlap could be detected in May 1998. Based on 9 specific isozymes (Table 2), introgression in this area could be detected at a rate of 2 % wild beet individuals (13 of 594 examined Californian plants), which were morphologically similar to *B. macrocarpa*, but had isozyme alleles specific to *B. vulgaris*.

CONCLUSION

There is genetic evidence that Californian wild beets belong to two different taxa, and have at least three different origins. We found wild beet evolved from (1) escaped Swiss chard or Red beet, (2) *B. macrocarpa*, presumably introduced from Spain, and (3) hybridization of *B. vulgaris* with introduced *B. macrocarpa*. Although wild sea beet probably played some role in the origin of California wild beets, our genetic information is insufficient to determine that extent to which hybridization of cultivated beet with sea beet and/or direct introduction of sea beet from Europe contributed to contemporary *B. vulgaris*-type wild beets in California.

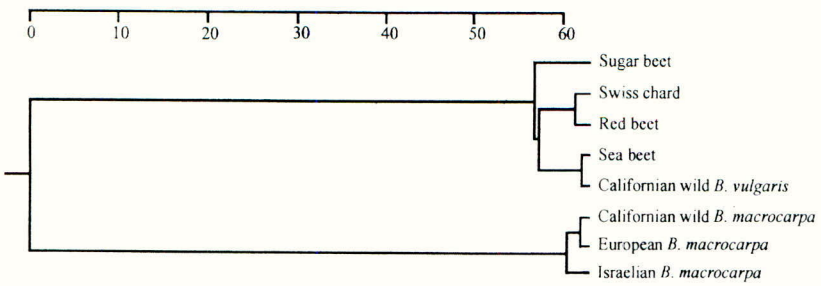

Figure 2. UPGMA dendrogram based Nei's (1978) genetic distance method of allele frequencies for 13 isozymes of *B. vulgaris* (see Table 1).

Engineered cultivars grown in California, especially the Imperial Valley, would have an increased probability of gene escape to wild relatives if their bolting properties were similar or higher than tendencies of the current cultivars planted in this area. Because of this increased tendency for beets to bolt in this area, the impact of an engineered trait escaping into wild populations would have to be assessed.

Table 2. Mean allele frequencies for 13 isozymes of genus *Beta*. Isozyme alleles specific to *B. vulgaris* which were found in 2% of Californian *B. macrocarpa* individuals, are printed bolt. (Origin of *B. macrocarpa*: I = California, II = Europe, III = Israel)

		Sugar beet	Swiss chard	Red beet	Sea beet	Wild beet California	*B.macro-carpa*-I	*B.macro-carpa*-II	*B.macro-carpa*-III
Mdh1	-1	0.765	0.525	0.827	0.885	0.760	0.692	0.726	0.961
	-2	0.157	0.443	0.005	0.023	0.152	**0.001**	0.000	0.000
	-3	0.078	0.032	0.168	0.092	0.088	0.307	0.274	0.039
Mdh2	-1	0.536	0.000	0.004	0.019	0.080	**0.006**	0.000	0.000
	-2	0.464	0.993	0.965	0.924	0.912	**0.002**	0.000	0.000
	-3	0.000	0.000	0.000	0.000	0.000	0.992	1.000	1.000
	-4	0.000	0.007	0.031	0.057	0.000	0.000	0.000	0.000
Aco	-1	0.914	0.176	0.254	0.728	0.612	1.000	1.000	1.000
	-2	0.086	0.824	0.746	0.272	0.388	0.000	0.000	0.000
Skd	-1	0.036	0.000	0.061	0.055	0.024	**0.045**	0.000	0.000
	-2	0.005	0.000	0.010	0.082	0.024	0.946	0.988	1.000
	-3	0.826	0.973	0.822	0.781	0.841	0.009	0.012	0.000
	-4	0.133	0.027	0.107	0.082	0.111	0.000	0.000	0.000
Lap	-1	0.390	0.725	0.551	0.821	0.791	**0.001**	0.000	0.000
	-2	0.610	0.275	0.449	0.179	0.210	**0.008**	0.000	0.000
	-3	0.000	0.000	0.000	0.000	0.000	0.991	1.000	1.000
Tpi1	-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Tpi2	-1	0.000	0.000	0.000	0.064	0.000	0.000	0.000	0.000
	-2	0.971	1.000	0.911	0.878	1.000	0.000	0.000	0.000
	-3	0.029	0.000	0.089	0.057	0.000	0.000	0.000	0.000
Aat1	-1	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	-2	0.533	0.500	0.496	0.570	0.515	0.005	0.000	0.096
	-3	0.000	0.000	0.004	0.210	0.067	0.995	1.000	0.904
	-4	0.466	0.500	0.500	0.220	0.415	0.000	0.000	0.000
Aat2	-1	0.000	0.000	0.000	0.000	1.000	1.000	0.985	1.000
	-2	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000
Udp	-1	0.556	0.482	0.831	0.180	0.520	0.000	0.000	0.000
	-2	0.444	0.518	0.169	0.820	0.480	1.000	1.000	1.000
Pgm1	-1	0.000	0.000	0.000	0.052	0.000	0.984	0.988	1.000
	-2	0.998	1.000	1.000	0.930	0.995	0.016	0.012	0.000
	-3	0.002	0.000	0.000	0.019	0.005	0.000	0.000	0.000
Pgm2	-1	0.000	0.000	0.000	0.013	0.000	0.992	0.933	0.519
	-2	0.266	0.028	0.009	0.109	0.069	0.003	0.067	0.000
	-3	0.686	0.964	0.991	0.828	0.902	0.005	0.000	0.481
	-4	0.048	0.008	0.000	0.050	0.029	**0.001**	0.000	0.000
Gdh	-1	0.824	0.933	1.000	0.742	0.198	**0.002**	0.000	0.000
	-2	0.176	0.067	0.000	0.215	0.000	**0.008**	0.000	0.000
	-3	0.000	0.000	0.000	0.042	0.000	0.989	1.000	1.000

ACKNOWLEDGEMENTS

We thank the following for assistance during early phases of this project: K Meyerholz (KWS Einbeck), J H Griffin, D C Melin (Spreckels-Holly Sugar Corporation), J R Stander (Betaseed), L Panella (Fort Collins), R Levellen and G Wisler (USDA-ARS Salinas), B Ertter (UC Berkeley), S. Chaney (Channel Islands National Park), D. Cudney, C Koehler, A Sanders, R Whitkus (UC Riverside), S Boyd (Rancho Santa Ana Botanic Garden), M Hoefs (Wrigley Botanic Garden Avalon), P Schuyler (Catalina Island Conservancy), D Wilken, S Junak (Santa Barbara Botanic Garden), M Beauchamp (Pacific Southwest Biological Services Inc.), J Gibson (San Diego Natural History Museum), W L Bretz (UC Irvine Natural Reserve System), C Morak, M Pohl-Orf, I Schuphan (RWTH Aachen), L Frese (FAL Braunschweig), S Kaffka and R Norris (UC Davis). This study was supported by University of California Competitive Grant (#1997-980069).

REFERENCES

Abe J; Tsuda CH (1987). Genetic analysis for allozyme variation in the section *Vulgares*, genus *Beta*. Japanese Journal of Breeding **37**, 253-261.

Bartsch D; Schmidt M (1997). Influence of sugar beet breeding on populations of *Beta vulgaris* ssp. *maritima* in Italy. *Journal of Vegetation Science* **8**, 81-84.

Boudry P; Mörchen M; Saumitou-Laprade P; Vernet P; Van Dijk H (1993). The origin and evolution of weed beets: consequences for the breeding and release of herbicide-resistant transgenic sugar-beets. *Theoretical and Applied Genetics* **87**, 471-478.

Devlin B; Ellstrand NC (1989). Transmission genetics of isozyme loci in *Raphanus sativus* (Brassicaceae): stress depended non-Mendelian segregation. *American Journal of Botany* **76**, 40-46.

Heywood JS (1980). *Genetic correlates of edaphic differentiation and endemism in Gaillardia*. PhD thesis. University of Texas, Austin.

Letschert JPW (1993) *Beta* section *Beta*: biogeographical patterns of variation and taxonomy. *Wageningen Agricultural University Papers* **93**,: 153pp.

McFarlane JS (1975). Naturally occuring hybrids between sugarbeet and *Beta macrocarpa* in the Imperial Valley of California. *Journal of the American Society of Sugar Beet Technology* (Fort Collins) **18**, 245-251.

Nagamine T; Catty JP; Ford-Lloyd BV (1989). Phenotypic polymorphism and allele differentiation of allozymes in fodder beet, multigerm sugar beet and monogerm sugar beet. *Theoretical and Applied Genetics* **77**, 711-720.

O'Malley DM; Wheeler NE; Guries RP (1980). A manual for starch gel electrophoresis. *University of Wisconsin Madison Department of Forestry Staff Paper Series* **11**.

Raybould, AF; Gray AJ (1993). Genetically modified crops and hybridization with wild elatives: a UK perspective. *Journal of Applied Ecology* **30**, 199-219.

Rieseberg LH; Soltis DE (1989). Assessing the utility of allozyme number for determining ploidal level: evidence from *Helianthus* and *Heliomeris* (Asteraceae). *Aliso* **12**, 277-286.

Weeden NF; Wendel JF (1989). Genetics of plant allozymes. In: *Allozymes in plant biology*, eds DE Soltis & PS Soltis PS. pp 46-72. Dioscorides Press.

Wendel JF, Weeden NF (1989) Visualization and interpretation of plant allozymes. In: *Allozymes in plant biology*, eds DE Soltis & PS Soltis PS. pp 5-45. Dioscorides Press

Evaluating the risk of transgene spread from *Brassica napus* to related species

R Pinder, N Al-Kaff, M Kreike, P Dale
John Innes Centre, Norwich, NR4 7UH UK.

ABSTRACT

There is a need to evaluate the likelihood of the sexual spread of transgenes from genetically modified crops to wild species. We attempt to estimate this by crossing a transgenic (hemizygous) line of oilseed rape to weed species with which it has varying degrees of sexual compatibility. The offspring were selfed and backcrossed in order to examine the inheritance of the transgene in subsequent generations. We found that while the transgene was inherited in a normal Mendelian fashion for the first generation, most of the offspring were infertile. However, analysis of those that are fertile could be very informative in estimating the risk of transgene spread in different genetic backgrounds.

INTRODUCTION

Oilseed rape (*Brassica napus oleifera* AACC 2n=38) is an important oil and fodder crop in the UK. There is increasing commercial pressure to develop genetically modified lines, with for example, herbicide tolerance, disease resistance, and improved oil quality. *Brassica napus* is classed as self-fertile, but shows a degree of out-breeding. It is both insect and wind pollinated. *Sinapis arvensis* (SarSar 2n=18) is classed as an out-breeder. Chromosome pairing studies show it has some chromosomal homoeology to *B. napus*, as does *Raphanus raphanistrum* (RrRr 2n=18) (Mizushima 1980). *Sinapis arvensis* and *Raphanus raphanistrum* have been shown to be weeds in areas of *B. napus* cultivation. *B. rapa* (AA 2n=20) and *B. juncea* (AABB 2n =36) can also be found as weeds (Chevre *et al.* 1996). Thus these plants are potential hybridization partners with *B. napus* . The possibility of hybridization between *B. napus* and some of its more or less compatible relatives has been reviewed (Scheffler and Dale, 1994). There is a possibility that the 'escape' of such transgenes into the wider environment could lead to the development of, for example, herbicide-tolerant weeds that are able to survive treatment by particular herbicides. There may also be other effects from the presence of such transgenes in the gene pool of weed species. These four annual species were all used with *B. napus* (also an annual) in a crossing programme designed to generate both backcrossed and selfed hybrid lines. An embryo rescue procedure was used to obtain hybrids between *B.napus* and the poorly compatible species (*S. arvensis* and *R. raphanistrum*); and chromosome number was determined for these hybrids.

MATERIALS AND METHODS

All plants were grown in a containment glasshouse. All hybridizations were made by hand-pollination of emasculated stigmas. Self-seeding was performed by bagging the entire plant.
Plants used in the crossing program: At least four ecotypes of each of the above species were collected from different sources. (Table 1). The *B. napus* (dihaploid line W10) contained a single, stable copy of the *bar* and *npt II* genes, both were driven by the cauliflower mosaic virus 35S

promoter, and terminated by the octopine synthase terminator. The number of seed obtained per cross, as well as the proportion of viable offspring containing the transgene, was measured.

Table 1. Code number and source of the wild species used in the crossing program with transgenic *B. napus*.

Plant species	Chromosome number	Ecotype code number	Sources
Brassica juncea	(2n=36)	NAK1	Dr. R. Mithen, JIC, UK.
		NAK2	Dr. R. Jorgensen, NL., Denmark
107*		NAK3	C. Gomez-Campo, Spain
108*		NAK4	C. Gomez-Campo, Spain.
Brassica rapa	(2n=20)	NAK5	Dr. R. Jorgensen, NL., Denmark
133*		NAK6	C. Gomez-Campo, Spain
		NAK7	Dr. R. Mithen, JIC, UK.
		NAK8	Dr. R. Mithen, JIC, UK.
		NAK25	Dr. A. Meikle, SAC, UK.
Brassica napus	(2n=38)	NAK9	C. Gomez-Campo, Spain
		NAK11	Dr. Philip Dale , JIC, UK.
		NAK12	Dr. Philip Dale , JIC, UK.
		NAK13	Dr. Philip Dale , JIC, UK.
Raphanus raphanistrum	(2n=18)	NAK14	Herbiseed Ltd., UK
511*		NAK15	C. Gomez-Campo, Spain
512*		NAK16	C. Gomez-Campo, Spain
513*		NAK17	C. Gomez-Campo, Spain
Sinapis arvensis	(2n=18)	NAK18	Herbiseed Ltd., UK
		NAK19	Dr. R. Jorgensen, NL., Denmark
		NAK20	Dr. R. Jorgensen, NL., Denmark
537*		NAK21	C. Gomez-Campo, Spain
538*		NAK22	C. Gomez-Campo, Spain
		NAK23	Dr. A. Meikle, SAC, UK
		NAK24	Dr. A. Meikle, SAC, UK

* Germplasm collection catalogue number, Madrid, 1995

Embryo rescue: Hybrids generated by crossing with ecotype numbers 14 to 25 were subjected to embryo-rescue. Whole pods were removed from plants 5-10 days after pollination, then sterilised in 20% sodium hypochlorite solution (BDH) containing 5 drops of Tween 20 (Sigma) per 20 ml for 5 minutes. Pods were then washed three times with single-distilled water for 5 minutes. The pod pedicle was embedded in Nitsch medium containing 2.14 g Nitsch medium concentrate (Imperial Laboratories), 0.3 g casein hydrolysate (Oxoid), 15 g sucrose (BDH), 15 g glucose (BDH) pH 5.8 with KOH, made up to 1 litre, then 2.5 g Gelrite (Imperial Laboratories). Developed ovules were removed to MS medium containing 4.41 g MS medium (Imperial Laboratories), 10 g sucrose (BDH), pH 5.8 with KOH, made up to 1 litre, then 2.5 g Gelrite. Pods and ovules were grown under artificial light, with a 16 h photoperiod at temperature of 25 °C during light and 20 °C during dark. Developed plants were moved to Jiffy pots and transferred to the glasshouse.

Transgene detection: Initially, presence of the transgene was assayed by polymerase chain reaction for both the *npt II* and *bar* genes, but recently this has been superseded by the much faster *bar* colour assay, which relies on the expression of the *bar* gene to allow ammonia metabolism in a pH sensitive indicating medium. (D'Halluin *et al.*1992).

Bar colour assay: 5mm leaf discs were placed in microtitre plates, on liquid MS medium containing 3% sucrose (BDH), 20 mgL^{-1} phosphinothricin (Duchefa), 50 mgL^{-1} chlorophenol red (Sigma), 25 mgL^{-1} nystatin (Duchefa), 500 mgL^{-1} carbenicillin (Duchefa). The plates were sealed with transparent film, and placed at 30 °C for 16 hours.

Chromosome number: Root tips, excised in the morning, were incubated at 18 °C in 0.05 % colchicine (Sigma), then fixed in 3:1 ethanol: acetic acid for 3-7 days at –20 °C. Fixed root tips were digested in 2% cellulase (Onozuka RS), 1.5% macerozyme R200, 0.3% pectolyase Y-23 (Genetic Research Instruments) in 1mM EDTA (BDH) for 45 minutes at 37 °C, then spread on slides in a drop of fixative (Fukui *et al.*1991). After staining with Giemsa (BDH) for 2 hours, the slides were examined at X 400 magnification. At least 10 metaphase nuclei per slide were counted in order to estimate chromosome number.

RESULTS

The results of the initial pollinations are presented in Table 2.

Chromosome numbers were determined for a small number of hybrids between *B. napus* x *Raphanus raphanistrum*, *B. napus* x *Sinapis arvensis* and *B. napus* x *B. rapa*. Each of the hybrids tested had an estimated chromosome number of 28, apart from one from *B. napus* x *Raphanus raphanistrum* (NAK14), which had 56 chromosomes.

Percentage of F1 hybrids giving rise to viable F2 seeds described in Table 3.

No crosses between *B. napus and R. raphanistrum* or between *B. napus* and *S. arvensis* gave fertile hybrids. The hybrids have been maintained vegetatively by taking cuttings for over 2 years. Pollination was allowed in a contained glasshouse, but no seeds were set (Table 3).

Table 2. Results of initial pollinations.

Species	Ecotype crossed with *B. napus*	Number of buds pollinated	Number of hybrids	Efficiency	No. Offspring tested *bar* positive.	No. offspring tested *bar* negative
B. juncea	NAK 1	24	67*	31	11	13
	NAK 2	27	122	46	75	59
	NAK 3	37	187	46	22	27
	NAK 4	16	24*	31	22	5
B. rapa	NAK 5	19	104	60	30	36
	NAK 6	17	64	41	34	20
	NAK 7	37	156	40	34	17
	NAK 8	8	23*	12	1	5
	NAK 25	65	13*	98	10	3
B. napus	NAK 11	22	166	80		
	NAK 12	43	450*	87	4	3
	NAK 13	14	64	80	9	9
R. raphanistrum	NAK 14	81	1*	40	1	0
	NAK 15	68	4*	21	4	0
S. arvensis	NAK 18	38	0	79		
	NAK 19	71	0	42		
	NAK 20	80	1*	40		
	NAK 22	30	0	40		
	NAK 23	81	1*	21	0	1
	NAK 24	101	0	34		

-* Sample too small for chi-squared analysis.

- Number of putative hybrid seeds generated is given for samples 1-13; number of plants generated by embryo rescue is given for plants 14-25. Efficiency is defined as percentage of pollinated buds producing pods containing either seeds (1-13) or embryos suitable for embryo rescue (14-25).

Table 3. Percentage of F1 hybrids giving rise to viable F2 seeds.

B. napus x weed hybrid	% giving F2	*B. napus* x weed hybrid	% giving F2
NAK 1 (*B. juncea*)	4	NAK 11(*napus*)	nt
NAK 2 (*B. juncea*)	2	NAK 12(*napus*)	4
NAK 3 (*B. juncea*)	10	NAK 13(*napus*)	34
NAK 4 (*B. juncea*)	25	NAK 14 (*R. raphanistrum*)	0
NAK 5 (*B. rapa*)	30*	NAK 15 (*R. raphanistrum*)	0
NAK 6 (*B. rapa*)	nt	NAK 23 (*S. arvensis*)	0
NAK 7 (*B. rapa*)	33*	NAK 25 (*B. rapa*)	32
NAK 8 (*B. rapa*)	nt		

- *Samples gave at least 3 plants which produced F3 generation.
- nt: not tested.
-
-

DISCUSSION

Hybridizations between distantly related species:

Our poor success in generating *B.napus x S.arvensis* hybrids was not unexpected. Kerlan *et al.* (1992) had some success in generating such hybrids but only when using *B.napus* as the female parent. Lefol *et al.* (1996) estimated the probability of generating a natural hybrid between wild *S. arvensis* and *B. napus*, based on flowering period overlap, hybridization rate and hybrid reproduction. They concluded that, for the Burgundy region of France this was about 1 in 10^{10} pollinations and, extrapolating for the whole of Western Europe, that this would result in about 1000 such hybrids per year. The success of those hybrids will depend on their sexual fertility which, from the evidence available, is likely to be very low.

Hybridization with more closely related species:

Mikkelsen *et al.* (1996a) report that crossing transgenic *B. napus* with *B. rapa* can create fertile, transgenic offspring after 2 generations of hybridization and backcrossing. This is confirmed by our results with the *B. rapa* and *B. juncea* hybrids. The selfing data show that for 3 ecotypes (one B. *juncea* and two *B. rapa*) there is evidence that such hybrids might persist. Backcross data are still being generated and at present there is no indication that the transgene is inherited at a higher rate than expected. Indeed, in one *B. napus* x *B. juncea* hybrid the transgene was entirely lost in the backcross population (data not shown). This might imply that the transgene is located on the C-genome, and has been lost in meiotic pairing. This result is in contrast to a comparable study by Frello *et al.* (1995) who found the transgene in approximately half of such progeny. They concluded that gene transfer from *B. napus* to *B. juncea* is possible. Mikkelsen *et al.* (1996b) hypothesized that 'safe' areas of the chromosome might be identified for transgene insertion. This could reduce the opportunity for transgene introgression to from *B. napus* to *B. rapa* and *B. juncea*, but they concluded from the analysis of transmission of 33 predominantly C-genome-linked *B. napus* RAPD markers from *B. napus x B. juncea* hybrids back to *B. juncea*, that this was unlikely. However, Metz *et al.* (1997) provide evidence to support this hypothesis. On backcrossing a transgenic *B. napus* x *B. rapa* hybrid to *B. rapa*, there was a low frequency of transmission of the transgene. They concluded that the transgene was located on the C-genome, and that location on either the A or the C genome would influence the probability of transfer to related species.

CONCLUSION

Further work needs to be done to evaluate the risk of transgene spread from *B napus* to related species. It is likely that if transgene flow occurs, it will be to the more closely related species, *B. rapa* and *B. juncea*. The persistence of a herbicide tolerance gene in the absence of herbicide selection pressure is undetermined. There is evidence that the specific chromosomal location of the transgene will affect the persistence of the transgene in a population. Long term backcrossing data would be useful to estimate the persistence of a transgene in a wild population.

ACKNOWLEDGMENTS

We are grateful to Mr Mike Hill and Mr Damien Alger for assistance in the glasshouse.
The project was funded by MAFF, project number CSA 2915.

REFERENCES

Chevre A M; Eber F; Kerlan M C; Barret P; Festoc G; Vallee P; Renard M (1996). Interspecific gene flow as a component of risk assessment for transgenic brassicas. *Acta Horticulturae* **407,** 169-179.

D'Halluin K D; Block M; Denecke J; Janssens J; Leemans J; Reynaerts A; Botterman J (1992). The bar gene as a selectable and screenable marker in plant engineering. *Methods in Enzymology* **216**, 415-426.

Frello S; Hansen K R; Jensen J; Jorgensen R B (1995). Inheritance of rapeseed (*Brassica napus*) specific RAPD markers and a transgene in the cross *B. juncea* x *(B. juncea x B. napus)*. *Theoretical and Applied Genetics* **91**, 236-241.

Fukui K; Iijima K (1991). Somatic chromosome map of rice by imaging methods. *Theoretical and Applied Genetics* **81**, 589-596.

Kerlan M C; Chevre A M; Eber F; Baranger A, Renard M (1992). Risk assessment of outcrossing of transgenic rapeseed to related species. *Euphytica* **62**, 145-153.

Lefol E; Danielou V; Darmency H (1996). Predicting hybridization between transgenic oilseed rape and wild mustard. *Field Crops Research* **45**, 153-161

Mikkelsen T R; Andersen B; Jorgensen R B (1996a). The risk of crop transgene spread. *Nature* **380**, 3.

Mikkelsen T R; Jensen J; Jorgensen R K (1996b). Inheritance of oilseed rape *(Brassica napus)* RAPD markers in a backcross progeny with *B. campestris*. *Theoretical and Applied Genetics* **95,** 442-450.

Mizushima U (1980). Genome analysis in *Brassica* and allied genera. In: *Brassica crops and wild allies*, eds S Tsunoda; K Hinata; C Gomez-Campo, pp 89-108. Japan Scientific Societies Press, Tokyo.

Scheffler J; Dale P J (1994). Opportunities for gene transfer from transgenic oilseed rape (*Brassica napus*) to related species. *Transgenic Research* **3**, 263-278.

Estimation of allele frequencies for *Bacillus thuringiensis* resistance in diamondback moth, *Plutella xylostella* and cotton bollworm, *Helicoverpa armigera* : An isofemale line (F_2) approach

M Ahmad, R Roush
Department of Applied & Molecular Ecology, The University of Adelaide, Waite Campus, Glen Osmond, SA 5064, Australia

ABSTRACT

The detection of resistance while still at low frequencies has been identified as a regulatory issue for transgenic crops. Recently, it has been assumed that the frequency of resistance in unselected populations is about 10^{-2}-10^{-4}. Using a leaf-dip bioassay method, 4 of 943 (=3772 alleles) isofemale lines (IFLs) were detected carriers of low level resistance to *Bacillus thuringiensis* (Bt) toxins in diamondback moth, *Plutella xylostella*. The frequency of alleles for Bt resistance found in this study is much lower than estimated elsewhere. Currently, transgenic cotton plants expressing Cry1Ac protein are being used to screen for Bt resistance alleles in field populations of cotton bollworm, *Helicoverpa armigera*. We are still looking to detect a resistant IFL after screening 347 lines. This also supports our hypothesis that the frequency of Bt resistance alleles in natural populations is lower than reported recently for other bollworms.

INTRODUCTION

The current decade has been earmarked as an era of transgenic crops. Transgenic plants offer a novel approach to insect pest control especially against those pests which normally escape sprays. It is widely believed that transgenic crops have the potential to replace chemicals to a large extent, eg., transgenic cotton can reduce overall insecticide use world-wide by at least 10-15% (Fitt *et al.*, 1994; Roush, 1994).

The most critical concern for these transgenic crops and *Bacillus thuringiensis* (Bt) sprays is the evolution of resistance in the targeted insect pests. Diamondback moth (DBM), *Plutella xylostella*, a major pest of cruciferous vegetables, and cotton bollworm (CBW), *Helicoverpa armigera*, a major pest of cotton, are major targets of Bt sprays and transgenic crops, respectively. Resistance to Bt toxins in field populations of DBM has already been documented from many parts of the world (Tang *et al.*, 1997; Tabashnik *et al.*, 1992; Hama *et al.*, 1992). Transgenic cotton may exert higher selection pressure in target insect pests due to continuous expression of insecticidal proteins. Therefore, planting transgenic cotton on a large scale has the potential of selecting resistance to Bt toxins in insect pests (Kaiser, 1996). Wide-spread evolution of resistance to Bt toxins would eliminate them as a valuable pest control tool (Roush & Shelton, 1997).

Before selection, the resistance alleles are carried mainly in heterozygous (SR) individuals in accordance with Hardy-Weinberg law (p^2: 2pq: q^2) (Dobzhansky, 1955). Because routine bioassays are inefficient to detect recessive alleles in the heterozygous form, eg., many cases of Bt resistance (Tabashnik *et al.*, 1997), any technique that has the potential to improve the precision of detecting resistance would be very attractive.

The frequency of resistance alleles is one of the key factors in the effectiveness of the more promising resistance management tactics for Bt crops (Roush, 1997). Change in allele frequencies is also the key indicator of the effectiveness of a resistance management program. Most resistance management models have been based on theoretical estimates of initial allele frequencies because of scanty empirical data (Gould, 1998). The initial allele frequency for Bt resistance has become highly controversial with recently published estimates as high as 10^{-3} (Gould *et al.*, 1997; Tabashnik *et al.*, 1997). Since the alleles conferring resistance to Bt are predominantly recessive, we used an IFL technique in which insects are inbred to an F_2 to estimate the frequencies of alleles for Bt resistance in the field populations. This technique increases homozygosity and allows ready detection of recessive alleles. (Andow & Alstad, 1998).

The sample sizes needed for detection of resistance and its frequency are in inverse proportion. For example, at a frequency of 10% or more, a sample size of 100 individuals can detect resistance easily (at 95% probability level). A sample size of approximately 300 would be required for detecting a resistant individual at a frequency of 1%. When resistance frequencies are 0.1% or less, it would require sample size in thousands (Roush & Miller, 1986).

The main objective of this work was to estimate the initial frequency of alleles conferring resistance to Bt endotoxins in field populations of DBM and CBW. The implications of these results will be highly relevant for devising future resistance management strategies for biopesticides based on Bt endotoxins in general, and transgenic crops in particular.

METHODS AND MATERIALS

Isofemale lines

IFLs (single pair mating) of DBM and CBW were established by adults reared in the lab from field-collected eggs, larvae and pupae. The setting up of IFLs can be summarized as below:

Generation I	SS	X ↓	SR	(field population, in single pairs)
Generation II (F_1)	SR	X ↓	SR	(mating of siblings in mass crosses)
Generation III (F_2)	RR	SS	SR	(in lines carrying resistance, 1/16 (6%) offspring are RR)

The "SS" refers to homozygous susceptible, "SR" to heterozygotes, and "RR" to homozygous resistant. The single pair crosses of DBM were set up in clear plastic bag cages especially

designed for rearing IFLs. The plastic bags were shifted when F_1 moths emerged and wrapped around another fresh rape-seedling pot for oviposition. The larvae (F_2) were reared until 2nd - 3rd instar (~5 day old) and used for the bioassay studies.

In order to establish IFLs of CBW, one day-old male moths were paired with 2-3 day-old females in a rearing cage measuring 50x60x75 cm^3. Moths while mating were transferred gently into one litre plastic containers. Eggs were collected on kitchen paper towel and the F_1 larvae were reared on artificial diet. The moths were enclosed in cages made of clear plastic bags with tiny holes for aeration.

Plants

Canola (oilseed rape) and Chinese cabbage plants were grown in plastic pots (17.5 cm diameter) in the glass house. Usually, 7-10 day-old canola seedlings and four to six week-old Chinese cabbage plants were used for rearing and bioassays, respectively. Broccoli, *Brassica oleracea* subsp. *italica* (Green Comet-non-Bt and transgenic broccoli, expressing the Cry1Ac protein of Bt), plants were used in bioassays at Cornell University (New York). A lab resistant (Res) and a lab susceptible (G88) strain were also bioassayed for confirmation of resistance in IFLs detected from South Australia (SA).

Bt-cotton and non-Bt cotton were grown in a growth room for bioassaying CBW neonates (F_2). We used 4-6 week old plants which normally have an expression level of Cry proteins high enough to kill susceptible homozygotes (SS) and heterozygotes (SR).

Screening of IFL (F_2)

A leaf-dip bioassay method was adopted for screening Bt resistance alleles in DBM. Delfin WG (Sandoz®) containing 53,000 *Spodoptera* units per mg Bt Berliner subsp. *kurstaki* was used for screening IFL's of DBM (with a single diagnostic dose as well as multidose bioassays). On an average, 50 larvae (5 day old) from each IFL (F_2) were tested for Bt resistance. The surviving 4 of 943 IFLs from SA were tested again by using leaf-dip bioassays with a range of doses. The dose mortality data were analysed using POLO-PC (Russel *et al.*, 1977). These 4 IFLs were further tested on broccoli (expressing Cry1Ac) at Cornell University, New York.

CBW eggs from the caged F_1 adults were laid directly onto the transgenic cotton plants. When the neonates hatched, they fed directly on the plant and took a dose of Bt toxin. The survivors (if any) were saved for further confirmation of resistance.

RESULTS & DISCUSSION

Screening of diamondback moth

Each IFL allowed us to test four alleles since DBM is diploid. We tested 943 IFLs (=3772 alleles) using a diagnostic dose (@1ppm Bt *kurstaki*). The survivors were reared further and tested again to confirm the presence of resistance alleles. Twelve IFLs survived the diagnostic

dose initially. In order to eliminate false positives, we tested them again using the same diagnostic dose and consequently eight families were discarded.

Further testing of surviving IFL's of DBM

A range of doses were used to confirm the presence or absence of any major resistance genes among the surviving four families (IFL-448, IFL-563, IFL-574, IFL-712). The probit regression analysis of mortality data indicate a low level of resistance among the four IFLs detected from SA (Table 1; Figure 1). The resistance ratios calculated on the basis of LC_{50}s also indicate a low level of resistance. This was further confirmed when these IFLs were bioassayed with transgenic broccoli plants along with a highly resistant population (Res) (Tang *et al.*, 1997) and a susceptible strain (G88) at Cornell University. None of the IFLs from SA, even though they showed a low level of resistance in leaf-dip bioassays, could survive on the transgenic broccoli. The resistant population (Res) survived successfully (av. 87% and 93% for neonate and third instar larvae) on the transgenic broccoli (Figure 2). This has further strengthened our hypothesis that the frequency of alleles conferring high levels of Bt resistance is much lower than 10^{-3} in natural populations.

Table 1. Probit regression analysis of IFLs of DBM detected as carriers of low level of resistance from SA by using leaf dip bioassays.

Population	n[a]	Slope ± SE	LC_{50} mg a.i./l	LC_{90} mg a.i./l	RR[b]
IFL-448[c]	480	0.674 ± 0.07	0.12 (0.066-.205)	9.6 (4-30)	24
IFL-563	480	0.437 ± 0.06	0.082 (0.011-0.286)	71 (9-1314)	16.4
IFL-574	480	0.419 ± 0.06	0.07 (0.005-0.298)	80 (7-189429)	14
IFL-712	480	0.641 ± 0.07	0.11 (0.035-0.267)	11 (3-111)	22
GF[d]	480	0.568 ± 0.7	0.417 (0.17-0.964)	75 (19-929)	83.4
TW[e]	240	0.804 ± 0.21	0.013 (0-0.045)	0.5 (0.2-1.2)	2.6
LP[f]	240	0.547 ± 0.7	0.005 (0.002-0.010	1.1 (0.4-5.4)	1

[a] Number of larvae tested; [b] Ratio is LC_{50} divided by the lab susceptible population; [c] Isofemale line number; [d] Lab resistant population (originally from Gatton field areas, Queensland); [e] Field population (from Two Wells, SA); [f] SA lab susceptible population.

The evolution of resistance can be delayed significantly by adopting resistance management strategies. The low frequencies of Bt resistance alleles detected in DBM are quite promising for the future use of Bt sprays and for transgenic crops in SA.

Screening of cotton bollworm

We are still looking to detect a resistant family after testing 347 single families on transgenic cotton. The results obtained so far suggest that the frequency of alleles conferring Bt resistance

seems to be much lower than has been reported recently in other bollworms (eg., *Heliothis virescens).* This experiment is still under way.

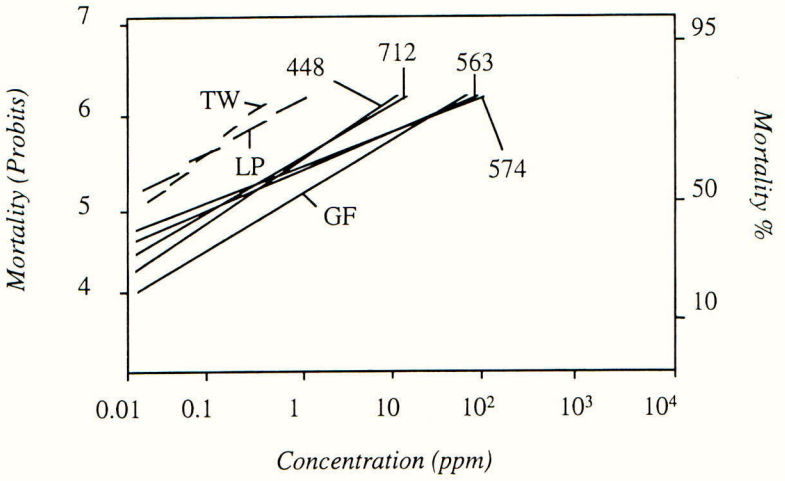

Figure 1. Genotypic responses of IFLs of DBM (448, 563, 574 and 712); lab susceptible (LP), resistant (GF) and a field population (TW) from SA.

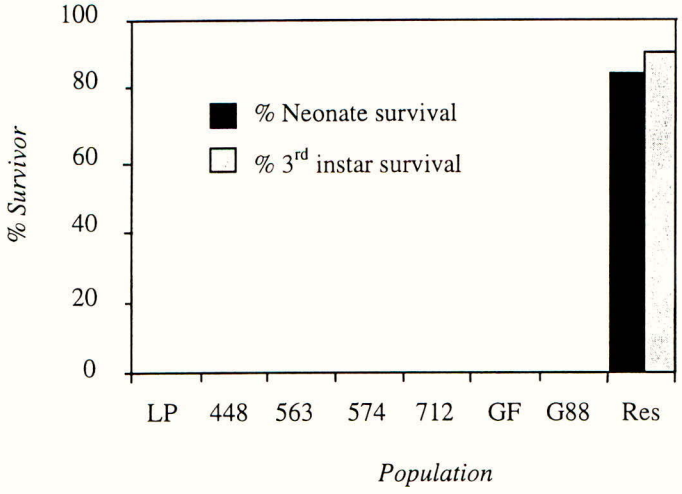

Figure 2. Response of neonate and third instar larvae of DBM to transgenic broccoli (Cry1Ac). LP refers to SA lab susceptible population; 448, 563, 574, and 712 are IFLs; GF, SA resistant lab population; G88, New York lab susceptible strain; Res, New York lab resistant strain (originally from Florida).

ACKNOWLEDGEMENTS

We thank G. Fitt, C. Tann, N. Forrester (New South Wales) and M. Cahill (Queensland) for supplying *H. armigera*.

REFERENCES

Andow D A; Alstad D N (1998). F_2 screen for rare resistance alleles. *Journal of Economic Entomology,* **91**, 572-578.

Dobzhansky, T (1955). *Evolution, genetics, and man*. John Wiley & Sons: New York.

Fitt G P; Mares C L; Llewellyn D J (1994). Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia. *Biocontrol Science and Technology,* **4**, 535-548.

Gould, F (1998). Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. *Annual Review of Entomology,* **43**, 701-26.

Gould F; Anderson A; Jones A; Sumerford D; Heckel D G; Lopez J; Micinski S; Leonard R; Laster M (1997). Initial frequency of alleles for resistance to*Bacillus thuringiensis* toxins in field populations of*Heliothis virescens. Proceedings of the National Academy of Sciences of the United States of America,* **94**, 3519-3523.

Hama H; Suzuki K; Tanaka H (1992). Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). *Applied Entomology and Zoology,* **27**, 355-362.

Kaiser J (1996). Pests overwhelm Bt cotton crop. *Science,* **273**, 423.

Roush R (1997). Managing resistance to transgenic crops. In: *Advances in insect control: The role of transgenic plants,* eds. N. Carozzi and M. Koziel, pp. 271-295. Taylor & Francis Ltd: London.

Roush R T (1994). Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? *Biocontrol Science and Technology,* **4**, 501-516.

Roush R T; Miller G L (1986). Considerations for design of insecticide resistance monitoring programs. *Journal of Economic Entomology,* **79**, 293-298.

Roush R T; Shelton A M (1997). Assessing the odds - the emergence of resistance to Bt transgenic plants. *Nature Biotechnology,* **15**, 816-817.

Russel R. M; Robertson J L; Savin N E (1977). POLO: a new computer program for probit analysis. *Bulletin of Entomological Society of America,* **23**, 209-213.

Tabashnik B E; Liu Y B; Finson N; Masson L; Heckel D G (1997). One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. *Proceedings of the National Academy of Sciences of the United States of America,* **94**, 1640-1644.

Tabashnik B E; Schwartz J M; Finson N; Johnson M W (1992). Inheritance of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, **85**, 1046-1055.

Tang J D; Gilboa S; Roush R T; Shelton A M (1997). Inheritance, stability, and lack-of-fitness costs of field-selected resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae) from Florida. *Journal of Economic Entomology,* **90**, 732-741.