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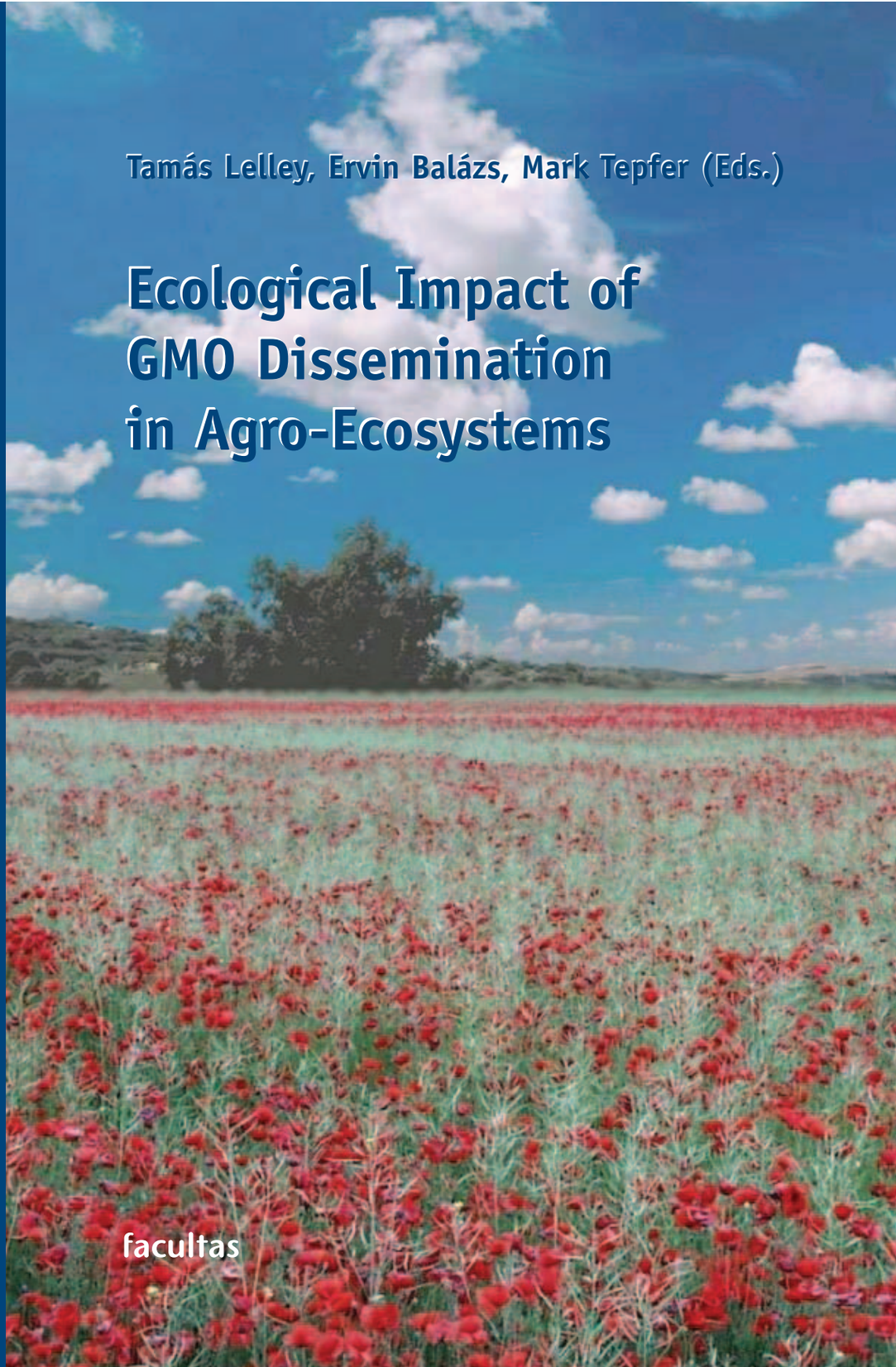

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Ecological Impact of GMO Dissemination in Agro-Ecosystems

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at an
International OECD Workshop held in
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Edited by
Tamás Lelley, Ervin Balázs, and Mark Tepfer

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PREFACE

These Proceedings evolved from the OECD Cooperative Research programme workshop on "Ecological impact of GMO Dissemination in Agro-ecosystems", held at Grossrussbach, Austria on 27-28 September 2002.

The OECD Cooperative research programme for "Biological Resource Management" has existed since 1990. It focuses on work in four specific areas, one of which is "New agricultural products for sustainable farming and industry" (Theme 1). This theme includes new approaches and possibilities for production of valuable materials and substances within agricultural systems, which could have large-scale effect at the farm level and for farming communities as a whole. It proposes to widen the variety of goods offered by the agricultural producers, and to encourage and increase opportunities to manage engineered crops, including raw materials for the fine chemical industry.

The topic areas include:

transgenic organisms in agriculture

new plant protection strategies for sustainable agriculture

engineered non-food crops

energy crops

production of specialty crops with potential wide-scale importance

novel ways to utilise and recycle agricultural wastes

molecular farming for speciality products.

The other themes are "Quality of animal products and safety of food", "Enhancing environmental quality in agricultural systems" and "Connecting scientific progress to sustainable and integrated agro-food systems".

The activities promoted by this programme are post-doctoral fellowships (announced annually) and the organisation of expert workshops (1-2 workshops per Theme per year). Currently participating countries in the programme are: Australia, Austria, Belgium, Canada, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Japan, Korea, Netherlands, New Zealand, Norway, Poland, Portugal, Slovak Republic, Spain, Sweden, Switzerland, Turkey, United Kingdom and the United States.

For further information about the OECD program, contact Dr. Françoise Coudert, Head of the Programme, Directorate for Food, Agriculture and Fisheries, OECD, 2 rue André-Pascal, F-75775, Paris Cedex France (e-mail: Francoise.Coudert@oecd.org), or consult the Internet site of the OECD (http//www.oecd.org/agr/prog)/.

The workshop on the dissemination of GMOs in agro-ecosystems was motivated by the increasing use of genetically modified organisms both in contained use and in deliberate releases. GMO use in agricultural settings is spectacularly increasing at the global scale (19 % in 2001 up to 51.7 million hectares), yet controversy remains concerning their potential impact in agro-ecosystems. One of the points that many believe requires further enquiry is the question of the fate of GMOs, and more precisely of the transgenes they bear, in the environment. Our major concern was to convene those experts who are world-wide most actively working in this field, in the expectation that on the basis of their latest results we will be able to separate facts from fictions, anticipating that this will be of use to the decision makers and legislators responsible for releases.

Grossrussbach, September 27, 2002.

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Bacillus thuringiensis

General concepts

Genetically modified microbes

DESIGNING IMPROVED GM BACTERIA FOR APPLICATION IN ENVIRONMENTAL BIOTECHNOLOGY

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Abstract

Rhizosphere competent fluorescent pseudomonads are ideal candidates for utilization in agri-industrial applications such as biocontrol and bioremediation. Direct links between biocontrol efficacy and production of anti-microbial compounds have emerged *via* utilization of recombinant DNA technology. Production of antimicrobial 2,4-diacetylphloroglucinol (Phl) is the central mechanism utilized by *P. fluorescens* F113 in biocontrol. Innovative design strategies based on reprogramming regulatory mechanisms *via* functional genomics can be employed to improve biocontrol efficacy of *Pseudomonas* inoculants. Furthermore Pseudomonads can be used as ideal carriers for polychlorinated biphenyl (PCB) degradative genes. Novel rhizoremediation derivatives of *P. fluorescens* F113 are being developed in which PCB degradative genes are regulated by specific root-exudate components to allow for controlled degradation in contaminated biosystems. However, public concerns as to the biosafety of genetically modified bacterial strains in the environment must be considered. Developments in molecular microbial technology have facilitated assessment as to the impact of bacterial inoculants on soil-borne non-target microbial communities. Genetically modified derivatives of *P. fluorescens* F113 had no significant influence on indigenous soil microbiota. *in vivo* expression technology can now be utilized to improve monitoring of both survival and activity of GM microbial inoculants in the environment. Signalling occurring between microbial population in the environment must also be considered in order to evaluate novel GM microbial inoculants. Unidentified signal molecules produced by the phytopathogen *Pythium* downregulate genes in *P. fluorescens*. A novel gene *espR*, influential in rhizosphere fitness has been identified in *P. fluorescens*. Innovative design systems based on genomic technologies can be adopted to improve GM bacteria facilitating biosafety and subsequent registration of microbial plant products used in environmental biotechnology applications such as biocontrol and bioremediation

Keywords: GMOs; Secondary metabolites; Biocontrol; Bioremediation; Biosafety; Plant Protection Products (PPPs); Microbial Signalling

Introduction

Soil exhibits a number of key environmental, social and economic functions, existing as a matrix for water, nutrients, gene pools, gas exchange and has a buffering capacity. Human activities such as industry and agriculture can lead to the deterioration of the soil, diminishing its fertility and subsequent function. Degradation processes are increasing across Europe and are being exacerbated by current changes in climatic conditions. The Sustainable Development Strategy presented by the EU Commission in 2001 (EU Commission 2001) emphasized that soil loss and decreasing fertility are degrading the viability of agricultural land and poses a real threat to sustainable development in environmental, social and economic contexts. This together with implementation of EU directives to reduce pesticide and fertilizer usage encourages adoption of alternative approaches for agri-industrial management. Utilization of microbial inoculants for sustainable agri-industrial applications such as phytostimulation, biofertilization, bioremediation and biocontrol has been the subject of a number of recent reviews (Bloemberg and Lugtenburg 2001; deLorenzo *et al*. 2001; Haas *et al*. 2001; Morrissey *et al*. 2002; Vance *et al*. 2000; Walsh *et al*. 2001). Limitations such as ecological fitness, low metabolite production, variability in inoculant delivery systems, and inconsistent performance in field applications have restricted the success of microbial inoculants as alternatives to conventional agri-industrial approaches. The advent of recombinant DNA technology has made it possible to utilize genetic modification strategies to potentially overcome these limitations previously displayed by first generation wild-type microbial inoculants. This review focuses on innovative design systems based on genomic technologies that can be adopted to improve GM pseudomonads and facilitate subsequent registration of microbial plant products used in environmental biotechnology applications such as biocontrol and bioremediation.

Designing improved GM microbial inoculants for agri-industrial applications

GM Biocontrol inoculants

Phenotypic and genotypic traits characteristic of the fluorescent soil-borne pseudomonads have distinguished this group of rhizobacteria as ideal candidates for biocontrol applications (Bangera and Thomashow 1999; Dowling and O'Gara 1994; Nowak-Thompson *et al*. 1999; Sharifi-Tehrani *et al*. 1998). Recombinant DNA technology has provided a direct link between biocontrol efficacy of soil-borne pseudomonads and secondary metabolite production (Bangera and Thomashow 1999; Chin-A-Woeng *et al*. 1998; Fenton *et al*. 1992; Keel *et al*. 1992; Thrane *et al*. 2000). Production of the secondary antifungal metabolite 2,4-diacetylphloroglucinol (Phl) by *Pseudomonas fluorescens* F113 is the central

mechanism in the inhibition of *Pythium ultimum* the causal agent of damping-off disease of sugarbeet (Shanahan *et al.* 1992). Increased frequency of Phl producing pseudomonads is evident in soils suppressive to Take-all disease of wheat (Raaijmakers *et al*. 1997).

Walsh *et al*. (2001) schematically illustrated design strategies that can be implemented to develop improved *Pseudomonas* biocontrol strains. One such strategy to enhance rhizosphere competence in GM microbial inoculants is the introduction of additional genes. Complexicity in the rhizosphere environment has lead to an array of genes being identified that are involved in ecological fitness of microbial inoculants. Genes identified as playing a role in rhizosphere competence of pseudomonads include *sss* genes (Dekkers *et al*. 1998) and *rhi* genes identified by *in vivo* expression technology (IVET) (Rainey 1999; Rainey and Preston 2000). Introduction of additional copies of *sss* colonisation genes into wildtype *Pseudomonas* conferred enhanced colonisation (Dekkers *et al*. 2000). Exploitation of functional genomics has made it possible to design improved biocontrol strains *via* genomic reprogramming of regulatory systems governing production of antifungal metabolites by *Pseudomonas* biocontrol strains. The Phl biosynthetic gene cluster (Fig 1.) encodes for the production of Phl in *P. fluorescens* F113. Regulation of Phl production occurs in *P. fluorescens* F113 by a pathway specific repressor PhlF at a transcriptional level and occurs in early log phase of growth *via* interaction of PhlF with a binding site *phO* located downstream of the *phlA* transcriptional start site (Abbas *et al*. 2002). In addition to this transcriptional control, Phl production is also regulated at the post-transcriptional level. This is mediated by interaction of a regulatory RNA molecule and a cognative repressor in *P. fluorescens* F113 (Aarons *et al*. 2000). Uncoupling transcriptional regulatory controls in GM *Pseudomonas* inoculants results in improved biocontrol efficacy exhibited by re-regulated strains (Abbas *et al*. 2002; Delany *et al*. 2000; Delany *et al*. 2001, Fig. 1). Mutation of *phlF* gene in *P. fluorescens* F113 enhanced Phl production *in vitro* during early log phase of growth and overexpression of the Phl biosynthetic genes resulted in over production of Phl. Enhanced biocontrol efficacy of GM Phl overproducing strains against *Pythium* was observed in soil-based microcosm trials (Delany *et al*. 2000). GM *P. fluorescens* Phl overproducing strains also protected sugarbeet against Rhizomania infection in soil-based microcosm trials (Resca *et al*. 2001)

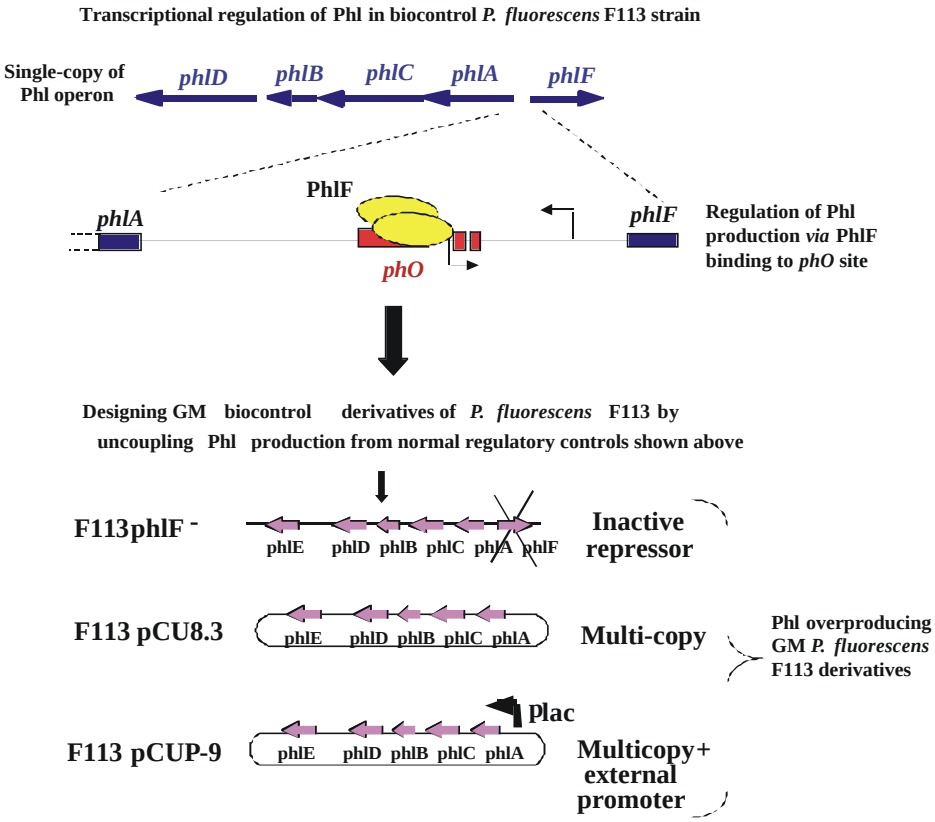

Fig. 1: Strategies utilized to design GM *P. fluorescens* F113 biocontrol inoculants. Transcriptional regulatory controls for Phl production in *P. fluorescens* F113 were uncoupled to produce GM Phl overproducing derivatives (adapted from Abbas *et al*. 2002; Delany *et al*. 2000; Delany *et al*. 2001).

GM bioremediation inoculants

Polychlorinated biphenyls (PCBs) are widely utilized in industrial applications and by 1980s 1.5 million tonnes had been produced worldwide (Abraham *et al*. 2002). Pervasive distribution of PCBs together with characteristic high chemical stability, poor degradation rates *in situ*, superhydrophobicity and toxicity make this class of compound a serious risk to the environment (Abraham *et al*. 2002). GM *P. fluorescens* HK44 strain for

bioremediation application was the first GMO field release conducted at Oakridge National University and University of Tennessee (Ripp *et al*. 2000; Sayler *et al*. 1999). *P. fluorescens* HK44 was genetically modified *by* introduction of a naphthalene catabolic plasmid together with a transposon-based bioluminescence producing *lux* gene fused with a promoter for naphlathane catabolic genes (Chatterjee and Merighen 1995). GM *P. fluorescens* strain HK44 displayed enhanced naphthalene gene expression, leading to boosted degradation rates of the contaminant. GM microbial inoculant survival can be unpredictable *in situ* so at the design stage consideration as to the metabolic load exerted on the bacterial strain due to introduction of foreign genetic elements must be assessed (Ripp *et al*. 2000; Sayler *et al*. 1999).

Ecological fitness of the GM *P. fluorescens* HK44 was not altered in the field, where the GM inoculant displayed persistence and competed well with the indigenous microbial populations (Ripp *et al*. 2000; Sayler *et al*. 1999). Under an EU-funded programme approaches that integrate plant/GEM systems for *in situ* bioremediation are being investigated. Plant root exudates have been shown to stimulate rhizosphere bacteria at levels of 10^6- 10^8 CFUs per g root. Plant exudates responsive promoters (P*ex*) have been identified (Biomerit Research Centre, unpublished data). *bph* genes were cloned from *Burkholderia cepacia* (LB400) and integrated into the chromosome of *P. fluorescens* F113, together with P*ex* promoters in order to design novel bioremediation inoculants with boosted PCB degradation capabilities. Fig. 2 illustrates the strategy utilized in reprogramming the *bcp* operon in *P. fluorescens* F113. This approach is being followed in parallel with evaluation of GM *Pseudomonas* strains carrying the *B. cepacia* LB400 *bph* operon under its native promoter (Brazil *et al*. 1995).

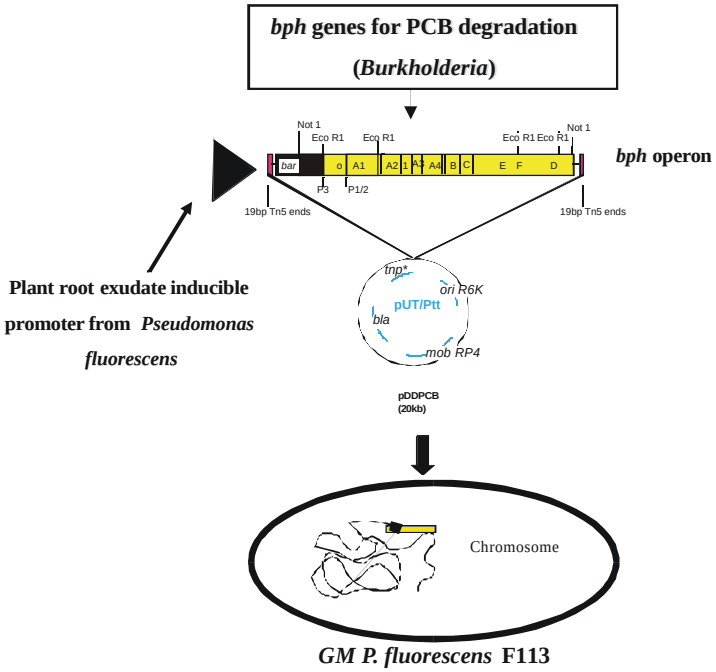

Fig. 2: Design strategy to produce GM bioremediation inoculants with boosted *bph* expression and subsequent enhanced PCB degradation capabilities (Brazil *et al*. 1995)

Biosafety assessment of GM microbial inoculants

Statutory requirements for deliberate release, registration and commercialisation of GM microbial inoculants as plant protection products are reviewed in detail by Morrissey *et al*. (2002). Environmental risk assessment of introduced GM microbial inoculants can be evaluated by assessing the impact of GM strains on non-target soil microbiota at the research stage followed by subsequent field-testing in ecosystems that could be affected by GMO release.

Base-line evaluation of wild-type microbial inoculants needs to be conducted prior to assessing GM inoculant impact. Field trials adopting a sugarbeet-clover rotation allowed for baseline evaluation of the impact of *P. fluorescens* F113 wildtype on key bacterial groups such as pseudomonads and *Rhizobium* (Moënne-Loccoz *et al*. 2001, 2002). *P. fluorescens* F113 WT colonised sugarbeet

roots successfully and had no influence on the number of resident culturable fluorescent pseudomonads (RCFP) either in the rhizosphere or rhizoplane. RCFP community structure was defined into 4 phylogenetic ARDRA groupings and RAPD analysis indicated a high level of strain diversity within these groups. Proportional distribution of RCFP within these defined ARDRA groupings and degree of strain diversity was not affected by *P. fluorescens* F113 WT applied as a seed inoculant. Mahaffee and Kloepper (1997) also showed that seed inoculation of cucumber roots with *P. fluorescens* 89B-27 did not have an impact on the genetic diversity of culturable aerobic bacteria in the rhizosphere. *P. fluorescens* F113Rif induced a shift in the phenotypic composition (based on carbon utilization) of RCFP. However, this shift was spatially restricted to the rhizoplane and occurred without influencing the RCFP diversity, and was not due to production of Phl (Moënne-Loccoz *et al*. 2001).

Rhizobium leguminosarum bv. *trifolii* prevalent in Irish agricultural soils (Meade *et al*. 1983) is an ideal bioindicator of potential perturbation caused by introduction of bacterial inoculant. Impact of *P. fluorescens* F113 WT on populations of *R. leguminosarum* bv. *trifolii* associated with clover nodules was assessed in the field (Moënne-Loccoz *et al*. 2002). A high degree of richness was prevalent in the *R. leguminosarum* bv. *trifolii* populations isolates from clover nodules where 47 different RAPD clusters were observed. Seed inoculation with *P. fluorescens* F113 lead to a 20% reduction in number of RAPD clusters observed and a 10% reduction in richness and evenness and resulted in enrichment of the dominant RAPD 1 cluster (Moënne-Loccoz *et al*. 2002). *Rhizobium* isolates tend to be more sensitive to Phl than fluorescent Pseudomonads **(**Natsch *et al*. 1998; Moënne-Loccoz *et al.* 2001). Introduction of Phl producing F113 enriched for isolates belonging to RAPD-1 cluster that were tolerant to Phl. Nodulation scores combining nodule frequency, distribution and size usually correlate with plant growth indices such as foliage biomass and nitrogen content (Brockwell *et al*. 1982). Despite the minor reduction in the diversity of *R. leguminosarum* populations in clover nodules due to *P. fluorescens* F113 WT applied as a seed inoculant no impact on nodulation scores, foliage biomass or nitrogen content was observed (Moënne-Loccoz *et al*. 1997; 1998). Therefore, the impact of *P. fluorescens* F113 WT on *Rhizobium* diversity did not appear to influence the functioning of the clover-*Rhizobium* symbiosis (Moënne-Loccoz *et al*. 1998; 2002).

IMPACT II, an EU funded project investigated the biosafety of *P. fluorescens* F113 genetically modified to overproduce Phl in field trials. Arbuscular mycorrhiza can also be used as a target model system for biosafety assessment of GMOs (Barea *et al*. 1996, 1998). Notification of field release of *P. fluorescens* F113 genetically modified to over produce Phl under the EU directive for deliberate release of GMOs (90/220/EC) occurred in Nov 1997 at Granada, Spain

with a vetch–maize crop rotation. Overproducing Phl *P. fluorescens* F113 derivative had no impact on number of diversity of key AM fungal groups, *Entrophospora infrequens*, *Glomus* spp. 1, 2, 3 or *G. mosseae*. The GM *P. fluorescens* F113 derivative also had no impact on mycorrhiza formation (Barea *et al*. unpublished). Notification for the field release (under EU directive 90/220/EC) of GM *P. fluorescens* F113 Phl over-producing derivative in Ravenna, Italy occurred in May 1998. GM *P. fluorescens* strain had no impact on the numbers of soil microbiota including culturable bacteria and microfungi and key rhizobacteria namely fluorescent pseudomonads and S*treptomycetes* (Resca *et al*. 2001).

Monitoring GM microbial inoculants *in situ*

The unpredictability of biocontrol and bioremediation microbial inoculants due to poor survival rates and variable bioavailability *in situ* highlights the need to have adequate monitoring strategies. Monitoring persistence and distribution of GM microbial inoculants in the environment also must be considered. Public concerns exist as to the use of antibiotic resistance markers as a method to determine survival and spatial and temporal distribution of GM microbial inoculants in the environment. There is now a de-facto moratorium on the use of such markers in Europe. Survival of bacteria and gene expression *in situ* for biocontrol applications can now be monitored using innovative fluorescent markers. Lübeck *et al*. (2000) showed the presence of *P. fluorescens* DR54 on sugarbeet rhizosphere using FISH technology**.** Incorporation of rhizosphere stable vectors such as broad-host range plasmids expressing fluorescent protein markers such as e-gfp, e-yfp, e-cfp or rfp have been constructed to track the presence of GM inoculants at the plant-soil interface (Bloemberg *et al*. 2000; Tombolini *et al*. 1997; Stuurman *et al*. 2000). Localisation, viability and activity of biocontrol P. fluorescens strain DR54-BN14 in barley rhizosphere was analysed successfully using gfp marker (Normander *et al*. 1999). Dual marker systems incorporating *gfp* and *lux*AB genes encoding green fluorescent protein and bacterial luciferase, respectively, promises to be a useful monitoring tool for presence and activity of GM bacterial strains in environmental samples. Unge and colleagues (1999) successfully monitored cell number and metabolic activity of *P. fluorescens* SBW25 in soil using *gfp/lux*AB marker system.

Bioremediation is a cost effective alternative to conventional incineration or landfill methods for clean-up of contaminated land (Swannell 1998; Zechendorf 1999). Monitoring the progress of pollutant degradation *via* expensive chemical methods can hamper economic viability of bioremediation approaches (Sayler and Ripp 2000). GM bioremediation inoculant *P. fluorescens* HK44 incorporated a transposon-based bioluminescence producing lux gene (Chattergee and

Merighen 1995). The bioluminescence of the GM HK44 inoculant can provide a novel strategy for continuous monitoring of bioavalibility and inoculant presence *in situ* (Sayler and Ripp 2000). Improvement of monitoring systems has the potential to enhancing economic viability of GM microbial inoculants used in both biocontrol and bioremediation applications.

Future considerations in the implementation of design strategies for GM microbial inoculants

IMPACT 1 and II EU-funded projects have provided evidence on biosafety of novel GM *P. fluorescens* inoculants for use in biocontrol applications. GM *P. fluorescens* inoculants displaying enhanced biocontrol efficacy had no ecological impact on non-target microorganisms in the environment. However, recent scientific findings concerning interactions between microbial populations in the soil have provided further insight into areas that need to be addressed in order to enhance the design and development of GM microbial inoculants for use in agri-industrial applications. Cell-density-dependent control of gene expression (quorum sensing, QS) has been identified as important in modulating production of secondary metabolites. Laue *et al.* (2000) reported that *P. fluorescens* F113 produces a number of signal molecules identified as *N*-acylhomoserine lactones. Bacterial signal molecules have the potential to play an ecologically significant role in biocontrol efficacy of *P. fluorescens*.

Another consideration is the interaction of target microbial populations in the soil and GM inoculants. Notz and colleagues (2002) provided evidence that *Fusarium oxysporum* strains producing fusaric acid altered Phl biosynthetic gene expression in biocontrol inoculant *P. fluorescens* CHAO both *in vitro* and in the rhizosphere. *P. ultimum* produces signal molecules that have the potential to downregulate gene in *P. fluorescens* F113 (Fedi *et al*. 1997). Using transposon mutagenesis, a number of ecologically important genes that are down-regulated by *Pythium* signal molecules have been identified. These include *gltB*, involved in nitrogen assimilation, and genes responsible for adaptation to changing environments such as *rrnB, rrnC* and *espR* (Morris *et al*. unpublished data; Smith *et al*. 1999). Addressing the role of signalling between soil microbial communities together with adoption of enhanced monitoring technology have the potential to enhance design strategies for GM inoculants. This potentially will facilitate future registration and commercialisation options for GM microbial inoculants with enhanced efficacy for utilization in agri-industrial applications such as biocontrol and bioremediation.

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IMPACT OF MARKER AND FUNCTIONAL GENES ON SOILS AND PLANTS

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Abstract

Determination of baseline perturbations of soil microbial communities and functions are illustrated. The impact of wild-type and genetically-modified microorganisms is usually small compared with conventional agricultural practices. The microorganisms can improve plant growth and nutrition, decrease plant stress and facilitate bioremediation. However, genetic modification can decrease the biological fitness of the organisms compared with the parent wild-type, potentially reducing environmental impact.

Keywords: Bioremediation, fitness, growth, nutrition, rhizosphere

Introduction

A major OECD conference on living modified organisms and the environment was held in late 2001 in Raleigh NC. The microbial aspects were covered there (Lynch 2002) and therefore this paper is a brief summary of that presentation.

Micro-organisms, have been used extensively in crop protection as well as bioremediation, and are seen as primary targets for genetic modification to improve performance. The rhizosphere is a site of particular interest because 40% of the plant's photosynthate captured is released by the roots as rhizodeposition products and therefore available to the soil biota (Lynch and Whipps, 1990). This is therefore the energy powerhouse of the soil ecosystem and is also the site at which introduced genes might exert greatest influence.

In the early investigations, some of the primary concern was whether there would be horizontal gene transfer from the introduced GMOs. In our own *in vitro* studies on plasmid exchange between rhizosphere bacteria *Enterobacter cloacae* one transconjugant was produced per 10^4 and 10^6 donors or recipients, but this was heavily dependent on the flow rate down the column and the distance from the hollow fibre (Pearce et al. 2001). This rate is comparable with the plasmid exchange frequencies which have been observed for other soil bacteria. However gene exchange has generally not been observed from chromosomally-borne genes. Plasmid fluidity between wild types is a natural occurrence, but antibiotic marker or functional genes

marking would have uncertain consequences and therefore it seems reasonable that chromosonal genetic modification is the safest option.

Traditionally monitoring of indigenous microbial populations in soil has centered on the enumeration of specific populations. However, for a significant perturbation to be measured, changes of between 100% and 300% (0.3 and 0.5 on a log scale) are necessary for the impact to be significant. Moreover, there is the problem of non-culturability. We have found the most effective way to quantitatively assess the effect of introduced GM organisms on culturables is to determine the *r* and *K* strategists from different habitats and to formulate an ecophysiological index (De Leij et al. 1994).

Marker Genes

The first release of a free-living GMO bacterium in the UK was carried out during 1993 and 1994 on spring wheat in a silt loam at Littlehampton, West Sussex, and on sugar beet in a heavy clay soil in Oxford. The bacterium *Pseudomonas fluorescens* SBW 25 was isolated from the phylloplane of sugar beet, but it was also shown to readily colonise the rhizosphere of sugar beet, as well as the phylloplane and rhizosphere of wheat. The marker genes (*lacZY* and *kan^{r}xylE*) were chosen to facilitate identification and detection of the GMO by simple culture methods and positioned 1 Mb apart on the 6.5Mb chromosome to ensure genotypic and phenotypic stability, as well as facilitating any gene exchange between microbial populations associated with the two crops. At the Ee site lacZY (4.0kb) was inserted and at the 6 site, *kan^r-xylE* (7.2kb) was inserted (Rainey and Bailey 1996).

The full account of the studies have been reviewed (De Leij et al. 1998a). In terms of impact the conclusions drawn are summarised in Table 1.

Table 1: Ecological Effects of *Pseudomonas fluorescens* SBW 25 EeZY-KX

Effect	Wheat	Sugar Beet
Survival and establishment	$>10^6$cfu^{-1}g root during season and 7 months after harvest	Up to 5 x 10^6cfu^{-1}g senescent leaves
Dissemination	Vertical > 45cm Lateral > 2m Colonised volunteer and resown plants and weeds	Vertical < 10 cm Lateral < 10cm Colonised volunteer and resown plants and weeds

There was no movement of the marker genes and only very small and transient effects on indigenous communities. Subsequent studies carried out (De Leij et al. 1998b) investigated the potential metabolic burden of the inserted genes on the ecological competence of a variety of constructs that were modified with the markers genes used in the release study strain of the bacterium. Whereas the kanamycin resistance did not seem to affect the

fitness of the organism, both of the other marker inserts did reduce ecological competence. The conclusion therefore is that even though the modified bacterium was competent in the field, the wild-type is even more competent.

Functional Genes

The DAPG Gene

Pseudomonas fluorescens F113 had been isolated from sugar beet and found to produce the antibiotic 2, 4-diacetylphloroglucinol (DAPG) (Shanahan et al. 1992). Besides being active against *Pythium* damping-off, it was also active against the potato soft-rot pathogen *Erwinia carotovora* subsp *atroseptica* and the potato cyst nematode *Globodera rostochiensis*. For comparative purposes, strain F113 G22 was constructed which is a Tn5::lacZY DAPG-negative derivative of F113 that does not have the ability to inhibit the growth of plant pathogenic fungi (Shanahan et al. 1992).

A study of the impact of the *Pseudomonas* strains on the rhizosphere was carried out. One of the main approaches was to determine the effect on the rhizosphere/soil enzymes N-acetyl glucosaminidase, chitobiosidase, acid and alkaline phosphatase, phosphodiesterase, aryl sulfatase and urease, being representative enzymes in the carbon, nitrogen, phosphorus and sulphur cycles in soil (Naseby and Lynch 1997). The results were published in a series of papers and are summarised in Table 2.

Table 2: Effect of *Pseudomonas fluorescens* on F113 producing the antibiotic DAPG on rhizosphere enzymes.

Plant	Increases	Decreases
Pea	alkaline phosphatase aryl sulphatase urease	B-glucosidase NAGase
Wheat	alkaline phosphatase	chitobiosidase aryl sulfatase urease

In a further series of studies the mineralization and uptake of ^{15}N-enriched wheat residues were studied (Brimecombe et al. 2000). Inoculation of pea seeds with *P. fluorescens* F113 or F113G22 increased mineralization and uptake of organic N in the rhizosphere. In contrast the inoculation of the same strains onto wheat seeds reduced mineralization and uptake. The explanation seems to be that inoculation of pea resulted in an increase in the number of nematodes and protozoa in the rhizosphere, but for wheat there was a decrease in the microfauna, which stimulate the mineralization of organic carbon. The inoculants in pea could catabolise nematicidal

compounds. This is therefore a clear benefit of the inoculants but took place irrespective of whether they had been modified or not.

As a further aspect of inoculation effects of nitrogen cycling in the rhizosphere, the impact on nodulation of peas was studied. Nodulation was increased, but only with the DAPG-producing strain of the bacterium (Andrade et al. 1998). Thus this beneficial effect was deleted by the genetic modification of the wild-type. In our most recent studies using lysimeters we have found that bacterial effects on nitrate-leaching were independent of genetic modification, and that the bacterial effects were smaller than those caused by stimulated ploughing (Bell-Perkins and Lynch, unpublished). Generally it seems likely conventional agricultural practices are likely to have more impact on soil function.

ACC Deaminase

Ethylene has a range of effects on plants. Most notably it is the classical inhibitor of root growth in flooded soils, either from endogenous root production causing arenchyma (air spaces) to form in roots, or from exogenous microbial sources. 1-aminocyclopropane-1-carboxylate (ACC) is synthesised in roots and transported to plant shoots where it is converted to ethylene by ACC oxidase. The synthesis of ethylene can be inhibited by the enzyme ACC deaminase. The ACC deaminase has been found in a range of strains of rhizosphere bacteria (*Enterobacter cloacae*, *Pseudomonas* spp, *Kluyvera ascorbata*) which appear to promote plant growth by inhibiting ethylene stress (Burd et al. 1998; Grichko and Glick 2001; Wang et al. 2000). The plants not only become flood-tolerant, but the de-stressing effect enable them to accumulate heavy metals and therefore become potential agents of bioremediation. Thus microbial inoculants with abilities to enhance bioremediation might become a very exciting new initiative to improve the soil environment.

Conclusions

Baseline ecology needs to be established to determine perturbation effects. Some conclusions which can be drawn from the studies thus far can be summarised as:

?? Gene products are better indicators of change than populations.

?? Gene exchange is mainly mediated by plasmids.

?? Field impacts of GMOs are generally smaller than impacts of conventional agricultural practices.

?? GMOs can carry metabolic loads which reduce ecological fitness.

?? GMOs can influence microbe/faunal interactions which regulate plant nutrition.

?? ACC deaminase can decrease plant stress and facilitate bioremediation.

The study of GM micro-organisms applied to the environment has greatly extended our knowledge base in microbial ecology and the use of molecular methods will continue to extend that base. However, with the lack of ecological fitness of many GM strains increasingly found, and the public concern of the use of GM organisms in the environment, the selection and application of new and improved wild-type strains from the environment with our new knowledge base seems to offer the greatest potential for commercial exploitation.

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DISSEMINATION OF GENETICALLY ENGINEERED MICROORGANISMS IN TERRESTRIAL ECOSYSTEMS – CASE STUDIES FOR IDENTIFYING RISK POTENTIALS

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Abstract

The vast majority of genetically engineered organisms are microorganisms, mainly bacteria. They are engineered for different purposes. The majority is used for cloning and expression of genes to study their structure and function. Other genetically engineered microorganisms (GMOs) are used as vectors to transfer genes from one into another organism. On the other hand, GMOs are developed for biotechnological applications. In the latter context microorganisms may be designed to work in closed systems, i.e., bioreactors, or in the environment, e.g., as biofertilizers or biocontrol agents. Before GMOs are constructed and used, it is important to consider their potential hazard for the environment. Due to the different properties and applications of GMOs, an environmental risk assessment should be based on a case-by-case evaluation. Here we report on case studies that have been performed in our laboratory during the last decade to identify risk potentials as a consequence of non-deliberate and deliberate releases of GMOs. Risk relevant aspects covered by our studies relate to the following aspects: (1) fitness changes of engineered versus non-engineered strains and their survival in soil, (2) expression of recombinant genes in soil, (3) displacement of indigenous by recombinant bacteria in ecological niches, (4) resilience to remove GMOs from soil, and (5) gene transfer from non-indigenous to indigenous bacteria. The results and conclusions from our case studies should contribute to a safer GMO risk-to-benefit analyses in the future. It should be noted that especially small scale field releases with non-hazardous marker-gene tagged soil bacteria were highly useful for gathering information about the ecological fate of a specific bacterial strain. Such information is important for future biosafety assessments of GMOs.

Keywords: GMO, field releases, risk assessment, biosafety

Diversity of genetically engineered microorganisms (GMOs) and their target environments

GMOs for laboratory, production and field applications

Genetic engineering is a laboratory method and tool to add specific genes to the genome of another organism or remove genes from a genome. The vast majority of genetic engineering work is done with microorganisms, mainly *Escherichia coli* strains or with yeast, the latter as a model eukaryotic organism. Even for investigations that have no specific interest in microbiology, microorganisms are used for gene cloning in order to sequence genes or to use them as expression systems for the study of activities and gene regulation.

Other microorganisms, however, are constructed to survive in large scale bioreactors or even in the environment, at least for a certain period of time. Different types of GMO applications and their environmental range are indicated in Figure 1. It should be emphasized that most GMOs are those engineered for laboratory use only.

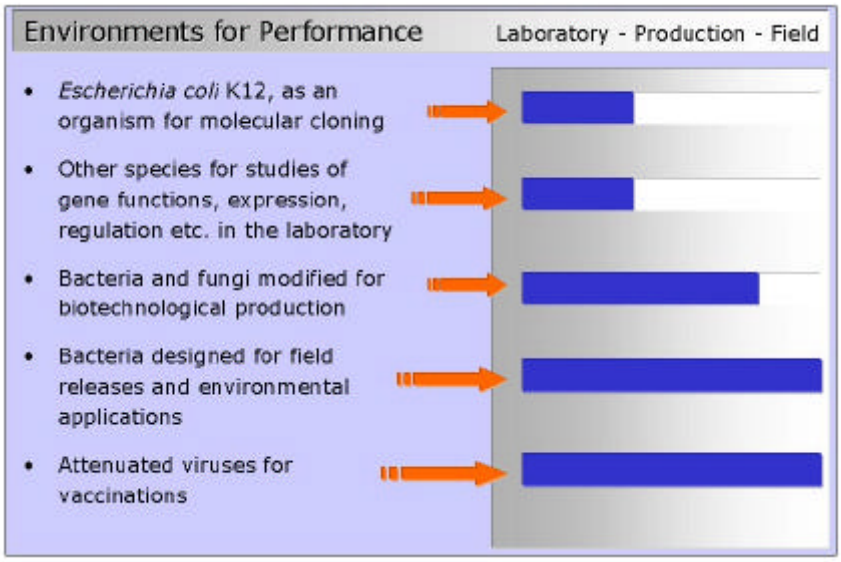

Fig. 1: Target environments for genetically engineered microorganisms

Environmental range of GMOs

The major ”work horse“ for genetic engineering are bacteria derived from the strain *E. coli*-K12. *E. coli* are gut bacteria that grow fast under laboratory conditions. *E. coli* K12 and its derived strains are not able to modify and destroy foreign DNA. In contrast to their wild-type relatives, *E. coli* K12

strains are non-pathogenic and they require special growth factors. The need for additional growth factors limits their capacity to grow outside of the laboratory.

Genetic engineering at the laboratory scale is also done with other bacterial species, since *E. coli* cannot be a model for all microorganisms. To date, there is a broad range of knowledge to genetically engineer bacteria of many groups, such as pseudomonads, rhizobia, bacilli, and actinobacteria. In addition to bacteria, yeast and Archaea are also accessible to genetic engineering techniques.

For waste disposal, organisms of the category “pure laboratory GMO” and their DNA are normally inactivated by heating. A release of GMOs into the environment would be unintended. For genetic engineering laboratories of a “no or low risk” category, it cannot be completely excluded that small numbers of microorganisms may in fact be accidentally released into the environment, e.g. by sticking to clothes or shoes that have been contaminated with single or few bacterial cells. However, there are no reports that any of such events would have been detected or even caused environmental effects.

A different kind or risk is generated when microorganisms are cultivated at a large scale in bioreactors. Like in a laboratory batch culture, bacteria are normally cultivated in media that provide ideal conditions for growth and for most organisms, it is unlikely that they would survive in environmental matrices. The release of microorganisms from bioreactors and production plants is normally controlled and unlikely. However, in case of accidents, such as leaking of a bioreactor, larger numbers of cells would be released and a risk analysis should consider this aspect.

Genetic engineering also offers the potential to enhance interactions between microorganisms and their environment. For example, rhizosphere colonizing bacteria can be modified in their rate of producing metabolites or signal compounds that stimulate plant growth (see contribution by F. O’Gara in this book). Such bacteria may be useable as biofertilizers in the future. Similar engineering strategies can be applied to develop inoculants which act as biocontrol agents or which are able to degrade pollutants, i.e., polyaromatic or chlorinated hydrocarbons. Also, viruses have been attenuated by genetic engineering and released in France and Belgium as baits into the environment to immunize foxes against rabies (see www.rki.de for information about field releases).

If GMOs are deliberately released into agroecosystems or into polluted environments, the organisms need to survive and express their phenotype in order to “do their job”. However, after the objective of the release has been achieved, GMO are not wanted any more, as they are, in most cases, not indigenous. Molecular engineering strategies have been applied to limit the rate of survival of a GMO, e.g., by using mutants with a lower capacity to survive in competition to indigenous microorganisms or by incorporating suicide genes (Knudsen et al. 1995; Selbitschka et al. 1992).

Depending on the targeted environment of a GMO, a risk to benefit analysis may look very different. For their environmental biosafety, it seems highly important to evaluate the potential of an organism to survive in their targeted environment and their capacity to spread into neighboring non-target environments. Also, it would be useful to know if the recombinant genes are expressed in soil and to characterize the probability of gene transfer to indigenous microorganisms. For a laboratory strain, survival and recombinant gene expression in the environment would be an undesired trait, whereas for a biofertilizer, it is mandatory that it survives and expresses genes in the context of their targeted application. Even the transfer of a recombinant gene cannot always be regarded as a risk, as it also may have a beneficial effect on indigenous microorganisms, e.g., by conferring the capability to degrade an otherwise persistent pollutant.

Non-deliberate releases of biotechnological production strains

For a case study on non-deliberate releases we selected four different species of microorganisms with a potential to be used in large scale bioreactors as GMO. We chose two yeast species, *Hansenula polymorpha* (Tebbe 2002) and *Saccharomyces cerevisiae*, and two bacterial species, *Corynebacterium glutamicum* and *Zymomonas mobilis*. For each species, we selected the non-engineered parental strain and one or several strains engineered with a gene encoding for aprotinin, a polypeptide of pharmaceutical value which was originally isolated from bovine lungs. According to the amino acid sequence of the peptide, a nucleotide sequence was derived and synthesized in the laboratory. The studies were conducted in confined laboratory systems, i.e., in batch incubations with soil samples and in soil microcosms. The work was part of a larger research initiative, which also covered other habitats, such as fresh and marine water as well as waste water treatment systems (Tebbe et al. 1994a; Tebbe et al. 1994b).

Survival of GMOs in soil

Each species was inoculated at a density of 10^6 cells per g of soil. The survival was studied in pre-sterilized soil, which allowed to follow the titer of both GMO and parental strains over a decrease rate of five orders of magnitude. In fact, no difference was detectable in the surival rates of the GMO and their parental strains. As expected, the population decline reflected the environmental range of the species. *Zymomonas mobilis*, an anaerobic bacterium which prefers high levels of carbohydrates as carbon sources, completely died off immediately upon exposure to soil. At a lower rate, but also very quickly, both yeasts decreased in cell numbers in soil, with *S. cerevisiae* showing higher rates of decline than *H. polymorpha*. As expected, *C. glutamicum*, an aerobic gram-positive species that can be isolated from soil, survived best. Over four weeks of monitoring the titer remained quite stable at a level of 10^5 cells per g of soil (measured by cultivation on non-selective agar-plates as colony forming units, CFU) (Vahjen et al. 1997).

In non-sterile soil, GMOs were monitored by cultivation and colony hybridization. Rates of survival were much lower than observed in the sterile systems, except for *Z. mobilis*. Survival rates of *Z. mobilis* could not be lower, anyway, due to its inability to survive in such an environment. Again, *C. glutamicum* survived at higher rates than *H. polymorpha*, and *H. polymorpha* at higher rates than *S. cerevisiae* (Vahjen et al. 1997). The lower persistence of GMOs in non-sterile systems can be explained (1) by the presence of protozoa, which feed on microorganisms, and (2) by the presence of indigenous microorganisms that compete for nutrients and colonization sites in the soil matrix. Low temperatures increased survival, indicating the importance of biological processes.

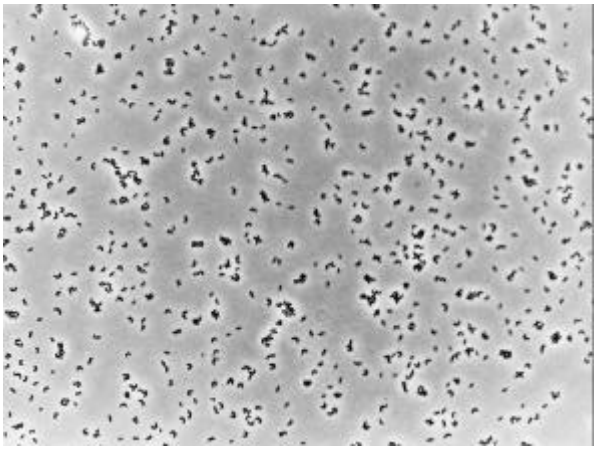

Fig. 2: *Corynbacterium glutamicum*; length of each cell is approx. 4 µm

Competition between GMOs and their parental strains

Competition experiments between GMO and their parental strains were also carried out in microcosms with sterile soil. For each experiment, 50 % of the inoculant was GMO, and 50 % the parental strain. Immediately after inoculation and during the decrease of the populations, the proportion of GMOs decreased for all three species investigated (*Z. mobilis* was not analyzed). After four weeks, less than 5 % of the remaining populations were GMOs, indicating that genetic engineering and expression of the aprotinin gene in fact decreased the environmental fitness of the microorganisms (Vahjen et al. 1997).

A surprising result, however, was found with *C. glutamicum*, which carried the aprotinin gene on a multi-copy plasmid. From the remaining 5 % surviving the inital competition experiment, we isolated cells, cultivated them and reinoculated them in the same type of competition experiments. It turned out that these GMO in fact were as competitive as the non-engineered strains with percentages of 35 to almost 50 % GMO after four weeks of incubation

(Vahjen et al. 1997). The molecular reason of this result was not further analyzed by it is likely that the selection process in soil may have had an effect of the plasmid host relationship, a phenomen that has been observed in more detailed analyses with *E. coli* in laboratory systems (Bouma and Lenski 1988). On the other hand, the fitness of *C. glutamicum* GMOs in our studies was never above that of the non-engineered parental strain.

Recombinant gene expression in soil

Recombinant gene expression in soil was investigated for both yeast species. The activity of the genes was studied at the transcriptional and at the translational levels. A new protocol for extracting and quantifying mRNA from soil was developed and applied, and the aprotinin molecule itself was quantified by an enzyme-linked immunosorbent assay (ELISA). Transcripts of the aprotinin gene were detected by Reverse Transcriptase-PCR and another susequent PCR. The ELISA was highly sensitive, as it allowed to detect as small amounts as 45 pg per g of soil. By means of their genetic modification, the GMO *S. cerevisiae* constitutively produced the aprotinin peptide whereas in *H. polymorpha* the gene was under control of a methanol-inducible promoter. Experiments were conducted in sterile and non-sterile soil, to analyze the importance of indigenous soil biota, present only in the non-sterile soil, on the persistence of the recombinant gene product (Tebbe et al. 1995).

After inoculation of 10^7 cells per g of soil with *S. cerevisiae* (GMO), recombinant gene expression could be detected over a period of 4 days. At that time the titer of GMO had decreased by three orders of magnitude in the non-sterile soil. In soil with *H. polymorpha*, no expression was observed in non-amended soils, soils with additional nutrients or even soils which received the inducer methanol. However, if soil was amended with both, methanol and nutrients, recombinant gene expression was detectable. Thus, under natural conditions no aprotinin gene expression would be expected. In sterile soil with *S. cerevisiae*, the aprotinin molecule accumulated to a concentration of 12 ng g^{-1} of soil. An initial increase in aprotinin was also found in non-sterile soil inoculated with *S. cerevisiae* or induced cells of *H. polymorpha*, but this initial increase to 8 to 10 ng g^{-1} of soil was followed by a decrease to levels below the threshold of detection, after 2 weeks of incubation (Tebbe et al. 1995). Thus, the peptide aprotinin was obviously degraded by soil microorganisms.

Effect on indigenous microorganisms

Community-level physiological profiles (CLPPs) are indicators for the composition of heterotrophic, aerobic soil microorganisms. The method is based in the inoculation of a microtiter plate with 95 different carbon sources and degradation patterns of these substrates upon inoculation with microbial cell consortia extracted from soil, are recorded. With aprotinin additions, a

stimulation of amino acid and organic acid utilization was observed. With a higher aprotinin concentration, this effect lasted longer than with a lower concentration (Vahjen et al. 1995). This result was another clear indicator that indigenous soil microorganisms degraded the recombinant gene product quickly. Interestingly, the CLPP profiles slowed down when the GMO or their non-engineered parental strains were inoculated. This effect lasted several days, until there was no difference to untreated controls again. Differences between engineered and non-engineered were not observed.

Gene transfer

Soil is a heterogeneous habitat and most studies on recombinant gene transfer were unable to detect gene transfer in bulk soil. In contrast, higher rates of transfer of mobile genetic elements (plasmids) were observed in the immediate vicinity of plant roots. These habitats, the rhizospheres, are characterized by higher levels of nutrients and higher metabolic activities of soil microorganisms. Our hypothesis was that there are also other micro-habitats which would enhance gene transfer in soil and we decided to analyze the effect of small soil animals. We chose Collembola (soil microarthropods, "springtails") and earthworms for our study.

C. glutamicum and other bacterial donor strains with recombinant antibiotic resistance plasmids were fed to *Folsomia candida* (Fig. 3), a soil inhabiting microarthropod. The experiments were conducted in petri dish microcosms with water agar on its surface. Each microcosm received 50 specimens of *F. candida* and the bacteria were placed in the center of the plate on a nutrient agar cube. Each or every other day the *F. candida* were transferred to fresh microcosms and the feces was analyzed for the occurrence of transcipients, i.e., indigenous bacteria that had acquired the recombinant plasmid. Donor counter-selection was achieved by cultivating indigenous bacteria on an agar with antibiotics to which the donor was sensitive. In addition, the transcipient agar was amended with the antibiotic to which resistance was encoded on the recombinant plasmid (Hoffmann et al. 1998).

Preliminary results indicated that the plasmid pUN1 of *C. glutamicum* was in fact transferred to a large number of indigenous bacteria in the gut of *F. candida*. The plasmid pUN1 is a multi-copy, broad host range, non-conjugative and non-mobilizable cloning vector. A thorough genetic analysis of the trancipients, however, demonstrated that the putative transcipients were false-positives, probably due to co-cultivation of donor cells on top of recipient cells.

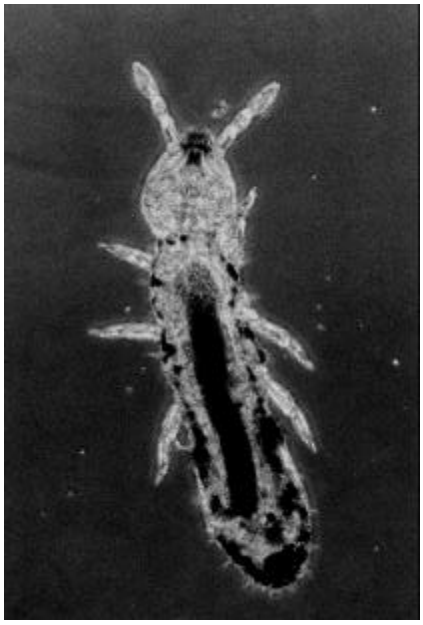

Fig. 3: *Folsomia candida* (Collembola), length 2 mm. The species served as a model organism for investigating stimulated gene transfer between bacteria in the gut of a soil invertebrate (Photography by H. Borkott, Braunschweig, Germany)

On the other hand, the transfer of conjugative broad and narrow host range plasmids from *E. coli* donor strains to various gram-negative gut bacteria was confirmed (Hoffmann et al. 1998). Also, mobilizable plasmids were transferred when the mobilizing plasmid was present in the donor cell (Hoffmann et al. 1998). The positive effect of collembolan gut passage on the transfer from a non-indigenous bacterium (*E. coli*) to indigenous bacteria was corroborated with another species, *Onychiurus fimatus* (Hoffmann et al. 1999). In addition, we could demonstrate that the feeding activities of the earthworm *Lumbricus rubellus* also promoted the transfer of plasmids from non-indigenous donor bacteria to soil bacteria (Thimm et al. 2001). The studies underline the importance to consider specific ecological niches, and not just bulk soil, in order to evaluate risks of GMOs in terrestrial ecosystems.

Field studies with *Sinorhizobium meliloti*

Sinorhizobium meliloti (formerly: *Rhizobium meliloti*) strains were chosen as model organisms to study the fate and ecological effects of a soil bacterium in context with deliberate releases (Tebbe et al. 1998). *S. meliloti*

is capable of forming root nodules on the legume alfalfa (*Medicago sativa*), and mediates fixation of atmospheric nitrogen. The use of bacterial inoculants in conjunction with cultivation of leguminous plants has a long tradition and there is clear evidence that *S. meliloti* is not pathogenic to any organism. Under the initiative of Alfred Pühler (University of Bielefeld) we carried out the first deliberate field release of GMOs in Germany at our Research Center for Agriculture in Braunschweig. Permission was obtained from the Robert-Koch Institute, Berlin (www.rki.de). The genetic modification of the released strains consisted in the chromosomal insertion of the luciferase marker-gene (*luc*) which provided nothing but a highly sensitive detection system for bioluminescent cells under laboratory conditions (Selbitschka et al. 1992). Other marker-systems that can be used to monitor bacteria in soil are *gfp* or *gusA* (Ramos et al. 2002). The *luc* gene in our study was inserted into a neutral chromosomal place (strain L33) or into the *recA* gene, the latter resulting in a $RecA^-$ phenotype (strain L1) (Dammann-Kalinowski et al. 1996). The field releases were conducted in a collaboration of Pühler's group and our group in Braunschweig.

Step by step: from the laboratory to the field

Prior to the field release, the performance of both strains was evaluated in the laboratory by comparing both strains for their capacity to survive in soil and nodulate alfalfa (Hagen et al. 1997). Later, a two year study was performed in soil columns under greenhouse conditions (Fig. 4) before field releases were performed (Schwieger et al. 1997). The field releases consisted of a GMO inoculation onto 8 field lysimeters, and half a year later of inoculation of 10 field plots, each with an area of 9 m^2. The strategy from the laboratory to the field was step-by-step, in order to detect possible non-anticipated effects, which would have required a reevaluation of risks associated with the prospective field releases.

The data collected before the field release were also used for the application for the field releases at the RKI. None of the experimental investigations in the laboratory and or greenhouse indicated any risk. A large number of data on the potential persistence of the GMO strains could be collected and the genetic stability and usefulness of *luc* as a marker-gene for this type of studies was proven.

A.

B.

Fig. 4: Greenhouse with soil columns that were used before field release to study *S. meliloti* L33 and L1. Length of a soil column (A.) approx. 1 m, diameter 33 cm (B.)

Persistence at the field site

Field lysimeters with a design similar to the green house soil columns shown in Figure 4 were inoculated with 10^7 cells per g in the upper 5 cm of the soil horizon. The study demonstrated that the vast majority of GMO remained in the upper soil horizons and almost no cells were transported by rain water through the soil profile of 80 cm depth within a monitoring period of two years (Schwieger et al. 2000 a). Both strains, L33 and L1 survived equally well in presence of the host plant, alfalfa. However, when the host plant was removed from the lysimeters, there was an indication that the recA-deficient strain L1 survived only at lower rates in bulk soil than its non-deficient counterpart L33 (Schwieger et al. 2000 a).

Field plots were inoculated with approx. 10^6 *S. meliloti* cells per g of soil in the ploughing layer and on the same day, alfalfa was seeded. As observed for the field lysimeters, the cell titer of the GMO decreases by 2 two 3 orders

of magnitude in the bulk soil within the first weeks after inoculation. The monitoring of the GMO titers is continuing until today, more than 7 years after the release. GMOs can still be detected on most inoculated field plots (unpublished results). A clear difference between the survival rates of L33 and L1 was not observed (unpublished results).

Colonization of rhizospheres and effect on indigenous bacteria

With the growth of alfalfa, we observed the occurrence of several weeds on the inoculated plots, with *Chenopodium album* being the most dominant in the first three months after the beginning of the experiment. We analyzed the diversity of bacteria in rhizospheres from alfalfa and *C. album* on L33 inoculated and on non-inoculated field plots, respectively. Samples were taken 12 weeks after the field release and a total of 1,200 bacterial colonies growing on a specific rhizosphere-optimized agar were characterized and grouped by amplified ribosomal restriction analysis (ARDRA). The 25 most dominant ARDRA groups, which comprised 859 isolates were further classified by rRNA gene sequencing of representative isolates (Schwieger and Tebbe 2000 b).

The isolated rhizosphere bacteria belonged to different phylogenetic groups, with *Proteobacteria* from the alpha-, and gamma-subgroups, and bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group being the most dominant ones. The most dominant group was represented by the genus *Variovorax*.

Inoculation with *S. meliloti* L33 did not affect the rhizosphere composition of *C. album* plants, but those of alfalfa were in fact altered. On inoculated plots, the inoculant itself was detected and also larger numbers of other bacteria from the alpha-subgroup (*Rhizobium* and related). On the other hand, the number of *Proteobacteria* from the gamma-subgroup was much higher on non-inculated plots in rhizospheres of alfalfa. The most dominant member was *Acinetobacter calcoaceticus*, which was almost not found in alfalfa rhizospheres from inoculated plots (Schwieger and Tebbe 2000b). Thus, inoculation shifted the community composition towards the inoculant and its related species. Most likely this effect was transient. A shift in a community may not have any relevance for detecting an ecological risk, as most soil functions are provided by many different bacteria.

How can established S. meliloti L33 be eliminated from soil?

Currently we are evaluating the efficiency of various strategies to reduce the number of *S. meliloti* L33 cells from soil. The analyses are done in microcosm greenhouse experiments. Treatments that are evaluated comprise the addition of herbicides, crop rotation, inoculation of non-engineered *S. meliloti* strains and the total removal of plants. Preliminary results indicate that removal of plants and herbicide applications are more efficient means

than crop rotation or inoculation with competitive strains (Miethling and Tebbe, unpublished).

The efficiency of wild-type inoculation to reduce the number of GMOs is also evaluated at our field site in Braunschweig. Our data have relevance for recommending procedures to eliminate GMOs from soil. They also should be useful to understand how bacteria in general persist in soil and how microbial communities may be changed for the benefit of agricultural practices, e.g., by eliminating pathogenic microorganisms from a soil.

Importance of small-scale field releases

Greenhouse studies are important to characterize certain aspects of the environmental behaviour of a GMO or other microorganisms, but the complex situation that is encountered in the field is hard to be mimiced. Therefore, small scale field releases are an invaluable tool in the context of studying interactions between a GMO and its environment. Provided that a GMO is safe, i.e., non-pathogenic and only tagged with a marker-gene, i.e., *luc*, a small scale field release can give new insights into microbial ecology and biosafety matters, as demonstrated with our studies.

The number of small scale field releases in Germany and other European countries is still low, and one reason is that the requirements for obtaining permissions for such studies are high and not achievable without a significant financial effort. It should be considered if small scale field studies with non-pathogenic marker-gene tagged soil bacteria really need such rigourous regulation, since additional experience with such systems would increase the safety of handling, monitoring and evaluating GMOs in the environment.

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WHAT DID WE LEARN FROM 24 FIELD RELEASES OF GMMS IN ITALY?

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Abstract

The deliberate release of genetically modified microorganisms (GMMs) stems from the advancements during the last two decades of recombinant DNA technology, and has shown its potential for environmental applications in bioremediation, biocontrol of plant pathogens and plant growth promotion. The modification of bacterial strains specifically designed to allow appropriate monitoring and risk assessment started in our lab on the eve of '90s, and their evaluation continued at the microcosm and green-houses. The field releases in Italy started in 1994, and involved nitrogen-fixing nodule-forming rhizobia, plant growth promoting azospirilli, and plant protecting pseudomonads. All 24 releases (1994-1999) were carried out by our groups according to existing European legislation, and have provided useful information on environmental impact characterization and biosafety-related aspects of GMMs. In some instances, post-release monitoring is still being done after eight years at the site of release.

Different marker genes/reporter systems were used for *Rhizobium leguminosarum*, *Azospirillum brasilense* and *Pseudomonas fluorescens*. They include *lac*ZY, *gus*A, Mer^R*lac*Z and *luc*. No introduced antibiotic resistance genes were used, well in advance of the recent EC Directive 18/2001. The genetic modifications involved agronomically relevant, as well as non-relevant traits, and the crops used included peas, soybean, faba bean, sorghum and sugarbeet.

Based on published results, and on existing strict regulations, it appears that environmental impact characterization represents an important element of a case-by-case evaluation of the biological agent to be used at the field level. A contribution towards mathematical modeling of risk assessment of field-released biological agents is presented and discussed.

Keywords: GMM, field release, biosafety, risk assessment, modelling.

The field releases

The field release of GMMs is a relative recent activity. The first releases were about 15 years ago starting with the Australian No-gall Tra$^-$ *Agrobacterium tumefaciens* (used as a plant protection product) and aimed at testing the agronomic performances and the safety of improved microorganisms. At present, the use of GMMs in agriculture is of limited economic relevance in the European Union, while in both Western and Eastern countries it covers considerably larger acreage. The past decade has seen a dramatic advance in the development of new tools to improve microorganisms with selected useful genetic traits (Davison 2002a) and to approach several biosafety-related aspects in microbial ecology, biodegradation of xenobiotics and pollutants, and biocontrol of plant diseases (Davison 2002b; Giovannetti et al. 2002).

In Italy the modification of bacterial strains specifically designed to approach biosafety-related questions before and during the field use, started in 1988 with the construction of multipurpose gene cartridges based on a novel synthetic promoter for high level gene expression in gram-negative bacteria (Giacomini et al. 1994), and with studies on the marker/reporter stability and selectivity (Corich et al. 2001). These studies continued at the microcosm level to define the fate of genetically modified rhizobia during the long-term storage of commercial inoculants (Corich et al. 1996; Nuti et al. 2002). Finally, field releases were designed and carried out to evaluate the environmental impact of genetically modified soil/seed inoculants (Casella et al. 2002; Corich et al. 1995; Corich et al. 2000; Nuti et al. 1996; Resca et al. 2001; (M. Basaglia, S. Casella, F. O'Gara, J. Vanderleyden, U. Peruch, M. Nuti, unpublished data) In some instances genetically modified plants were used, in experiments designed at the greenhouse level, to assess the effects of antifungal proteins with respect to symbiotic and pathogenic fungi . In this case the antifungal Dm-AMPI protein from *Dahlia merckii* Lehm, expressed in *Solanum melongena* is released in root exudates and differentially affects symbiotic *Glomus mosseae* and the pathogenic fungus *Verticillium albo-atrum* (A. Turrini, C. Sbrana, L. Pitto, M. Ruffini Castiglione, L. Giorgetti, R. Briganti, T. Bracci, M. Evangelista, M.P. Nuti, M. Giovannetti, unpublished data) For some of the field released bacterial inoculants, monitoring is still being done after eight years at the site of release.

Tables 1, 2 and 3 summarize the released GMMs, along with the marker gene and other modifications, as well as the purpose of the release. The genetic modifications involved agronomically relevant as well as non-relevant traits, and the crops used included peas, soybean, faba bean, sorghum and sugarbeet. All the releases carried out in the period 1994 - 1999, were done according to the existing European legislation, and aimed at elucidating the effect of genetically modified inoculants on crops, while the resident microbiota was compared to that of wild type strains. Many of the

field releases were done on a surface of about or less than 1 ha, a minority of tests was done on small plots of 1 m^2.

Table 1: Field releases of GMMs in 1994

Released GMMs	Year	Plant	Marker gene	Other modifications	Purpose(s) of the release
Pseudomonas fluorescens F113 *lacZY*	1994	Soybean	*lacZY*	no	Evaluation of the marker gene in the field and ecological impact BCA against *Pythium*
Pseudomonas fluorescens F113 *lacZY*	1994	Soybean	*lacZY*	no	Evaluation of the marker gene in the field and ecological impact and rotation in 1995 BCA against *Pythium*
Azospirillum brasilense 245 *lacZ*	1994	Sorghum	*lacZ*	no	Evaluation of the marker gene in the field and ecological impact
Azospirillum brasilense Sp6 *gusA*	1994	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact
Azospirillum brasilense Sp6 IAA++ *gusA*	1994	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact
Rhizobium leguminosarum bv.*viciae* 1110	1994	Pea	Mer^R*lacZ*	no	Stability, evaluation of the marker gene in the field and ecological impact
Rhizobium leguminosarum bv.*viciae* 1111	1994	Pea	Mer^R*lacZ*	no	Stability, evaluation of the marker gene in the field and ecological impact
Rhizobium leguminosarum bv.*viciae* 1112	1994	Pea	Mer^R*lacZ*	no	Stability, evaluation of the marker gene in the field and ecological impact

Table 2: Field releases of GMMs in 1995-1997

Released GMMs	Year	Plant	Marker gene	Other modifications	Purpose(s) of the release
Rhizobium leguminosarum bv.viciae *1114*	1995	*Vicia faba*	Mer^R*lacZ*	no	Stability, evaluation of the marker gene in the field and ecological impact
Rhizobium leguminosarum bv.*viciae 1114*	1995	Pea	Mer^R*lacZ*	no	Stability, evaluation of the marker gene in the field and ecological impact
Pseudomonas fluorescens F113 *lacZY*	1995	Soybean	*lacZY*	no	Evaluation of the marker gene in the field and ecological impact BCA against *Phythium*
Azospirillum brasilense 245 *lacZ*	1995	Sorghum	*lacZ*	no	Evaluation of the marker gene in the field and ecological impact
Azospirillum brasilense Sp6 *gusA*	1995	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact
Azospirillum brasilense Sp6 IAA^+ *gusA*	1995	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact
Azospirillum brasilense 245 *lacZ*	1995	Sorghum	*lacZ*	no	Evaluation of the marker gene in the field and ecological impact after rotation
Azospirillum brasilense Sp6 *gusA*	1995	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact after rotation
Azospirillum brasilense Sp6 IAA^+ *gusA*	1995	Sorghum	*gusA*	no	Evaluation of the marker gene in the field and ecological impact after rotation
Pseudomonas fluorescens F113 *lacZY* in rhizomania infested soil	1997	Sugarbeet	*lacZY*	no	Evaluation of the marker gene in the field, ecological impact, effect on rhizomania BCA against *Polymyxa betae*
Pseudomonas fluorescens F113 *lacZY* in rhizomania non infested soil	1997	Sugarbeet	*lacZY*	no	Evaluation of the marker gene in the field, ecological impact. BCA against *Polymyxa betae*

Table 3: Field releases of GMMs in 1999

Released GMMs	Year	Plant	Marker gene	Other modifications	Purpose(s) of the release
Pseudomonas fluorescens F113Rif pCUGP In rhizomania infested soil	1999	Sugarbeet	*gusA*	Yes: The strain iperproduces DAPG	Evaluation of the marker gene in the field, ecological impact, effect on rhizomania through the control of the vector *Polymyxa betae*
Pseudomonas fluorescens F113Rif pCUGP in rhizomania non infested soil	1999	Sugarbeet	*gusA*	Yes: The strain iperproduces DAPG	Evaluation of the marker gene in the field, ecological impact, effect on rhizomania through the control of the vector *Polymyxa betae*
Azospirillum brasilense Sp245 *luc* IAA^-	1999	Sorghum	*luc*	Yes. The strain produces low level of IAA	Evaluation of the marker gene in the field, ecological impact, effect of functional modification on sorghum
Azospirillum brasilense Sp245 *luc*	1999	Sorghum	*luc*	No	Evaluation of the marker gene in the field, ecological impact
Azospirillum brasilense Sp245 *luc* IAA^+	1999	Sorghum	*luc*	Yes. The strain produce high level of IAA	Evaluation of the marker gene in the field, ecological impact effect of functional modification on sorghum

The biosafety-related relevant aspects

Different marker genes/reporter systems were used for *R. leguminosarum*, *A. brasilense* and *P. fluorescens*. They include *lac*ZY, *gus*A, Mer^R*lac*Z and *luc*. No introduced antibiotic resistance genes were used, well in advance of the recent EC Directive 18/2001. In 1999 GMMs carrying marker genes and modifications in functional genes (i.e. for the production of indole-3-acetic acid and 2,4-diacetylphluoroglucinol) were field-released aiming at verifying ecological behaviour and plant protection performances.

Among the relevant outcome of these studies there are the following:

(1) genetic modifications, such as the introduction of marker genes and functional modification, affects neither the ecological fitness nor the impact of the GMM on culturable microbial populations.

(2) although an initial expected increase of population density of the GMM has been observed, the introduced seed inoculants remained at 10^2-10^3 cfu per gram of soil or below the detection limit a few weeks or months after release. In the case of aggressive soil/root colonizers (i.e. *Pseudomonas fluorescens* F113 *lacZY*), one year after release, the GMM's population density was below detection limits under non selective conditions.

(3) other ecological parameters (like soil microbial biomass, denitrification and soil respiration) were analyzed but only small and transient or no differences were found.

(4) the genetic modification consisting of agronomically relevant traits may lead to the expected agronomically beneficial effects

(5) in some cases, the beneficial effects of the GMM observed at the lab, microcosm and green-house levels were not confirmed when the microbe was released in the field (e.g. as a biocontrol agent). These results were probably due to the influence of biotic/abiotic factors, like the competition with resident soil microbiota, to the action of predators, or to intense rainfalls that occurred during some field releases. When the environment was modified in order to create selective conditions that would favour bacteria bearing the introduced genes (e.g. amended with lactose for strains carrying highly-expressed *lacZ*), their persistence was significantly extended. For -galactosidase overexpressing rhizobia, in terms of recoverable colony forming GMMs four years after the release, trophic selection proved at least tenfold more effective than plant host presence and nearly a hundred times superior when compared to totally neutral environmental conditions consisting in bare soil neither sown with legumes nor supplemented with lactose (V. Corich, A. Squartini, M. Nuti, unpublished data).

Based on published results and on existing strict regulations, it appears that environmental impact characterization represents an important element of case-by-case evaluation of the biological agent to be used at field level.

Towards modelling risk assessment

In 2001, two Directives were introduced (Commission Directive 2001/36/EC of 16.05.2001 and Commission Directive 2001/18/EC of 12.05.2001) which regulate plant protection products formerly known as “biocontrol agents” and the deliberate release of GMOs, respectively. Both Directives are innovative and courageous in that they introduce stringent rules in these matters. Among the innovative aspects, both Directives require an accurate environmental impact evaluation within the framework of a detailed risk assessment. This in turn will allow the regulatory bodies to identify a risk profile for the introduced biological entity, which essentially will describe:

(a) which microbiological hazards are causing the environmental/ health problem and the difficulty in controlling them,

(b) the source of the hazard from the entire chain,

(c) the available data on prevalence and numbers of the organism in question,

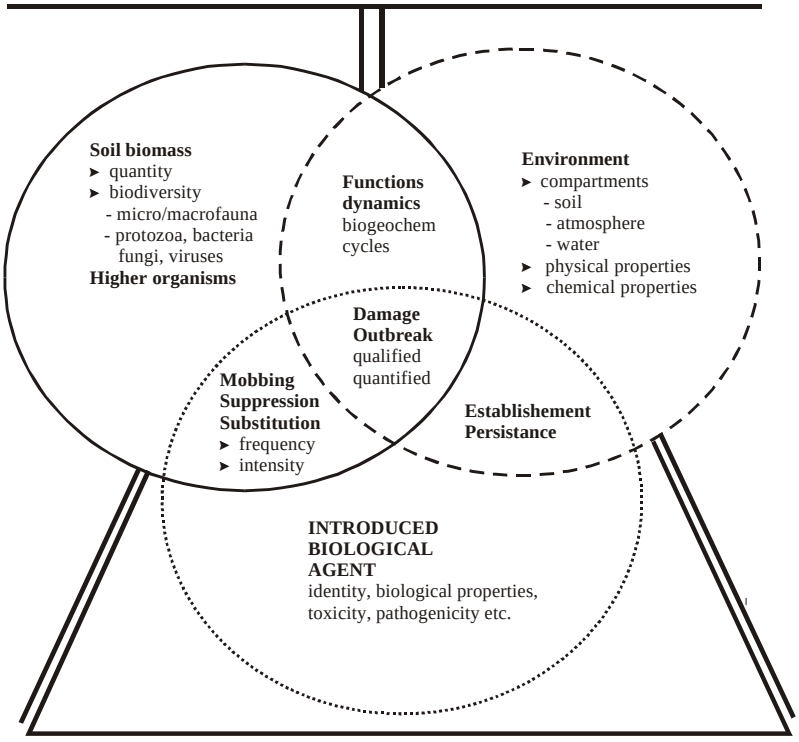
(d) what is expected to be at risk,

(e) preventive measures and distribution of risks/benefits.

It is clear that to identify the risk profile one of the most relevant steps will be the exposure assessment, which is an estimation of how likely an individual or a population will be exposed to a microorganism (plant protection product, GMM, or other). To implement and describe the risk profile we can use essentially the following approaches: (a) observation, which has the advantage of being process-, product-, and microbe-specific, although it is a rather time consuming and expensive approach; (b) lab testing or simulation, which allows the elucidation of different functions, but it is neither too precise nor specific; (c) mathematical modeling, which gives rise to predictive microbiology. In Figure 1 we present a diagram, which could be used in view of modeling risk assessment for GMMs.

While the presence of a GMM, through the channel of the primary production, cannot be excluded a priori, and is to be evaluated with respect to human health, modeling risk assessment of a GMM from an environmental perspective requires an approach which takes into consideration the interaction of three main components: (a) the introduced biological agent, (b) the environment compartments viewed as physical-chemical properties, and (c) the biophase of the environment, from microbes to human beings.

The primary-level interaction of the GMMs in the environment, considered for its physical-chemical properties, is the distribution in terms of establishment and persistence of the biological agent in the various compartments, i.e. soil, atmospheres and water phase. The second primary-level interaction is between the environment and its biophase. The latter is considered in terms of quantity and biodiversity and embraces micro-mesofauna, microbial biomass (protozoa, bacteria, fungi, viruses) and higher organisms (flora and fauna, and human being). This interaction gives rise to the dynamics of functions, i.e. biochemical cycles (of which the turnover of organic matter has a prevalent role with respect to the possible environmental impact of the released GMMs). The third primary-level interaction defines the frequency and intensity of the introduced organism (aggressive colonization or “mobbing”, suppression and population substitution). The secondary level interactions will be among the primary-level ones described above, and this will ultimately lead to the qualification and quantification of the outbreaks of environmental damage.



ENVIRONMENTAL DAMAGE: any harmful modification of physical, chemical, biological properties of any compartments of the environment, soil, water, atmosphere, biosphere,
The modification can be: **immediate or delayed, reversible or irreversible**

Fig. 1: Towards modelling risk assessment for genetically modified microorganisms (GMMs)

Although deterministic as well as stochastic models may have their limitations, there is an increasing trend to look for innovative approaches for modeling, including probabilistic neural network approaches for modeling and classification of bacterial growth/no-growth data (Hajmeer and Basheer 2002). Mathematical modeling to describe human exposure to food-borne pathogens in the food chain from farm to fork has recently been proposed at the EU level (Directorate SANCO 2002).

In summary, the numerous field releases of GMMs in Italy as well as in other Member States of the European Union, or else outside the EU, have provided in the last decade useful information on the behavior, fate and impact of GMMs on target and non-target organisms. This “observation” approach can be used has a valuable tool to implement and describe the risk profile for GMMs, on a “case by case” base as requested by the current

Directives. The tremendous advance of knowledge of cell biology and molecular techniques can also be used in the "simulation approach" to implement and describe the risk profile. Predictive microbiology can nowadays take advantage of the rapidly expanding knowledge of the "mathematical modeling approach" to implement and describe the risk profile for genetically modified microorganisms to be used in agriculture as plant stimulators or protection products, and in bioremediation of xenobiotics and pollutants.

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Gene flow in genetically modified plants

GENE FLOW IN HERBICIDE-RESISTANT CANOLA (*BRASSICA NAPUS*): THE CANADIAN EXPERIENCE

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Abstract

Herbicide-resistant *B. napus* comprises over 85% of the *B. napus* grown in Canada. Because Canadian producers have been growing herbicide-resistant varieties since 1996, *B. napus* presents a unique test case for novel gene flow from crop plants. Gene flow is possible by both seed and/or pollen escape. Certified conventional canola has been shown to contain one or more types of herbicide-resistant canola seed in excess of the 0.25% allowed by Canadian seed regulation standards. It is expected that seed grown by the producer would have similar or higher levels of contamination. Admixture is common where seed is handled as a bulk commodity, but its impact on potential gene flow is not well understood. Volunteer canola is a common weed and persists in the seed bank in relatively high densities for four to five years. Persistence of volunteers extends the potential for gene flow and lessens our ability to eradicate a transgene after environmental release. Gene flow via pollen movement occurs at low frequencies. The distribution of outcrossing is skewed, with a high frequency of cross-pollination close to the pollen source, and a rapid drop distal to the pollen source. Because of insect vectors or wind, there is a low frequency of cross-pollination over long distances from the pollen source. It is difficult to establish a distance at which zero cross-pollination occurs. Inter-specific hybridization is a less likely source of gene flow and the consequences of introgression of herbicide resistance in related weeds will be restricted to the agro-ecosystem. While Canadian experience with *B. napus* has shown abundant opportunity for gene flow, the environmental consequences of gene flow must be weighed relative to the positive impact of changes to agronomic practice, including reduced herbicide and fuel usage.

Keywords: multiple-resistant, volunteer crops, *Brassica napus*, *Brassica rapa*, hybridization, introgression, admixture

Agronomic Context

Two species of canola are grown in Canada: *Brassica napus* and *B. rapa*. *B. napus* forms the majority of the canola grown, comprising over 90% of the 4.5 million ha grown in 2001. Prior to the introduction of herbicide-resistant varieties, few herbicide options were available. Soil-applied herbicides had a limited weed spectra and efficacy, and efficacy was influenced by environmental conditions. In addition, soil-applied herbicides required incorporation, limiting adoption of environmentally beneficial direct-seeding practices. Available post-emergence products had narrow broadleaf weed spectra, resulting in extensive yield losses due to weed competition. Weed and disease control necessitated crop rotations which limited producers to a single canola crop within a four-year rotation. Therefore, when herbicide-resistant *B. napus* varieties became available and acceptable in the majority of Canadian canola markets, they were rapidly and widely adopted. Herbicide-resistant canola now occupies more than 85% of the *B. napus* ha in Western Canada (Table 1).

Currently, four herbicide-resistant *B. napus* types are available to producers: glyphosate-, glufosinate- and bromoxynil-resistant canola, all created using transgenic techniques; and imidazolinone-resistant canola, produced by mutagenesis (Table 1). In Canada, all four types are regulated identically as 'plants with novel traits' (PNTs). The Canadian Food Inspection Agency defines PNTs as "plant varieties/genotypes that are not considered substantially equivalent, in terms of their specific use and safety both for environment and for human health, to plants of the same species in Canada, having regard to weediness potential, gene flow, plant pest potential, impact on non-target organisms and impact on biodiversity. PNTs may be produced by conventional breeding, mutagenesis, or more commonly, by recombinant DNA techniques" (Canadian Food Inspection Agency, 2002).

Approximately 15% of producers continue to grow *B. rapa*, or conventional canola, either because of satisfaction with the crop (e.g. early maturing *B. rapa*) and available conventional weed control, or a philosophical disagreement with the technology. This is predicted to remain relatively stable (Hartman, pers. communication).

Table 1: Herbicide-resistant *B. napus* currently grown in Western Canada

Release Year	Herbicide	Resistance	Seed Protection	Volunteer Control Measures	Market* (%)
1996	glufosinate	*pat or bar* transgene	Some hybrid varieties	Like conventional canola	15
1996	glyphosate	*epsps* + *gox, hor* transgenes	Seed use prohibited by contract	Chem-fallow and pre-seeding treatments require modification	55
1996	imidazolinones	ALS modified via mutagenesis	Producers free to re-seed	ALS inhibitors may not be used alone	15
2000	bromoxynil	Nitrilase , *Oxy* transgenes	Producers free to re-seed	Like conventional canola	<1

*2002 estimated

Gene flow can follow from several initial events. Inadvertent seed admixture during commercial production or within seed producer fields may permit cross-pollination. Shared seeding and harvesting equipment, and use of bulk storage facilities, may contribute to seed admixtures. Volunteer canola within subsequent production or seed producer fields can facilitate gene flow over time. Gene flow can occur via pollen between adjacent or nearby canola crops. Finally, gene flow may occur by inter-specific hybridization.

Gene Flow Facilitated by Admixture or Seed Source

The majority (80%) of producers purchase canola seed from seed producers who strive to preserve varietal integrity. Some varieties are hybrids, produced from two different parental varieties. Because hybrid vigor is diminished in subsequent generations by segregation, producers who use hybrids must continue to purchase seed. Similarly, 'Technology Use Agreements' required for glyphosate-resistant canola contractually prohibit producers from re-seeding the harvested crop. Finally, reduced weed seed contamination and seed pre-treated with insecticides and fungicides contribute to the trend of purchasing seed.

Varietal purity depends on the seed source. Canadian Seed Growers practice crop rotations to reduce volunteers (see below), and maintain a minimum 100 m isolation distance between different varieties of *B. napus* and between *B. napus* and *B. rapa*. Certified seed fields are visually inspected; however, the presence of herbicide resistance cannot be visually detected. In a recent study of 70 samples of conventional certified canola seed from several varieties and seed

sources, Downey and Beckie (2002) reported that three varieties of 14 tested exceeded the 0.25% maximum allowable contamination. A single conventional variety, grown by 10 different seed producers, showed high levels of contamination by the glyphosate and glufosinate resistance genes, (0.40% and 0.29%, respectively). Contamination of a single variety from many different seed producers suggests that seed was contaminated in the breeding nursery, rather than by the seed producer. Where seed stocks were uncontaminated, the 100 m isolation distance was sufficient to maintain varietal purity standards. A similar study of conventional varieties by researchers at the University of Manitoba (Friesen, Nelson and Van Acker, pers. communication) has indicated the level of herbicide resistance contamination in conventional seed lots exceeded the 0.25% limit in approximately half of the 27 samples tested. Researchers have assumed that the seed purity standards for the presence of other varieties in pedigree seed lots, as laid down by the Association of Official Seed Certifying Agencies (AOSCA) and the crop certification standards of the Canadian Seed Growers' Association could be directly applied to the presence of specific genes, rather than visual traits, as presently applied. However, even when purity standards for certified seed are met, it may still contain a low frequency of herbicide resistant seeds.

When certified seed reaches the producer, varietal separation varies directly with the care practiced by the producer. The same seeding equipment typically seeds all canola crops on a single multi-field farm, and in some cases, may handle several conventional and herbicide-resistant varieties. Thus, seed contamination from field to field with seeding equipment must be considered. Information is lacking on the frequency and extent of contamination from seeding and handling.

Harvested canola is handled as a bulk commodity. For internal and export purposes, PNT canola is not separated from conventional canola. Seed mixing following harvest is customary, unless seed is retained for re-planting. While most seed goes for crushing, there are ample opportunities for seed loss in the chain between production and crushing for oil.

Gene Flow Facilitated by Volunteer Canola

Volunteer *B. napus* is a common agricultural weed. In western Canada, it occurs in 11% of cropped fields and was ranked as 18th in relative abundance of all weeds (Beckie et al. 2001, Leeson et al. 2002, Légère et al. 2001). In eastern Canada, a survey of 131 fields in the canola-growing region found volunteer canola in 90% of fields (Simard et al. 2002). The presence of volunteer canola is under-reported in canola crops because of the difficulties in distinguishing volunteers from planted crop. Volunteer weed levels depend upon seed shatter at harvest, and management factors including seeding practices, crop rotation and

post-harvest practices. The type of herbicide resistance influences the success of chemical controls and the time effective controls can be applied (Table 1). All types of canola can be controlled by inexpensive auxinic herbicides such as 2,4-D and MCPA. Clearly, field records from previous crops become critical as weed control decisions become PNT specific.

Volunteer *B. napus* can persist for up to five years in the seed bank (Légère et al. 2001, Simard et al. 2002). Although density is reduced over time, after four years the average density is 0.2 to 0.5 plants m^{-2}. In the absence of herbicide selection, there is no difference in reproductive potential between resistant and susceptible varieties (reviewed in Warwick et al. 1999, Crawley 1992, Kumar et al. 1998; Snow et al. 1999). It seems likely that survival in the seed bank and intermittent replenishment by reproductive volunteers contribute to volunteer persistence. Volunteer canola has the potential to cross-pollinate with canola cropped in that field, or in adjacent fields. Volunteer plants extend the potential for gene flow spatially and temporally.

Gene Flow Facilitated by Pollen Movement

B. napus has inter-plant outcrossing rates of 21.8% (Rakow and Woods 1987). Outcrossing diminishes with distance. Under field conditions, the rate of outcrossing is less than 1% at 100 meters. However, assuming a 0.2% outcrossing rate in a field yielding 1400 kg ha^{-1} with a harvest loss of 5%, Downey (1999) estimated some 35,000 outcrossed seeds (3.5 seeds m^{-2}) would remain in the recipient field. Fall or spring frost and/or cultivation during seedbed preparation would kill most, but not all, of the plants.

Outcrossing, combined with volunteer survival, extends gene flow potential over space and time. In 1998, a field in Alberta was investigated to determine the cause of glyphosate-resistant volunteer canola where no glyphosate-resistant crop had been grown (Hall et al. 2000). In 1997, glufosinate- and imidazolinone-resistant canola was sown in different portions of the field. Seed shatter and harvest deposited canola seeds, seeds germinated in 1998 and some volunteers were not controlled by glyphosate. Since glyphosate-resistant canola was grown in an adjacent field, 22 m distant, glyphosate resistance gene movement via pollen was investigated as the cause. Progeny from volunteers were collected and tested for resistance. Glyphosate-resistant progeny were identified over 500 meters from the pollen source. Progeny from volunteers were resistant to glyphosate/imidazolinone, imidazolinone/glufosinate, or glyphosate/glufosinate and a small number were resistant to all three herbicides.

In a broader study, 11 commercial fields in Saskatchewan were examined in 1999 to measure pollen flow between adjacent fields of glyphosate- and glufosinate-resistant canola (Beckie et al. 2001). Seed was collected from 0 to

800 m along a transect perpendicular to the field border. Frequency of double resistance diminished rapidly at 50 m from the pollen source, but a low level of pollen flow was apparent up to 400 m from the source. In 2000, frequency and distribution of double-resistant volunteers and effect on gene flow were assessed; gene flow as a result of pollen flow in 1999 was detected to the limits of the study area (800 m). The level of cross- pollination between adjacent commercial fields is supported by a recent study in Australia where low frequency of pollen movement was detected over 3 km away from source fields (Rieger et al. 2002).

These studies suggest gene stacking of herbicide resistance in volunteers is likely a common occurrence. However, to date, multiple-resistant volunteers have caused little consternation to the average western Canadian producer, with the exception of producers that elect not to grow transgenic canola. Unexpected resistance, resistant volunteer canola growing where that type of canola has not been sown, is occasionally noticed where glyphosate was used alone in chemical fallow or prior to seeding or where an ALS inhibitor was used alone in pulse crops. If noticed, they are usually controlled with auxinic herbicides.

For organic producers, the consequences of pollen flow are significant. Because of zero tolerance for transgenes in organically grown Canadian canola, the purity and marketability of the organic canola crop is put at risk. In the traditional canola growing regions, organically grown canola is effectively excluded because of the potential for gene flow from neighboring crops. This issue is now before the Canadian courts.

Gene Flow Facilitated by Hybridization and Introgression

Brassica crop species can cross-pollinate with wild or weedy relatives and, therefore, have the potential to influence the evolution of wild plants (Ellstrand et al. 1999). *B. napus* (AACC) contains the AA genome from *B. rapa* and the CC genome from *B. oleracea*. *B. rapa* is both a canola crop and widely distributed weedy species in many parts of the world (reviewed in Warwick et al. 2000). In Denmark, *B. napus* and *B. rapa* spontaneously hybridize (J?rgensen and Andersen 1994; Landbo et al. 1996) at a frequency of 9 to 93%, depending on the degree of isolation of *B. rapa* plants from each other. Hansen et al. (2001) has subsequently demonstrated introgression between *B. napus* and a wild population of *B. rapa*. While the F_1 hybrids were more fit than *B. rapa*, both backcrosses and F_2s had a reduced fitness compared to the original wild parent (Hauser et al. 1998 a and b). Reduced fitness of hybrids will slow, but not prevent, introgression of *B. napus* genes.

Brassica napus co-exists in Canada with several weedy relatives (Table 2). Studies examining the potential for and actual occurrence of gene flow from *B. napus* to *B. rapa*, *Erucastrum gallicum*, *Raphanus raphanistrum* and *Sinapsis*

arvensis in commercial HR canola fields in Canada are currently in progress. Where the weed relatives occur in cropped fields, a herbicide-resistant hybrid might have a significant fitness advantage, and thus, introgression (the stable incorporation into the genome) is possible. The potential for hybridization and introgression will depend on a myriad of factors, including hybridization frequency, distribution and abundance of weeds, concurrent flowering time, fitness of hybrids, and selection for novel traits. However rare, the scale of potential pollen flow and the evolutionary time frame allow us to predict these events will occur.

Since these events are predicted to occur, a response plan to eradicate hybrids with novel traits should be developed. It would be appropriate to use procedures now in place for restricted weeds (eradication followed by monitoring) to eliminate with these hybrids when they are located.

Table 2: Canadian crop and weed species related to *B. napus*, their distribution, potential for hybridization and appropriate references.

Species	Distribution	Hybridization Potential	Reference
Brassica rapa	Crop, and widely distributed weed – infrequent, naturalized in E and crop volunteer in W	High (9-93%) Introgression	J?rgensen and Andersen 1994; Landbo et al. 1996 Hansen et al. 2001
Brassica juncea	Crop	Moderate (3%)	Bing et al. 1996
Raphanus raphanistrum	Widely distributed weed in E, rare in W	Very low Very low	Chèvre et al. 2000 Rieger et al. 2001
Sinapsis arvensis	Widely distributed weed	Very low	Moyes et al. 2002
Erucastrum gallicum	Locally abundant weed	Very low	Lefol et al. 1997

Summary

Gene flow from herbicide-resistant canola varieties does not threaten the acceptability of Canadian canola oil or products, as customers currently accept all herbicide-resistant canola. The average producer successfully uses herbicide-resistant canola varieties with few tribulations and substantial perceived benefits (Canola Council of Canada 2001).

The impact of gene flow on the environment must be weighed against potential benefits due to a modified agronomic system. Herbicide-resistant canola allowed producers to: reduce annual herbicide usage by 6,000 tonnes; reduce tillage, previously required for weed control and incorporation of soil-applied herbicides; and consequently, reduce fuel consumption by 3.2 million liters (Canola Council of Canada 2001). While experiences with herbicide-resistant *B. napus* provide ample evidence that widespread gene flow will occur, to date there have been few negative economic or environmental consequences. Decisions on future traits must continue to be based on the specific crop, trait and agronomic environment.

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GENE FLOW FROM OILSEED RAPE (*BRASSICA NAPUS*) AND BEET (*BETA VULGARIS*) TO WILD RELATIVES: EFFECTS OF HERBICIDE TOLERANT CULTIVARS

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Abstract

Transgenes may be transferred from genetically modified (GM) crops to the wider environment through crosses with compatible wild or weedy relatives. For oilseed rape (*Brassica napus)* we found extensive transfer of nuclear as well as plastid DNA (cpDNA) to *Brassica rapa* in environments with poor weed control. In most conventionally managed fields with oilseed rape as crop and the wild recipient as a weed, the introgression was insignificant or not detected. The extent of gene flow from the crop apparently depends on the agricultural practice and weed control: When the species coexist over several growth seasons introgression is likely to take place. Some of the plants with markers from both species were introgressed beyond the stage of F_1-hybrids. The results also show, that oilseed rape plastids will be captured by *B. rapa* and therefore transplastomic oilseed rape with transgenes engineered into the chloroplast DNA will not prevent dispersal of these genes to the weedy relative. Field and laboratory experiments show that fitness of interspecific F_1 hybrids and backcross plants with *B. rapa* were variable but could be as high as and even higher than the fitness of the parental species. For cultivated beets (*Beta vulgaris* ssp. *vulgaris*) we analyzed the winter survival of F_1 hybrids with the wild sea beet. We found that the F_1 hybrids had a cold tolerance in between that of the two parental subspecies. Apparently introgression between cultivated beet and sea beet is not infrequent and the survival of the introgressed plants at places with poor weed control seems likely. In the light of our results we discuss the perspectives of growing herbicide tolerant oilseed rape and beet.

Keywords: *Brassica*, *Beta*, interspecific hybridization, introgression, fitness, transgenes, herbicide tolerance.

Introduction

Flow of transgenes by hybridization between genetically modified crops and wild relatives is not necessarily a risk to the environment. The consequences to natural and cultivated ecosystems certainly depend on the

inserted traits. Therefore the effects are most appropriately addressed by a targeted risk analysis of the transgenic plant in its proper environment. However, for the pre- and post-commercial risk evaluation regulators always request baseline knowledge about the extent of gene flow between the crop and related recipients as well as survival of the resulting progeny. One way of generating this knowledge is to monitor the extent of gene flow from crop to wild relatives that has already occurred under natural conditions, and to study the fitness of the introgressed plants. In the this paper we present some of our data on gene flow and fitness analysis in *Brassica* and *Beta* and evaluate the results in the light of future cultivation of GM herbicide tolerant oilseed rape and beet in Europe.

Results and discussion

Brassica

Introgression between oilseed rape and B. rapa in an organic field

In an organic field of barley and legumes in eastern Denmark some morphologically deviating *Brassica* plants were observed together with weedy oilseed rape (*B. napus*, 2n=38 genomes AACC) and *B. rapa* (2n=20, genomes AA). At flowering all *Brassica* plants were collected from a 3 m^2 plot. Leaf material from a total of 102 plants was analysed using AFLP markers specific to *B. napus* or *B. rapa*. The development of these species-specific markers was described in Hansen et al. (2001). Among the 24 AFLP markers used in the analysis three were specific to *B. rapa* (one monomorphic and two polymorphic markers) and 21 to oilseed rape (17 monomorphic and four polymorphic markers), 18 of which were positioned on the C-genome. In parallel with the analysis of the natural population the inheritance of the markers was studied in offspring from controlled crosses: F_1 interspecific hybrids and first and second backcross generations with *B. rapa*. *B. rapa* was the female in the controlled crosses, and as all AFLP markers specific to oilseed rape were transferred to offspring plants, these marker were judged to be nuclear.

We revealed a pronounced introgression in the natural weedy population (Figure 1). 45 plants were introgressed having both oilseed rape and *B. rapa* specific markers. Among the remaining 57 plants, seven had only *B. napus* specific markers and 50 plants had only *B. rapa* markers. Figure 1 gives the distribution of oilseed rape markers in the 102 plants.

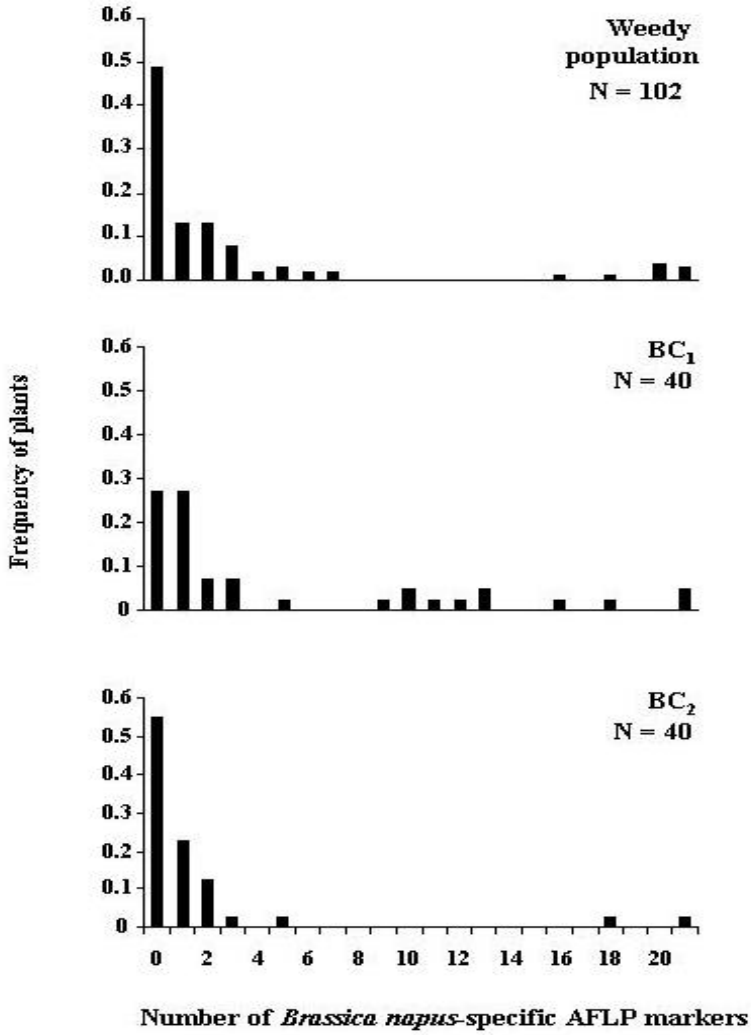

Figure 1: Distribution of oilseed rape specific AFLP markers in the weedy population of *B. rapa* found together with oilseed rape in an organic field (top), in the BC_1 generation (middle) and BC_2 generation (bottom) from controlled crosses.

The monomorphic markers showed that there was only one first generation hybrids (F_1) among the analyzed plants. Infrequency of F_1 hybrids could be due to a small number of oilseed rape plants compared to *B. rapa*. We assume that the natural population had maintained it self since 1987, the last time oilseed rape was cultivated in this field. The proportion of oilseed rape probably decreased since then as *B. rapa* has a better survival over time due to pronounced seed dormancy (Landbo and Jørgensen 1997). The long

existence of the mixed population suggests that some of the plants with DNA markers from both species were advanced generations of introgressed plants**.** This was confirmed by comparing the marker distribution in the natural population with distribution of the very same markers in the BC_1 and BC_2 generation from the controlled crosses (Fig. 1). As the marker distribution in BC_2 plants resembled the marker complement in the natural population, we tentatively conclude that the latter was introgressed beyond the BC_1 generation. The chromosome number was counted in 15 offspring plants from five introgressed females in the natural population. Offspring chromosome numbers were 20-26, and they had from 1 to 12 C-genome specific markers. The presence of plants with C-genome markers and 20 chromosomes suggests that recombination had taken place between the C-genome of oilseed rape and the A-genome of *B. rapa*.

Introgression between oilseed rape and B. rapa in conventionally managed field

The inefficient weed control in the organic field probably accounts for the high frequency of introgressed plants. In accordance with this the frequency of introgressed plants from conventionally grown oilseed rape fields was much lower. In 2450 plants from seeds harvested in eight populations of weedy *B. rapa* found in conventional managed fields we only detected two plants introgressed beyond the F_1-stage and 81 F_1 hybrids. The introgressed plants had more than six *B. napus* specific AFLP markers in addition to the *B. rapa* markers. For the majority of these plants the results were obtained from RAPD and isoenzyme data and only a few hundred plants were analyzed by AFLP using the same markers as in the organic field. As the AFLP provided more markers than the other marker techniques the frequency of introgressed plants might have been underestimated. On the other hand the frequency of introgressed plants might also have been overestimated as most of the plants analysed were reared in growth chambers from seeds harvested on *B. rapa* in the field. The survival of introgressed plants to the adult and reproductive stage is probably lower in nature. Figure 2 compares the data on introgressed plants from the organic and the conventional fields.

For UK environments a low frequency of transgene dispersal from oilseed rape to *B. rapa* was predicted from findings of a low number of F_1 hybrids germinated from seed harvested on *B. rapa* in natural populations found along rivers (Scott and Wilkinson 1998). The true wild *B. rapa* in UK rarely occur together with oilseed rape minimizing the risk of gene flow, but weedy populations of *B. rapa* have now been found also in UK and they await further analysis of introgression from oilseed rape (Mike Wilkinson, pers. comm.). Our data from the Danish populations demonstrate that introgression between oilseed rape and *B. rapa* can be considerable when the two species are found in long existing mixed populations. Once a transgene has been introgressed to weedy *B. rapa* in the field the potential for gene dispersal to other populations of *B. rapa* in disturbed environments is enhanced.

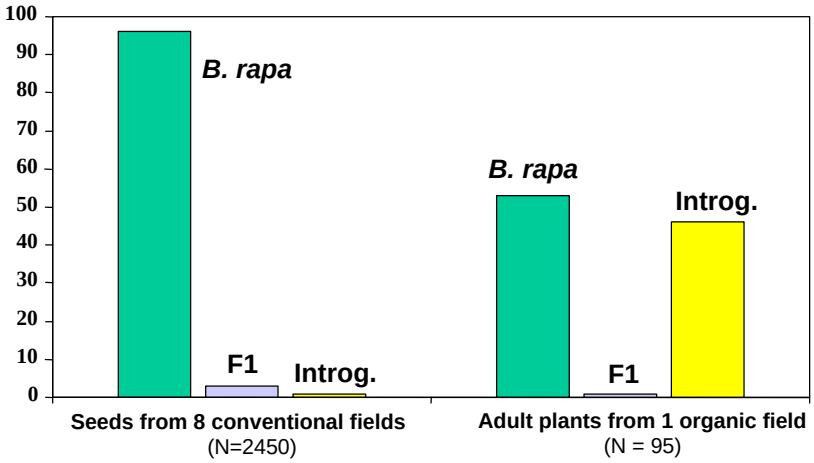

Figure 2: Frequency of *B. rapa*, F1 and introgressed plants from mixed populations of oilseed rape and *B. rapa*.

Fitness of introgressed plants

We analysed the fitness of different generation of introgressed plants in field and growth chamber experiments. Hauser et al. 2002 showed, that seed set of F_1 hybrids between *B. napus* and *B. rapa* was dependent on the environmental conditions. The F_1 hybrids could produce more seeds than *B. rapa*, but also less seeds dependent on the number and densities of parents and backcross plants in the population. As to the F_2 and BC_1 with *B. rapa*, Hauser et al. (1998) found large variation in fitness with a smaller fraction of these plants being just as fit as *B. rapa* and *B. napus*. In a further advanced backcross programme, BC_1 plants were selected for being *B. rapa*-like and these plants backcrossed to *B. rapa*. The reproductive fitness of the resulting BC_3 generation was as great as that for *B. rapa* (Snow et al. 1999). The BC_3 generation segregated 1:1 for a *bar* gene providing glyphosinate resistance. There was no difference in the seed set and survival of the GM siblings compared to the non-GM siblings when the herbicide was not applied indicating that a cost associated with the transgene was negligible. The overall conclusion from our fitness data is that given the "right" environment a part of the introgressed plants will be as fit as their wild parent and occasionally also as fit as the crop. This supports our finding of persistent populations of introgressed plants occurring spontaneously in the agro-ecosystem (mentioned above).

Transfer of plastid encoded genes from oilseed rape to B. rapa

In *Brassica* like most other angiosperms chloroplasts are not transmitted by the pollen (Corriveau and Coleman 1988). Therefore, transplastomic

oilseed rape where the transgene is engineered into the plastid genome, can only disperse the new genes through the seed (Daniell et al. 1998; Scott and Wilkinson 1999). The origin of the chloroplast DNA was analysed in 91 of the plants from the natural population in the organic field. The analysis was performed by PCR using primers to non-coding regions of the cpDNA (Taberlet et al. 1991). The primers amplify species-specific markers for oilseed rape and for *B. rapa*. The analysis assigned the chloroplast of the plants to either oilseed rape- or *B. rapa*-type and this information was compared to their nuclear AFLP-fingerprint. The cpDNA analysis showed that besides the seven *B. napus*-like plants, one *B. rapa*-like plants and two introgressed plants carried the oilseed rape chloroplast. In a huge and persisting weedy *B. rapa* population in a conventionally managed field, we found that 9 of the 23 plants analyzed carried oilseed rape chloroplasts. The AFLP analysis of these plants failed to detect any oilseed rape specific nuclear markers but an AFLP marker segregating together with the *B. rapa* cytoplasm was also missing, supporting that chloroplast capture had taken place in these plants. Our results from these long-lasting populations of oilseed rape and *B. rapa* indicate that transgenes positioned in the chloroplast DNA of *B. napus* will be captured by *B. rapa*. If oilseed rape and subsequently the F_1 hybrid are females in the crosses, fertile *B. rapa*-like plants with 20 chromosomes can be produced after just two generations (Mikkelsen et al. 1996), a pathway for production of transplastomic *B. rapa*. Scott and Wilkinson (1999) analysed the chloroplast inheritance in 47 F_1 hybrids harvested on wild *B. rapa* and sired by oilseed rape. They only found plants with a *B. rapa* cytoplasm and concluded that transgene introgression from transplastomics would occur extremely rarely in populations of *B. rapa* found along river banks in UK.

Beta

The spontaneous introgression between cultivated beet (*Beta vulgaris* ssp. *vulgaris*) and sea beet (*Beta vulgaris* ssp. *maritima*) has been reported by several authors. The agronomic concerns from weed beets in sugar production areas is probable caused by the offspring from such introgression. In example Viard et al. (2002) and Boudry et al. (1993) analyzed the origin of the weed beets. They claimed that" in row weeds" were due to "accidental pollinations of seed-production plants" in the seed propagation areas in Southern Europe, while the out-row-weeds were derived from the seed bank in the sugar production areas, and were a result of the intercrosses between all flowering plants in the vicinity (other out-row beets, in-row-weeds, variety bolters and wild sea beet). In the light of the weed beet origin and frequency it seems likely that a transgene will easily be transferred to these weedy types and perhaps also to the sea beet populations in the area.

The main area for sugar production in Denmark is close to populations of sea beet, ssp. *maritima* along the cost. Crop bolters and weed beets in the fields therefore have a potential for hybridization with the sea beet nearby.

Vernalization is required for flowering of the Danish populations of sea beet as it is for the F_1 hybrids. One of the key factors for beet survival in northern Europe is apparently cold tolerance. Pohl-Orf et al. (1999) showed that the survival of cultivated beet was tightly linked to the temperature. The lower the temperature the lower the survival, the least survival was found in the most northern environments. Therefore survival was tested in a Danish environment over winter. F_1 hybrids and the two parents, cultivated beet and sea beet were grown over winter at a exposed field where the average temperature was below zero for several weeks. The number of survivors was recorded in May the following year. None of the cultivated beets survived; the survival of the sea beet and F_1 hybrids were 18% and 10% respectively. The results show that some of the F_1 hybrids can survive temperatures substantially below zero indicating that they have the potential for persistence over winter and flowering the following year. This is a likely scenario in places where weed control is inefficient, however, in an agro-ecosystem with weed control the problem with the introgressed weed beets will probably not increase due to a better winter survival in the introgressed plants.

Effects of growing herbicide tolerant oilseed rape and beet

Our gene flow analysis is based on natural DNA markers of the oilseed rape nucleus and plastids. These markers are supposed to be selectively neutral. There is no reason to believe that transgenes will be introgressed differently from these markers. However, the rate of transgene transfer may be increased due to selection in favour of the transgenic traits, which will be the case for herbicide tolerance in the agro-ecosystem (Snow et al. 1999). In conclusion, given the right environment oilseed rape is apparently a potent donor of genes to *B. rapa* and introgressed plants survive and reproduce in the natural populations. Experiments in non-selective environments suggest that the fitness of some of the backcross plants will be equivalent to that of the wild parent. Under certain environmental conditions the reproductive fitness of interspecific F_1 hybrids will be even greater than that of both parents.

We have shown that integration of genes in the plastid DNA or on C-chromosomes of oilseed rape will not provide safeguards towards the natural introgression. However, the frequency of gene transfer can be reduced by efficient control of related weeds – i.e. by efficient herbicide control. Therefore the development of herbicide tolerant *B. rapa* will be slow in the conventionally managed fields and herbicide tolerant cultivars will provide easy and secure management of *B. rapa* over many years. As many farmers convert to agricultural practices with no or low herbicide usage weed problems may increase. The newly started organic production of oilseed rape may promote the presence of mixed populations of oilseed rape and *B. rapa* in succeeding crops. Transgenes from neighboring GM oilseed rape may be transferred to *B. rapa* and *B. napus* in these mixed populations. This scenario may give rise to problems in relation to the other types of transgenes being

stacked in the weedy populations and problems in relation to treshold values of GM in the organic production.

As introgressed plants between cultivated beet and sea beet already cause problems in many beet fields over Europe, it seems likely that weed beets will acquire the herbicide tolerance from a GM variety rather quickly. Probably the worst consequence of that would be that the herbicide was rendered inefficient for weed beet control – a situation much like the one we have today before the introduction of herbicide tolerant cultivars. The GM-herbicide will still be effective against other weedy species in the field. In a GM-beet field herbicide will be applied only when needed, and recent reports suggest that the optimal management of herbicide tolerant beets is band spraying over the rows. In this way the frequency of other weedy species will increase out-row, increasing biodiversity in the field but also increasing inter-weed competition. This could reduce the abundance of out-row weed beets. The winter survival of F_1 hybrids would only be a fitness-enhancing trait in places with poor weed control and poor competition from other weeds. In conclusion the cultivation of GM herbicide tolerant beets could provide environmental benefits if the herbicide in question is less toxic to the environment than the ones presently applied in non-GM fields. As herbicide application is highly unlikely at the seashores where ssp. *maritima* grows, there will not be any direct effects from transfer of the herbicide tolerance to sea beet.

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TRANSGENIC HERBICIDE-RESISTANT CROPS: WHAT MAKES THE DIFFERENCE?

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Abstract

Herbicide resistant crops contribute to the development of the chemical weed control. Aims, tools and achievements are examined from an historical point of view. Genetic engineering is the last, powerful technique used for that purpose. However, in contrast to the other tools of plant breeding, genetic engineering enables a kind of new crop domestication process which consequences extend beyond the botanical family frontiers. Therefore, it is not only necessary to study the herbicide resistance trait for itself, but also to investigate the impact of genetic engineering on biodiversity and plant evolution.

Keywords: Plant breeding, Transgenic, Herbicide resistance, Gene flow, Wild populations

Introduction

To determine what could be new features or scaled up phenomena in agriculture if using a transgenic herbicide resistant variety, and what could be the impact on environment, it seems necessary to review the origin of chemical weed control and its relationship with plant breeding. Fighting against weeds in arable fields is probably as old as crop domestication. In old times, hand weeding, then mechanical cultivation were the only ways to control weeds. The use of chemicals to control weeds appeared at the beginning of the twentieth century and developed following the second World War. Meantime, "automatic" farmer's selection of crops was continuing, then plant breeding theories were proposed in the later two centuries and actual crop improvement achieved. However, the capacity of crop cultivar to tolerate or to compete against weeds was never seriously considered, which placed weed control outside the domain of plant breeding. Hence, when herbicides were released, the task of designing selective herbicides was naturally devoted to agrochemical companies only. New molecules with herbicidal activity were screened on samples of various crops to determine safety (=selectivity), and this property was assumed to be homogeneous within a crop species. Plant breeders took no account of that new property and even forget to check it at each step of the selection process, which resulted, in some cases, in the release of varieties susceptible to herbicides commonly used on the crop (e.g. Barrentine et al. 1982).

Therefore, the responsibility of the innovation was due to the chemist who proposed the herbicide-crop pair.

Differential responses to herbicide among current cultivars were soon observed in the field, not only minor variations due to plant morphology or vigor, but also major genetic effects: e.g. triazines in maize (Grogan et al. 1963), barban in barley (Hayes et al. 1965), triazines in rape and mustard (Karim and Bradshaw 1968), chlorotoluron in wheat (Tottman at al. 1975), metoxuron in wheat (Lupton and Oliver, 1976), metribuzine in soybean (Edwards et al. 1976), EPTC in maize, etc. While variability permitted genetic improvement for enhanced herbicide tolerance, little was done. Research was relegated to foot-note of plant breeding and results were inconclusive (see references in Darmency 1994a). It was noteworthy that several papers were entitled "inheritance of sensitivity…", which suggest tolerance was just a curiosity trait, and no deliberate selection was considered. Very few authors discussed the implication of varietal tolerance to chemicals in plant breeding and weed control (Hayes et al. 1965). The idea that herbicide resistance could dramatically increase through simple breeding became a reality in the late 1970's when several weed species were identified that were 1000 fold resistant to atrazine.

Resistance was due to mutation of the chloroplast gene *psbA*. The release of an atrazine resistant oilseed rape variety, after a cross with a resistant wild relative (Beversdorf et al. 1980), laid the foundation for efficient genetic improvement of herbicide resistance in plant breeding. At the same time, the introduction of several efficient breeding techniques facilitated further work, and breeders were encouraged by agrochemical industries to work on herbicide resistance. Finally, genetic engineering promised easy transfer and expression of foreign genes in plants, especially when a transgene alone was responsible of clear-cut plant response as in the case of dominant-inherited herbicide-resistance genes.

We first examine the objectives and tools for breeding herbicide resistant varieties. Secondly, we discuss the possible environmental impact due to herbicide resistance. Since herbicide resistance can be obtained from conventional techniques of breeding, the consequences specific to genetic engineering are also addressed.

Why and how creating herbicide resistant varieties?

Objectives

In the 1960's, when a new efficient herbicide was released, it was very attractive to expand its use on other crops. Therefore, the earliest research consisted of screening among accessions to select for tolerant lines, however detected variability was generally limited and research ceased. In a few instances, research was undertaken because a given herbicide injured some cultivars while being safe for others, as 2,4-D on flax (Stafford et al. 1968). Genetic improvement was also evaluated on secondary effects of herbicides.

For instance, high doses of atrazine on maize resulted in toxicity in subsequent susceptible crop, like wheat. Therefore, plant breeders attempted to enhance the tolerance of susceptible crops to residual herbicides (Gournay et al. 1975).

With time, the need to improve the strategy of chemical control increased and new ideas for the use of herbicide resistant varieties were proposed. Some were to solve acute problems like the occurrence of Solaneae in a tomato field, wild Brassiceae in oilseed rape, weedy beet in sugar beet, etc., because all wild species within a botanical family share similar herbicide resistance as their domesticated counterparts. Similarly, the use of non-selective herbicides as glyphosate and glufosinate often appeared to be the only tool to control weeds with no other herbicide control. Resistant crops, therefore, would allow control of those troublesome weeds. There are also cases of crops for which no efficient selective herbicide has been developed because they do not represent a market large enough to justify the research expenses of a new molecule (Wang and Darmency 1997). The question of parasitic weeds could also be solved since for the first time a resistant crop host could survive the treatment while translocating herbicide through the phloem (Surov et al. 1997). Creating a diversity of resistant varieties was suggested to facilitate herbicide rotations for delaying the selection of resistant weeds (Gressel 1993). Varietal purity of a resistant variety could be increased by destroying susceptible volunteers in the field (e.g. 0 versus 00 oilseed rape to maintain low levels of erucic acid and glucosinolate). This concept could apply to producing true hybrid varieties by preserving resistant seed parents while killing susceptible pollinator, or to grow homogeneous varieties by preserving resistant hybrids while killing seedlings from self-pollinated seeds when male sterility is not 100% (Beversdorf et al. 1987).

Other objectives were simply the extension of the chemical treatment, such as to allow increased rate of herbicide for a wider range of killed weed species. There was also the possibility to free the farmer from concerns with growth stage of the crop and the weed by increasing the tolerance difference between them. Paradoxically, such a flexibility of herbicide use could allow applying integrated pest management (IPM), which fulfils with environmental concerns and enhances the possibility of alternative weed control. In addition, improvement of herbicide resistance could replace old molecules by more environmentally friendly herbicides. Finally, financial returns for agrochemical companies were obvious. Enabling the use of herbicides on previously susceptible varieties would increase market share for the herbicide and increase profitability. It could reduce research expenses for new products and extend the usefulness of toxicology tests developed for that herbicide. All these objectives hold true whatever the genetic technique used to create herbicide resistant varieties.

Achievements

Tools for breeding herbicide resistant varieties evolved with the time of the research. For instance, works published in the late 1960's dealing with 2,4-D and triazines used mass selection and crossing among numerous varieties. Cell culture was used in the 1970's, with and without mutagenesis, and finally genetic engineering in the 1980's. All plant breeding strategies continued to be used altogether. The method depends on the objectives and the material available, but also on the balance between work time versus quality of results. The use of conventional breeding using the wild genetic resources of a crop can result in a very efficient herbicide resistant variety, as the triazine resistant oilseed rape presently grown over more than one million hectare in Australia. In contrast, transfer of glutathione-S-transferase genes of maize by genetic engineering into other crops did not provide sufficient tolerance for the same herbicide family. In the case of the sulfonylureas and imidazolinones, two herbicide families inhibiting the acetolactate synthase, many breeding methods have been used in various crops, with varied success. While the first herbicide of the family was commercially released in 1982, the first papers reporting the set up of resistant varieties appeared as soon as 1987. Within a few years, resistant germplasm of soybeans, maize, sugar beet, oilseed rape, wheat, rice, cotton, lettuce and carrots was reported, and some led to commercial varieties. The techniques used involved selection among genetic resources (Mallory-Smith et al. 1993), selection in cell culture (Anderson and Georgeson 1989), pollen (Swanson et al. 1989), seeds (Newhouse et al. 1992) and cell culture mutagenesis (Sebastian and Chaleff 1987), and genetic engineering (Haughn et al. 1988). Additional sophistication involved both directed mutagenesis on extracted endogenous acetolactacte synthase genes and biolistic transformation (Rajasekaran et al. 1996). The most efficient resistances were not always those using genetic engineering, but often derived from the isolation of mutants in mutagenesized cell culture, genetic resources, and gene amplification through cell cycles under increasing selection pressure (Caretto et al. 1994).

For genetic engineering, most resistances genes were investigated and located in bacteria (Table 1), except for sulfonylureas because several plants provided early various efficient mutants at the acetolactate synthase gene. Yeast, algae and fungi mutants for some herbicide resistance are also known, which provides an abundant source of future breeding work. Even genes of mammals were indicated as possible tools to confer herbicide resistance. All are dominant genes and are easily identified on microorganism culture. Further modification of the candidate gene is often necessary to provide satisfactory level of resistance. For instance, the former *aroA* gene from *Salmonella* was not efficient enough to protect a crop against glyphosate, and it has been tentatively replaced by once, then twice mutated EPSP synthase genes from *Petunia*, and finally by over-expressed constructs CP4 of mutated EPSP synthase of bacteria linked to the chloroplast transit peptide of *Petunia* (Padgette et al. 1996). Numbers of mutant genes and organisms were

assayed, and also transformants having the best and stable expression had to be selected. Later on, the oxydoreductase gene *GOX* from *Pseudomonas* has been added, in some crops, to detoxify the herbicide.

Table 1: Transgenes conferring herbicide resistance (organisms from which they belong in brackets).

Herbicide	Mechanism of resistance	
	Target alteration	Herbicide detoxification
Asulam	*Sul (Escherichia)*	
Chlorotoluron		*CYP1A (rat)*
Chlorsulfuron	*csr1 (Arabidopsis),* *Sur (Nicotiana)* *Ahas3 (Brassica)*	
Dalapon		*dehal (Pseudomonas)*
2,4-D		*tfdA (Alcaligenes)*
Dinitroanilines	*EiRtua1 (Eleusine)*	
Diphenyl ether	*protox (Bacillus)*	
Glufosinate		*bar, pat (Streptomyces)*
Glyphosate	*aroA (Salmonella),* *GIO1 (Petunia)* *CP4 (bacteria)*	*GOX (Pseudomona)s*
Metolachlor		*GST (Zea)*
Norflurazon	*crtI (Erwinia)*	
Oxynils		*bxm (Klebsiella)*
Phenmediphan		*pcd (Arthrobacter)*

Although the release of transgenic herbicide resistant varieties dates from only seven years, they represent 95 % of the herbicide resistant varieties grown today. The area growing herbicide resistant varieties obtained through conventional tools, throughout the world, is less than two millions hectares. It includes triazines resistant oilseed rape, imidazolinones and sulfonylureas resistant maize, oilseed rape and wheat, and cyclohexanediones resistant maize. Transgenic varieties expressing herbicide resistance represent two third of the genetically modified plants grown over the world, that is around 35 million hectares. The quick adoption of transgenic varieties by farmers, since the first commercial release in 1996, indicates an immediate benefit perceived through decreasing the frequency of field treatment and allowing more flexibility.

Agro-environmental impact of the trait

Since creating a herbicide resistant variety is the same as creating a herbicide molecule selective of a crop, it should be expected that nothing would change. In both cases, there is a combination of herbicide and crop that has been agreed by regulatory authorities after studies of field efficiency and toxicity, etc. In both cases, the properties of the herbicides are similar: official requirements and farmers' wishes for a new herbicide are a toxicity level under an accepted threshold, low volatility, short environmental half-life, reduced leaching in soil, biological activity at low dose, efficacy on a broad spectrum of weeds, and used as post-emergence of the weeds. These arguments were delineated by the "pros" of transgenic varieties (Burnside 1996), but in fact, most of these issues do fit with recent herbicides and conventional varieties. In that perspective, using transgenic varieties is no more than continuing and sophisticating chemical weed control. This aspect fits well with the American concept of "familiarity". Indeed, crops resist to selected herbicides: 95% of the cultivated land is currently sprayed with herbicide in developed countries, natural variability of herbicide resistance occurs among varieties, and herbicide resistant varieties were already created using conventional techniques. Therefore, herbicides and crop surviving herbicides are familiar and transgenic varieties or not make no difference. For instance, prior to the growing of transgenic varieties, glyphosate, the chemical for which there is most resistant varieties, was already the most widely used product in the world, with around 11% of the market.

The logical conclusion should be to scrutinize the agro-environmental consequences of the chemical weed control in isolation. Benefits are allowing mechanization of agriculture, reducing soil erosion, saving working time, preserving the crop against weed competition, producing clean uncontaminated harvest, providing higher returns, etc. In contrast, the classical analyze of negative issues involves the chemical contamination of the environment, the soil pollution by long lived or soil-trapped residues, the contamination of ground water, long term toxicity and decrease of the fertility of the wild fauna, etc. Making more efficient chemical weed control could meet the goals both of reducing the damaging impact of agrochemicals upon the environment and providing an efficient weed control, while solving the questions indicated above. To be sure that the use of transgenic herbicide resistant varieties actually goes towards that way, environmental effects should be evaluated with relevant methods. The only tools available now are preliminary reports on herbicide use in experimental farms or in whole regions (Heimlich et al. 2000), but they are expressed in tonnage of product and not yet in any ecological unit, so that the effective beneficial impact cannot be estimated. On the dark side, one could say that a lot of money is devoted now to genetic engineering and field testing of these new varieties, which would reduce the research effort for alternate strategies and delay the set up of other kind of biological or ecological weed control. From an absolute point of view, this could be hardly ecologically acceptable

considering that transgenic herbicide resistant varieties always mean herbicide use.

Consequences specific to transgenic varieties

Impact due to over-use

Say that everything is the same, whatever the release or withdraws of transgenic herbicide resistant varieties, is not completely accurate because farmers' weed control practices could change slightly. Indeed, as the availability and diversity of chemical/crop variety options increase, new situations develop. Individual effects, although familiar, may have cumulative effects which are difficult to predict. Such effects include the occurrence of more types of volunteers growing in the following crops sprayed with the same herbicide. Volunteers already occur, but their susceptibility to other herbicides is known and this can be used to design appropriate management. In the case of transgenic volunteers, they can cross with plants from other resistant varieties, stacking herbicide resistance genes and reducing the options of chemical control. The first triple-resistant oilseed rape volunteer seeds were discovered in Canada at the end of the second season of growing transgenic varieties (Hall et al. 2000). In the past, crossing between volunteers or varieties was not the source of different patterns of herbicide resistance. One must notice that oilseed rape varieties now could be resistant to one or more of the following herbicides that were previously lethal: triazines, ioxynils, glyphosate, glufosinate, sulfonylureas and imidazolinones, which greatly decrease alternate option for management.

Another effect on the farming system is the occurrence of herbicide resistant weeds. Three weed species have evolved glyphosate resistance because of the repeated application of the same herbicide that selected for spontaneous resistant mutants. This has occurred with fifteen other herbicide families since the 1970's (Heap 2002). The release of resistant varieties for a crop grown in an area where glyphosate was not previously used, and the use of different herbicide dosages at different dates would open new areas and submit new flora to select for resistant weeds. One such case was observed in transgenic glyphosate resistant soybean in America where horseweed evolved spontaneous resistance after a few years only of glyphosate application (VanGessel 2001).

Finally, the use of the same resistance gene in several crop species would led to a widespread use of the same herbicide, allowing concurrence among herbicides not only within one crop but also among several crops. Where these herbicides are low priced, commodity products, over use might increase risk for the environment. Farmers will tend to use repeatedly the same inexpensive herbicide, even increasing dosage because it is cheap and not toxic for the crops in case of some weed becomes more troublesome. This will result in increasing the opportunity to select for new spontaneous resistant weeds, leaving more and more resistant volunteers of different crops

growing in the field, and increasing accumulation of residues of the same herbicide in the soil. In addition, a low price would make it adopted by farmers that otherwise should had no need to use transgenic plants because yield losses due to weeds are insignificant. Finally, a low price would render a new competing herbicide for the same crop comparatively more expensive and difficult to put on the market, which in turn would reduce the diversity of new agrochemical available for weed control in the future. This reduction of potential tools for farmers to fight against weeds could be soon a problem since numbers of agrochemicals will be withdrawn from the market according to the European regulation and there is no abundance of new molecules proposed by the industry. At last, one should point out that the unique option of total weed control using the unselective herbicides glyphosate and glufosinate has been lost since a series of crops display transgenic resistant varieties, resistant volunteers occur, and possibly gene flow will result in some resistant weeds. Such loss of efficiency of these herbicides could impair no-tillage practices and management of uncultivated areas, finally resulting in more environmental pollution due to inappropriate and enhanced product use.

Impact due to the genetic modification

Genetic engineering is a powerful tool to modify plant characteristics and achieve the goal of food and plant-derived goods supply. It allows transferring more than one dozen of transgenes encoding herbicide resistance (Table 1), while conventional plant breeding succeeded for only four herbicides. In those cases, and for most spontaneous resistant weeds, the resistance mechanisms are mainly target mutation which makes the mutant no longer susceptible. In the case of transgenic varieties, half the transgenes encodes for metabolic detoxification of the herbicide, which results in secondary products which have never been produced in the field before. An estimate of the eco-toxic impact of these products is needed.

Crops can cross with wild relatives, and the use of new research techniques has allowed many such cases to be documented (Ellstrand et al, 1999). In Europe, this includes wheat, oilseed rape, sugar beet, oats, alfalfa, carrots, chicory and lettuce. Most concerns are centered around the areas of origin of the crops, as maize, sunflower and squash in Central America. Introgression of an herbicide resistance gene simply renders weeds resistant to herbicides, thus jeopardizing the breeders' effort to provide new tools combating against weeds. There is a more important consequence of using genetic engineering. In the past, interspecific hybrids usually inherited no new advantage to resist against herbicides since all species within a botanical family share the same resistances (obviously with some exception such isoxaben for oilseed rape/wild radish). Therefore, crop/weed introgression was not significant since crop genes were likely to confer genetic drag. With a transgenic derived resistance, the trait is new for the whole botanical family, so that the hybrids display an additional adaptive trait. Even if the

physiological and reproductive barriers of interspecific hybrid are high, their survival is increased by 20 to 100 in the presence of the herbicide. With such advantage, they could spread, produce progeny and dominate the original taxa. The importance of biological novelty brought about by only one new gene has been illustrated in several case studies (Darmency 1994b). Therefore, genetic engineering for herbicide resistance could allow the evolution of hybrid forms, a feature never met in the past when using conventional breeders' tools. In addition, since there is a genetic variability among wild plants for their ability to cross with a related crop (Guéritaine and Darmency 2001), one can expect also selection for easy interspecific crossing, thus changing the genetic structure of wild plant populations. On the one hand, enhanced introgression could result in "super weeds" and invading forms. On the other hand, gene flow from a domesticated species toward a wild plant could overflow the species barriers and contribute to a loss of biodiversity through a lack of adaptive potential. One way to prevent transgene dispersal is to make impossible their expression in hybrids, for instance by using recessive or multi-genes in spite of dominant genes, but, unfortunately, most genetically engineered traits are dominant.

Once established into a wild species or a new plant form (e.g. amphiploids), the transgene is expressed independently of any control by man. Agrochemical remediation is possible, but genetic modification cannot be repaired! In the presence or absence of herbicide, engineered transgenic resistant varieties and introgressed wild plants express the resistance gene, which means that a protein product is released in the plant, and finally released in the field and in wild habitats. Nobody knows what kind of organism should be capable of using such protein as substrata for growth and reproduction, and what can be its impact on the plant and animal communities. Alterations of the environment, especially of soil microorganisms, could be transgene-specific and target population-specific, which deserves to assess the question case by case (Oger et al. 1997). In the absence of selection pressure, outside of the agro-environment or following herbicide withdraw, other questions are raised. If the gene has no adaptive cost, it can keep on being transmitted through generations and fixed in some wild populations. This is an opportunity for the gene to mutate and evolve. Very little is known about the stability of transgenes in the absence of selective pressure and control of presence and expression by man, especially if it is accidentally transferred to wild plant and submitted to genome recombination. There are no models predicting the possible function of recombined and mutated transgenes.

Conclusion

Engineering transgenic herbicide resistant varieties is not simply providing new toys for breeders and farmers: it provides useful tools in order to fit with agricultural necessity and solve some acute problems of weed control. Focusing only on the herbicide resistance trait makes possible to

conclude that there is no new concern or scaling up of environmental impact whether the varieties originate from conventional breeding or genetic engineering. One must keep in mind that creating resistant varieties contributes to chemical weed control, which means that it carries all defects of herbicide use. Risk of overuse of herbicides because transgenic varieties is a serious event, but it could be mitigated through educating farmers and providing guidelines for the best use of agriculture practices. However, we identified three issues depending specifically on the origin of the variety. First, interspecific crosses with related species take a new importance since the transgene is the source of new fitness not known in the past. Herbicide resistance often confers a 95-99 % increased survival, which allow hybrids to survive and reproduce, changing genetic structures in populations of wild species and modifying biodiversity. Second, genetic engineering opens a new era of plant domestication, using genes from various botanical family or microorganisms, and even animals, but this "a la carte" domestication could lead to further adaptive re-organization of genomes and alteration of transgenes, the impact of which is hard to predict. Finally, the behavior in the habitat of the transgene products and new herbicides metabolites has not been addressed seriously.

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Genetically modified virus-resistant plants

ECOLOGICAL RISKS OF TRANSGENIC VIRUS-RESISTANT CROPS

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Abstract

One ecological concern about the widespread commercial use of transgenic crops is that transgenes might move into natural populations. For transgenic virus resistant crops, there is potential for gene flow via two mechanisms: 1) recombination of trangenes with wildtype viruses that invade the plant; and 2) transgene introgression into populations of wild relatives of the crop. Both recombination and transgene introgression into wild plant populations appear to be highly probable in many plant virus systems. The consequences of gene flow are less well understood. Evaluation of the fitness impacts of transgenes in both virus populations or plant populations requires further research in natural populations.

Many viruses, like the aphid-transmitted barley yellow dwarf viruses (BYDVs), infect both agricultural crops and wild host plants. In addition to their crop hosts (wheat, oats, barley, rice, corn), BYDVs infect over 150 species of wild grasses. Data from recent field experiments with BYDVs in mixed host plant communities indicate that these viruses can have strong effects on host competitive abilities and host fitness and can significantly modify plant community attributes. Thus changes in virus properties due to recombination or changes in virus resistance due to the movement of viral transgenes could have important impacts on natural plant communities. In particular, the acquisition of transgenic resistance by exotic weeds like wild oats may have negative impacts on both agricultural and natural ecosystems.

Keywords: barley yellow dwarf virus, plant virus, viral transgene, virus resistance

Introduction

One of the ecological risks of the widespread adoption of transgenic virus resistance is the potential for the movement of viral genes from the transgenic crop into natural populations. Viral genes may move through the process of recombination between viral transgenes and invading viruses. Alternatively, viral transgenes may move from transgenic crops by means of pollen flow to wild relatives. A number of recent studies have measured virus recombination

and gene flow from crops to wild relatives in the field, and both processes appear likely to lead to the movement of virus-resistant transgenes (for reviews see Tepfer and Balázs 1997, Aaziz and Tepfer 1999b, Hammond et al. 1999, Power 2002). However, it is difficult to evaluate the hazards associated with this gene flow in the absence of a better understanding of the impacts of viruses in natural plant communities. A comprehensive evaluation of the fitness impacts of transgenes in either virus populations or plant populations requires further research carried out in natural populations.

Gene flow via recombination

Recombination between invading wildtype viruses and transgenes has been demonstrated numerous times (see Aaziz and Tepfer 1999b, Hammond et al. 1999). Although most of the studies demonstrating such recombination have designed the experimental conditions to select for recombinants, a few studies have shown significant amounts of virus-transgene recombination even under conditions of moderate to weak selection (Wintermantel and Schoelz 1996, Borja et al. 1999). Similarly, Aaziz and Tepfer (1999a) showed that recombination among viruses can occur even under weak selection. In a monoculture of transgenic plants expressing viral genes, every infection with an invading virus provides the potential for recombination. This means that the number of recombinants is likely to rise, even if the rate of recombination is equal to or less than the rate of natural recombination among viruses (de Zoeten 1991).

Increased host range, modifications in virulence, and any changes in aphid transmission could provide a selective advantage that would allow the recombinant to spread. Research on cauliflower mosaic caulimovirus in transgenic tobacco has demonstrated expansions of viral host range and changes in symptom severity, an indicator of viral virulence, as a result of transgene-wildtype virus recombination (Schoelz and Wintermantel 1993). In direct competition with its parental wildtype virus, the recombinant was significantly more aggressive than the wildtype, outcompeting it as the infection proceeded (Kiraly et al. 1998). In another study, Borja et al. (1999) detected high levels of recombination between tomato bushy stunt tombusvirus and host transgenes under moderate selection. Again, recombinants, which required two recombination steps to become viable, rapidly dominated in infected plants. These studies illustrate the potential for significant impacts of transgenic virus resistance on the population biology of viruses in nature.

One recent study attempted to examine the probability of recombinant viruses arising under field conditions, by screening for changes in the characteristics of viruses found in infected transgenic potato plants. Over 6 years, Thomas et al. (1998) exposed 65,000 transgenic potatoes expressing potato leafroll virus coat

protein or replicase genes to field infections of wild-type viruses. Viruses found to be infecting transgenic plants were examined for changes in symptoms, serology, host range, and transmission characteristics, and no unusual variants were detected. Although this does not rule out recombination having occurred, it does suggest that the probability is rather low that recombinants with significant effects on virus fitness will persist in this system. Similarly, Fuchs et al. (1998, 1999) were unable to detect recombinant viruses in fields of transgenic squash or melon. These types of field studies are clearly a useful first step in assessing risk in the field. Unfortunately, low probability events are difficult to detect in the short term, but they may still result in significant ecological or evolutionary effects in the long term.

Another significant obstacle to predicting the likelihood of spread and persistence of transgene-virus recombinants is the paucity of data about competition between viruses in the field (Power 1996) and about the spread of viruses from crops to wild plant hosts. Although there have been numerous surveys for particular viruses in wild or weedy plants that are suspected of being reservoirs of viruses (Thresh 1980), apparently no studies have monitored the movement of viruses from crops to wild hosts.

Transgene introgression into natural plant populations

The potential for introgression of transgenes into natural populations of crop relatives is well documented (e.g., Linder et al. 1998, Bartsch et al. 1999, Ellstrand et al. 1999, Spencer and Snow 2001). The consequences of such gene flow will vary with the fitness of these hybrids and the particular effect of the transgene on plant fitness and performance.

Transgenic virus resistance may confer a selective advantage to wild relatives in cases where viruses cause significant fitness effects on plants in natural populations. There have been relatively few studies that examine the fitness consequences of viruses in natural plant populations, but several recent studies have demonstrated significant negative impacts of virus infection on the growth, survivorship, and reproduction of purslane, *Portulaca oleracea* (Friess and Maillet 1996), the wild composite *Eupatorium makinoi* (Funayama et al. 1997), wild cabbage, *Brassica oleracea* (Maskell et al. 1999), and the wild oat *Avena sativa* (Power 2002).

No published studies have explicitly examined the consequences for a virus resistance transgene in a wild relative. Spencer and Snow (2001) showed that introgression of transgenic virus resistance from cultivated yellow squash (*Cucurbita pepo*) into wild *C. pepo* was highly probable based on experiments in agricultural fields. However, the extent of virus pressure in natural populations of wild *C. pepo* is not well understood, so the fitness advantage of the transgene

in such populations is unclear. Bartsch et al. (2001) used field experiments to look at the fitness of hybrids of sugar beets (*Beta vulgaris* ssp. *vulgaris* var. *altissima*) and swiss chard (*Beta vulgaris* ssp. *vulgaris* var. *vulgaris*) with and without the viral transgene that has been inserted into sugar beet to increase resistance to beet necrotic yellow vein virus. Swiss chard was used as a surrogate for weedy beets, because it is a close relative of sugar beet that naturalizes easily. Under conditions of high virus pressure, Bartsch et al. (2001) found increased biomass of the transgenic hybrid relative to the nontransgenic hybrid or swiss chard parent. However, as in the case of wild squash, the extent of virus infestation in natural populations of wild or weedy beets is not well documented.

Ecological risks of transgenic resistance to barley yellow dwarf

We have been studying the impacts of the barley yellow dwarf viruses (BYDVs) on wild grasses in order to assess the potential hazards associated with movement of transgenic virus resistance from cultivated cereals to wild grasses. Transgene movement from cereal crops expressing transgenic resistance to BYDVs may pose particularly high risks because of the paucity of natural resistance to BYDVs in some wild relatives such as wild oats. Accumulating evidence suggests that both the probability of transgene transfer to wild relatives, and the fitness advantages of the transgenes, are likely to be high for some cereals targeted for transgenic BYDV resistance.

Of the crops likely to be engineered for resistance to BYDVs, oats (*Avena sativa*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) all hybridize with wild relatives in the U.S. and other parts of their range (Ellstrand et al. 1999, Seefeldt et al. 1998). Among these wild relatives, wild oats (*A. fatua*), squirreltail grass (*H. jubatum*), and jointed goatgrass (*Aegilops cylindrical)* are extremely troublesome weeds in the United States (Holm 1977). Although the crop species are largely self-pollinating, and out-crossing rates are considered to be low, even low rates of gene flow can have significant effects on the evolution of wild relatives, if the genes confer a selective advantage on the recipient (Kareiva et al. 1994).

Both wild oats and squirreltail grass, along with other wild species of *Avena* and *Hordeum*, become infected with BYDVs and show typical disease symptoms (Griesbach et al. 1990; A. Power, *unpublished data*), indicating that fitness may be reduced in infected plants. Our data show reduced biomass and seed production in infected wild oats (Power 2002), but data on fitness effects in *H. jubatum* are lacking. Although recent studies have documented significant gene flow from wheat to a wild relative, jointed goatgrass (Seefeldt et al. 1998, Zemetra et al. 1998, Guadagnunolo et al. 2001), the susceptibility of jointed

goatgrass to BYDV has not been studied. Therefore, it is not clear whether transgenic virus resistance would confer any fitness advantages to this grass.

Our work has largely focused on the impacts of BYDVs on wild oats and on its competitive relations with cultivated oats and other grasses, in order to predict the ecological and agronomic effects of movement of transgenic virus resistance from cultivated oats to wild oats (Power 2002). Our research suggests that BYDVs can cause a very significant reduction in the fitness of wild oats and can significantly affect its competitive relations with crops and other grasses (Power 2002). Data from recent field experiments with BYDVs in mixed host plant communities indicate that these viruses can have strong effects on host competitive abilities and host fitness and can significantly modify plant community attributes. For example, we have found that when virus pressure is high in experimental plant communities, evenness declines and dominance increases (C. Mitchell and A. Power, *unpublished data*).

The movement of transgenes for BYDV resistance into weedy annual grasses like wild oats or squirreltail grass poses two types of risk, one agronomic and the other ecological. In terms of agronomic risk, acquisition of BYDV resistance by these weeds may make them more significant competitors with cultivated cereals. Our experiments indicate that wild oats exert greater competitive pressure against cultivated oats, and have greater seed production, when they are not infected by BYDVs (Fig. 1; Power 2002). Greater seed production may lead to increased population sizes in later generations, resulting in an increase in crop losses associated with this weed species.

Increased fitness of wild oats or other wild species of *Avena* and *Hordeum* could also have significant impacts on natural plant communities. These introduced species already compete heavily with native grasses in natural California grasslands, causing declines in native grass populations

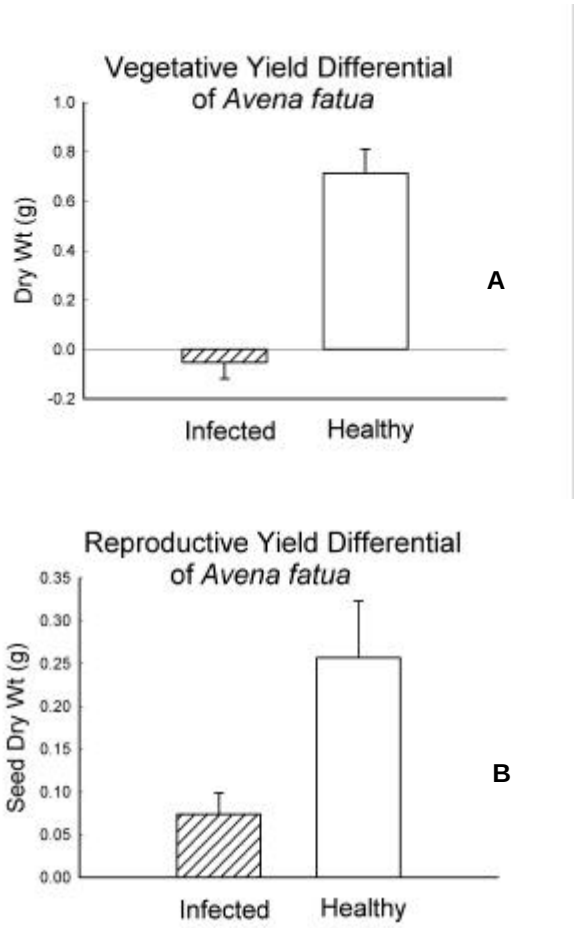

Fig. 1: Yield of healthy and BYDV-infected wild oats (*A. fatua*) when grown in a two-species mixture with domesticated oats (*Avena sativa*) [Power 2002]. The competitive pressure of wild oats on cultivated oats is significantly reduced ($P<0.05$) when wild oats are infected with BYDV. The acquisition of transgenic resistance by wild oats may result in greater competitive pressure on cultivated oats. A. Vegetative biomass. B. Seed yields. Error bars designate standard error of the mean.

and impeding current efforts at grassland restoration (Barbour et al. 1993, Dyer and Rice 1997). Increased fitness of wild species of *Avena* and *Hordeum* through the acquisition of transgenic resistance could result in the release of these species from ecological constraints normally imposed by infection with BYDVs (Schmitt and Linder 1994), resulting in significant negative impacts on native grassland ecosystems. Demonstrating the potential for ecological release will require a better understanding of the demographic consequences of BYDV in field populations of these grasses.

Understanding the influence on BYDVs on the competitive relations between wild oats and co-occurring native grasses, as well as crops, would allow us to better predict the potential consequences of any movement of transgenic BYDV resistance to wild relatives of grain crops. In a more general sense, it is clear that both changes in virus properties due to recombination and changes in virus resistance due to the movement of viral transgenes could have important impacts on natural plant communities as well as agroecosystems.

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CHARACTERISATION OF TRANSGENIC FRUIT TREES AND ANALYSES OF DIRECT AND INDIRECT BIOLOGICAL INTERACTIONS

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Abstract

In this paper the "Austrian situation" concerning field trials, and the legal framework for working with GMOs in contained systems and for deliberate release will be described. However, so far no field trials have been carried out in Austria. A short history of the failed applications is given. Meanwhile, activities at the regional level have started, to declare whole regions "GMO-free zones", a move conflicting with EU and Austrian Legislation.

Our work with GMOs started in the late 80s. In the second part of this paper an ongoing project "Characterisation of transgenic fruit trees and analyses of direct and indirect biological interactions", approved by the Austrian Ministeries of Science and Agriculture in 2000, will be described (www.boku.ac.at/sicherheitsforschung). Transgenic trees of the genus *Prunus* were selected as model organisms to study the performance of woody GMOs over a period of five years. Other institutes from our University were invited to submit projects on biosafety aspects of transgenic trees. Three of the submitted projects were selected for financial support. Experimental approaches and some results will be presented and discussed.

Keywords: transgenic woody fruit tree species, *Prunus*, virus resistance, marker genes, public perception, acceptance, step-by-step procedure, case-by-case evaluation

The Austrian situation

Legal aspects

The European directives 90/219 and 90/220 were converted into the Austrian Gene Technology Law 510/1994 which came into force on 01. 01. 1995 (http://www.bmbwk.gv.at). This law regulates work with GMOs in contained systems, deliberate release, gene analyses and gene therapy in humans. The law foresees the installation of a Gene Technology Commission and two Scientific Boards: one for work with GMOs in containment and one for deliberate release.

Modifications were made, according to 98/81/EC, in the Austrian Laws 73/1998, 98/2001 and 94/2002, introducing the issues of liability and public hearings, and regulating the position of public parties in cases of deliberate

releases (www.gentechnik.gv.at/gentechnik/gesetz/gesetznovell02.html). Special bylaws regulate work in contained systems, determining the criteria for safety classification of GMOs and the procedures at public hearings on deliberate releases:

www.gentechnik.gv.at/gentechnik/gesetz/Systemverordnung.html,

www.gentechnik.gv.at/gentechnik/gesetz/gesetz_freisetzung.html

www.gentechnik.gv.at/gentechnik/gesetz/Anhoerungvo.html

The directive modifying conditions for deliberate release and commercialisation, 2001/18/EG of March 2001 should have been converted into national laws before October 2002, which still has not been done in Austria.

Since the first attempt of field testing a GMO in Austria in 1996, the discussion was dominated by non-scientific arguments in public media. A Referendum on Gene Technology held in April 1997 requesting: (1) no food from the gene laboratory; (2) no deliberate release of GMOs in Austria; (3) no patent on life; received 1.2 million signatures.

In 1997 a national import ban was imposed on GM *Bt*-maize 176 (Ciba) resistant to lepidoptera and tolerant to glufosinate, in 1998 on GM *Bt*-maize MON 810 (Monsanto) resistant to lepidoptera and in 2000 on GM maize T25 (AgrEvo) tolerant to glufosinate.

Requests for field trials

The "Austrian situation" concerning field trials is not correctly presented in international databases like http://biotech.jrc.it/gmo.asp. or the OECD database on field trials. They present all “notifications of requests” which have to be circulated among Member States since the implementation of Directive 90/220/EEC (21 October 1991), but not the actually granted permits. In the first Report of the Gene Technology Commission to the National Council in December 1998 (covering the period from 01.01.1995 till 01.06. 1998) five requests for field trials are mentioned, which were either withdrawn by the applicant or were not granted.

?? In 1995 the Österreichisches Forschungszentrum Seibersdorf Ges.m.b.H requested a field trial for testing a transgenic potato carrying a cecropin gene to confer resistance to the storage pathogen *Erwinia*. The Report mentions that the request was discussed in three expert meetings, where finally no safety concerns were raised, but it was agreed to carry out the experiments in greenhouse under contained conditions.

?? The second request from the Österreichische Zuckerforschung Tulln GesmbH concerned a potato variety with a modified starch composition (amylose-free), not foreseen for human consumption. The Scientific Board for deliberate releases of GMOs agreed on 11. 04. 1996 that this experiment represent no hazard to humans and to

the environment and suggested criteria for monitoring the soil fauna. Since there were indications from the Ministry that the permit would be soon issued, that the legal deadline for reply (90 days) had elapsedby weeks (Prof. Ruckenbauer, pers. comm), and the optimum planting date for potatoes was over, the potatoes were planted. Within days the Ministry ordered the digging up of the potatoes, and NGOs took over the interpretation of the case. The Report says: Due to the illegal release by Zuckerforschung Tulln GesmbH the Competent Authorities denied the permission of the field trial. However, in a footnote the Report also states that on January 21, 1997 the rejection of the Minister was cancelled by the supreme administrative court upon an appeal by the applicant against the "Dig-out-order". But this information had no effect on public perception.

?? Since then, a second request of Zuckerforschung Tulln GesmbH for a field trial with the starch-modified potatoes, a request of TB Agrartechnik, Bad Vöslau, for a field trial with herbicide (glufosinate) resistant maize (T14 and T25) submitted on the 28. 12. 1995, and a request of Pioneer Seeds for a field trial with *Bt*-maize for lepidopteran resistance submitted on the 19. 12. 1997 were withdrawn by the applicants. No further expert consultation was necessary.

According to the second Report of the Gene Technology Commission to the National Council, in the years 1999 – 2002 there were no requests for permission for field trials with the competent authorities.

Recent developments in legal terms

In the meantime in Austria discussion is going on how to guarantee freedom of organic farming products from unwanted GMO contamination. Attempts are being made to declare entire regions GMO-free by regional laws, disregarding the fact that this contradicts EU legislation and even the Austrian Gene Technology Law (Stelzer et al. 2002).

The safety projects of the University of Agricultural Sciences

At the IAM, work started in 1988 on pathogen-mediated resistance in woody species, focussing on virus resistance breeding in fruit trees and grapevines. No control of these pathogens by chemical means exists (CABI/EPPO 1992), and the chemical control of their vector organisms, e.g. aphids, nematodes, etc. is ecologically highly questionable. Following the pathogen-mediated protection approach (Beachy et al. 1990, Lomonossoff 1995), we isolated the coat protein (CP) gene of the stone fruit pathogen *Plum pox virus* (PPV) (Laimer da Câmara Machado et al. 1992), *Prunus necrotic ringsport virus* (PNRSV) (Hammond et al. unpublished) and of four grapevine viruses: *Grapevine fanleaf virus* (GFLV) (including non-

translatable and truncated forms of the CP gene), *Arabis mosaic virus* (ArMV), *Grapevine virus A* (GVA), and *Grapevine virus B* (GVB) (Gölles et al. 1996, 2000, Minafra et al. 1998), and transformed different explants of different woody species.

However, beyond technical feasibility and environmental safety, public acceptance also needs to be considered. To build public confidence the project "Characterisation of transgenic fruit trees and analyses of direct and indirect biological interactions" was initiated to demonstrate the step-by-step principle of working with GMOs on the case of transgenic fruit trees (http://www.boku.ac.at/sicherheitsforschung/open-e.htm).

Other institutes from our University were invited to submit projects dealing with safety aspects of transgenic plants. From the submitted proposals the following three were selected: (1) "Biological and pomological investigation of the gradual release of transgenic fruit trees (apricot and japanese flowering cherry) in the Saranhouse and in field trials" submitted by the Institute of Pomology and Horticulture, (2) "Interactions between transgenic/non-transgenic *Prunus* ssp. and phytopathogens, aphids and natural enemies of aphids", Institute of Plant Protection and (3) "Leaf quality of transgenic and wild type apricot and cherry trees and its effect on the development of herbivores and their natural enemies", Institute of Forest Entomology, Forest Pathology and Forest Protection.

Worldwide efforts in the transformation of woody crop plants have yielded so far only a few examples with relatively low numbers of transgenic lines (see Oliveira et al. 1996). Few statistical data on deliberate releases exist (James and Krattiger 1996, Robert Koch Institute http://www.rki.de/GENTEC/FREISETZUNGEN, Ellis et al. 1993, 1996, 2002, Aphis 2002: http://www.aphis.usda.gov/bbep/bp.

The above listed projects are innovative from several perspectives: they study perennial plants, clarify whether the genetic modifications in woody plants remain stable over a prolonged period of time, and analyse their direct and indirect interactions with the environment. Better specific knowledge concerning virus diseases of woody plants is expected .

The plants

The following transgenic trees of the genus *Prunus* were selected as model organisms to study the performance of woody GMOs over a period of 5 years under screenhouse and field conditions:

Prunus subhirtella, japanese cherry (2n = 16, self sterile) used as cold tolerant rootstock. Two marker genes: *nptII*, conferring resistance to kanamycin and *uidA* = GUS, for histochemical detection and quantification (da Câmara Machado et al. 1995) were introduced. These plants are particularly useful because the activity of the transgene can be monitored over the years in different organs of the plant by qualitative and quantitative assays (Jefferson et al. 1987, Gallagher 1992).

P. armeniaca, apricot cv. Kecskemeter, (2n = 16, self fertile). The *nptII* gene conferring resistance to kanamycin and the coat protein gene of Plum Pox Virus (PPV cp) derived from a non-aphid transmissible strain of PPV-NAT were introduced (Laimer da Câmara Machado et al. 1992). The introduced sequences from the viral genome should confer resistance to PPV, a major disease of stone fruit trees.

The core project of the Plant Biotechnology Unit of the IAM

The core project is divided into two phases as depicted in Figure 1. In Phase 1 plants are studied for two years in a contained system, i.e. in a screenhouse, with nearly natural conditions. Phase 2 is a field trial which should last for three years. Under open field conditions the genetic stability of introduced genes and their interaction with other organisms will be analysed. However, this field trial requires a permit by the Competent Authorities according to the Gene Technology Law. Only when the permit for the field trial is granted, can Phase 2 start.

Molecular and biochemical characterisation of transgenic cherries and apricots in the screenhouse

Selected lines of apricot and cherry were planted in June 2000 in two compartments of an insect proof screenhouse. To verify the impact of horticultural practice e.g. grafting on different rootstocks, the following types of plants were planted: original transformants (A), T-buddings on the rootstock F12/1 (B), whip-and tongue grafts to F12/1 (C), and self-rooted cuttings (D). Phenological observations of the cherry plants during the vegetative growth phase showed normal development (Figures 2 and 3) without morphological differences between the transgenic and the non-transgenic control plants.

Monitoring GUS over two years confirmed that *in vivo* activity is lower than the previously measured *in vitro* activity. This is in accordance with data from the literature (Ellis et al. 1996, Seres et al. 1997). A consistent difference in expression level between the lines was observed. Moreover, during the growing season, GUS activity increased parallel to growth initiation in early spring and to new flush development. A correlation of the level of expression with seasonal climatic changes was not found.

To determine a possible influence of the copy number of insertions on the level of expression Southern analyses were carried out. The lines varied between 1, 2 and 3 integrated copies, but the copy number did not correlate with the level of expression.

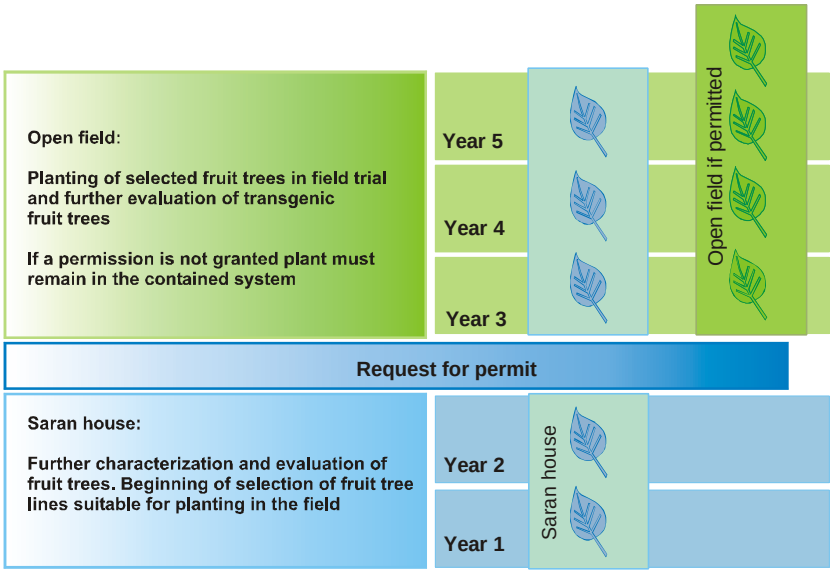

Fig. 1: Timetable foreseen for the stepwise procedure from contained environment to field conditions

Since the expression of the coat protein gene in apricots is below detection level, we carried out phenological studies, and just as in case of cherries, we found no difference in the development of the transgenic versus the non-transgenic lines.

Molecular analyses of protection against viruses in herbaceous and woody plants in the greenhouse

Considering recent models on post transcriptional gene silencing (PTGS) involved in pathogen-mediated protection (Dougherty and Parks 1995, Baulcombe 1996, English et al. 1996, Ratcliff and Baulcombe 1997, Waterhouse et al. 1998, 2001, Scorza et al. 2001), different sequences from the PPV genome were chosen for a comparison. The effectiveness in causing protection of different modified sequences of the PPV genome was tested in R1 and R2 plants of *Nicotiana benthamiana* transformed with the genes P1, CI, Nib and CP in translatable and untranslatable constructs under the control of single or double 35S promoters (Korte et al. 1995).

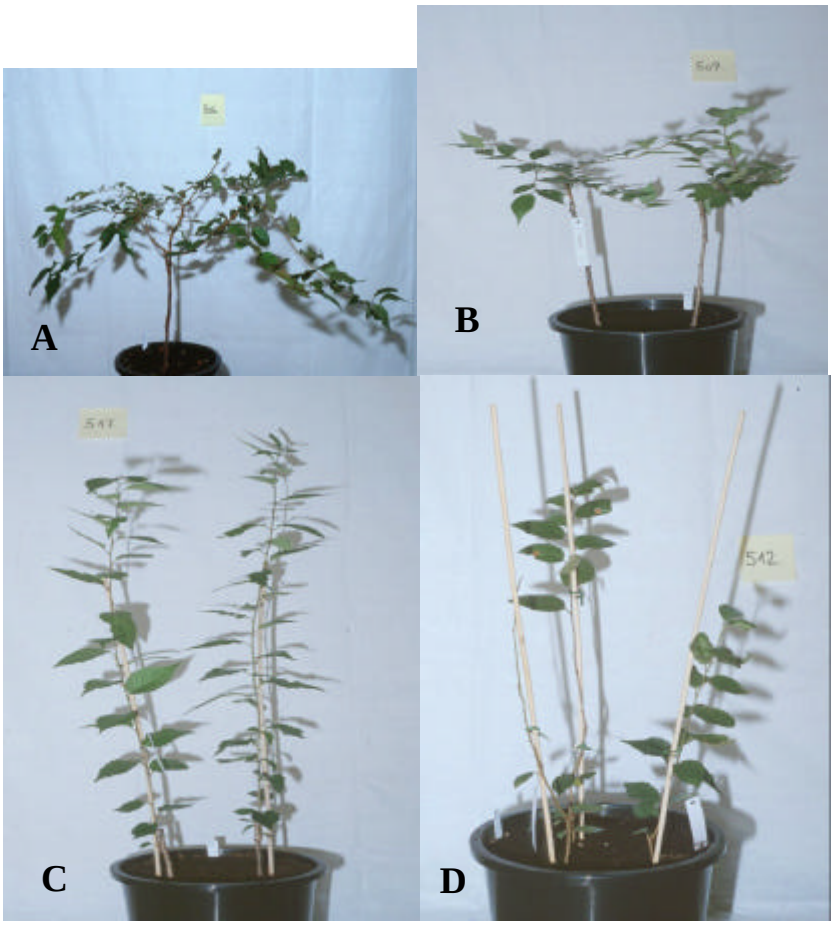

Fig. 2: Transgenic *Prunus* plants at planting time: original transformant (A), whip and tongue grafts (B), T-buddings (C), and self rooted cutting (D).

All sequences, either translatable or untranslatable, gave a certain level of protection. However, challenge infection experiments revealed a high variation in the effectiveness of the different constructs to protect against PPV. The CP sequences seem to give a consistently high rate of protection (Mendonça 2003).

We conducted a search for alternative regulatory sequences by expressing marker genes under the control of different promoters. Preliminary results show some potential of the *Mal d 1* promoter (Pühringer et al. 2000) in inducing GUS activity in transgenic *Nicotiana benthamiana* plants following infection with PPV (Mendonça 2003).

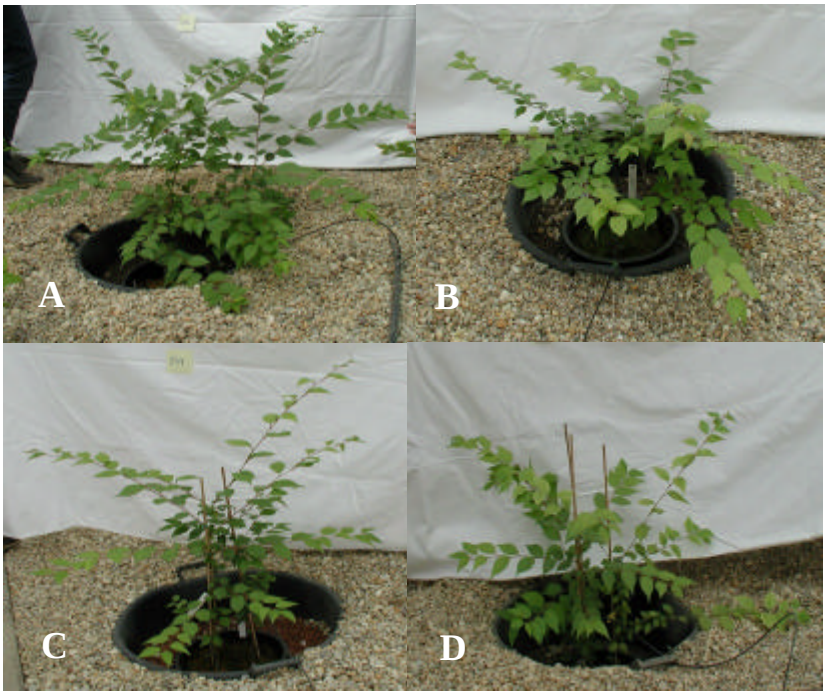

Fig. 3: Transgenic *Prunus* plants after 12 months under screenhouse conditions: original transformant (A), T-buddings (B), whip and tongue grafts (C), and self rooted cutting (D).

Transformation of woody plants with modified constructs for improved expression of different sequences

A major challenge currently is to overcome genotypic variation in response to regeneration conditions (Ellis et al. 2001). Different explants from different cultivars of plum and apricot are used to produce new transgenic lines with the transformation systems available at the IAM (Laimer da Câmara Machado et al. 1992, da Câmara Machado et al. 1995, Gölles et al. 2000). Since these experiments are long-term by the nature of the tree species involved, only preliminary data are available so far. The constructs that yield the best protection on herbaceous plants (see above) are being used for transforming woody plants.

Molecular characterisation of PPV isolates present in Austria to determine genetic variability

To determine the genetic variability of PPV currently present in Austria a collection of 181 isolates from different stone-fruit-growing regions from

orchards, wild sites and along the borders of the orchards was established. The collection comprises 27 isolates from *P. armeniaca*, 8 isolates from *P. spinosa*, 7 isolates from *P. myrobolan*, 11 isolates from *P. persica*, and 128 isolates from *P. domestica*.

Serological and molecular strain differentiation of isolates by PCR/RFLP in the region of the CP and NIb gene (Wetzel et al. 1991, Hammond et al. 1997) was carried out to distinguish M and D strains. Furthermore a selection of 54 isolates was studied by serology, applying 11 monoclonal antibodies, which in a Europe-wide study distinguished two geographic races of M-strains: a central European and a Mediterranean group (Myrta et al. 2001, Laimer et al. 2003).

The vast majority of the isolates analysed so far belong to the D-strain. Only a few M-isolates belonging to the Mediterranean group of M-types could be detected. This is in clear contrast to the distribution of strains found in Eastern and Central European countries (Laimer et al. 2003).

Multiplication of plant material

As described below, the other project teams have to be supplied with experimental material ranging from entire sets of transgenic and control plants to large amounts of virus-free and virus-infected leaf material for feeding experiments.

Elaboration and submission of a request for permit for a field trial

At present we are at the end of Phase 1 (Figure 1). The continuation of the work depends on the authorization of field trials. If permit is granted, the experiments initiated under screenhouse conditions will be continued under open field conditions, and will include the pomological, biochemical and molecular characterisation of selected cherry and apricot lines.

Accompanying projects

Other Institutes from our University are studying aspects related to biological safety of the transgenic plants. The Institute of Pomology and Horticulture in the project “Biological and pomological investigation of the gradual release of transgenic fruit trees (apricot and japanese flowering cherry) in the Saranhouse and in field trials” compares transgenic and non-transgenic fruit trees according to biological and pomological criteria. To evaluate the potential of gene flow, the impact of cross pollination in a crossing garden is investigated.

The Institute of Plant Protection in the project “Interactions between transgenic/non-transgenic *Prunus* ssp. and phytopathogens, aphids and natural enemies of aphids” studies possible effects of transgenic plants on animals feeding on different trophic levels and makes an ecological risk evaluation.

The Institute of Forest Entomology, Forest Pathology and Forest Protection in the project “Leaf quality of transgenic and wild type apricot and cherry trees and its effect on the development of herbivores and their natural enemies” studies possible effects of transgenic fruit trees on leaf composition and development and fertility of feeding pests and their natural enemies on the model system gypsy moth and parasitic wasp.

Information of the public

For information of the public, printed folders for lay and professional and an Internet webpage have been issued (www.boku.ac.at/Sicherheitsforschung). Seminars and other public presentations are organised.

The projects strictly observe conformity to the Austrian Gene Technology Law. The Austrian Academy of Sciences was requested to install an international expert commission to supervise and evaluate the progress of the work (http://www.boku.ac.at/sicherheitsforschung/trans-fr-e.htm).
This commission is informed at regular intervals.

Discussion

Agronomic traits which can be modified in fruit trees by a transgenic approach do not differ much from those addressed in other crop species. A “trait/construct dependent approach” as suggested by Metz and Nap (1997) seems to be the most appropriate way to evaluate risks and benefits of a genetically modified tree. Much potential exists in the optimisation of constructs, by limiting the expression of transgenes in time and space, e.g. in a certain tissue at a certain developmental stage of a plant.

For long-lived tree species however, new questions arise regarding the stability of the integration and expression of foreign genes (OECD 2002). Biosafety considerations include impact of transgene dispersion through pollen and unexpected effects on non target organisms.

These issues were discussed at several international meetings since 1997. As a result Schiemann et al. (2000) summarised the requirements for an acceptable transgenic plant as follows: The transgenic plant of the future should be resistant to pathogens, adapted to the location, should be high yielding, and should have transgenic sequences reduced to a minimum. No antibiotic resistance marker genes should be used, and expression of transgenes should be directable spatially and temporally.

To our understanding, there exists no contradiction between organic production and the use of certain GMOs. We hope that in a not too distant future, when a differentiation between traits will be made, GMOs can be integrated into organic production. This will avoid the unjustified exclusion of certain crops that may make an important contribution towards a healthier production of food (Ammann 1998).

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ECOLOGICAL CONSEQUENCES OF GENE FLOW FROM CULTIVARS TO WILD RELATIVES: RHIZOMANIA RESISTANCE GENES IN THE GENUS *BETA*

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Abstract

Conventional sugar beet (*Beta vulgaris* ssp. *vulgaris*) has been cultivated for 200 years. This cultivar has not shown any unwanted ecological effects despite the introduction and spread of this European species to the New World. The only realistic way of assessing the environmental effect of transgenic beets is a comparison with classically bred cultivars. In particular, we compared the ecological performance of rhizomania-resistant genotypes under various environmental conditions with regard to parameters such as competitiveness, winter hardiness and seed production. We found no difference in seedling performance even under virus infestation. We tested the competitive performance of beet against *Chenopodium album*, a common weed in sugar beet fields and young fallow. Field experiments carried out between 1993-2001 demonstrated that transgenic sugar beets grew better than virus-susceptible beets only when the virus was present. The difference between susceptible and resistant beets declined as more competing weeds were placed nearby. No differences were observed if the virus was absent. Some of our experiments focused on over-wintering of transgenic and non-transgenic sugar beet at different locations in Europe representing the range from mild to cold winters of the years 1994-1999. We found no survival differences even under virus infestation conditions. We also addressed the weed beet problem. Seed bolters pose problems to mechanical harvest machinery and cause reduced yields. In sugar beet fields they are therefore regarded as weeds. Early bolting and seed production in the first vegetation period is an important attribute for the ecological distribution of beet. At the end of its life cycle, beet seeds may have a higher chance to survive freezing temperatures in the Northern hemisphere. In addition, the development of an annual habit is also important for the weediness of beet in disturbed habitats such as agricultural fields. In one experiment, the transgenic genotype had a much "safer" performance due to its higher resistance to early pre-bolting than the isogenic control. Via gene flow, transgenic attributes can be transferred to all sexually compatible relatives. One prerequisite is sympatric growth of cultivars and their hybridisation partners. There are only a few plants that can cross with sugar beet: Swiss chard, Fodder beet, Table beet and wild *Beta* species belonging to the Section Vulgaris. No difference was found in the hybridisation

ability of transgenic, in comparison to non-transgenic controls. The resulting hybrids between sugar beet and relatives were also the subject of experiments investigating biosafety in terms of germination, competitiveness, winter hardiness and seed production. We found no special transgenic effect except a potential pleiotropic cost of resistance of some transformation events. Generally, transgenic sugar beet plants behave in an ecologically similar manner to non-GMPs if the modified trait confers a neutral advantage under environmental or experimental conditions. However, GMPs perform better than non-GMPs if the new phenotype is challenged by conditions ecologically advantageous to the modified trait. We demonstrated that rhizomania is absent in saline wild beet habitats, and concluded that this virus disease can not play an ecological role there. Only weed beet of infested sugar beet fields may benefit from resistance genes, whether they are of transgenic or classical bred origin. The future task is causal analytic monitoring – a combination of experimental biosafety research, ecological field observation and population genetic analysis.

Keywords: *Beta vulgaris*, biosafety, gene flow, monitoring, Rhizomania, virus resistant transgenic plants

Introduction

Gene flow per definition is the active or passive dispersal of genes via seed, pollen or clonal parts of a plant into the environment. Since risk is caused by both exposure and hazard, it is clear that biosafety research on environmental effects should not only target the probability of gene flow, but must also focus on the consequences (and potential hazards) of successful transgene flow to relatives of transgenic crops (Figure 1). Gene flow via seed or pollen is a basic biological principle in plant evolution. The ecological and genetic consequences of gene flow depend on the amount and direction of gene flow as well as on the fitness of hybrids (Bartsch et al. 2001). The assessment of potential risks has to be performed taking into account that conventional crops do also cross with wild plants (Saeglitz and Bartsch 2002). This means that biosafety research should address the phenotype (especially the fitness phenotype) of the transgenic hybrid in comparison with that of non-transgenic controls.

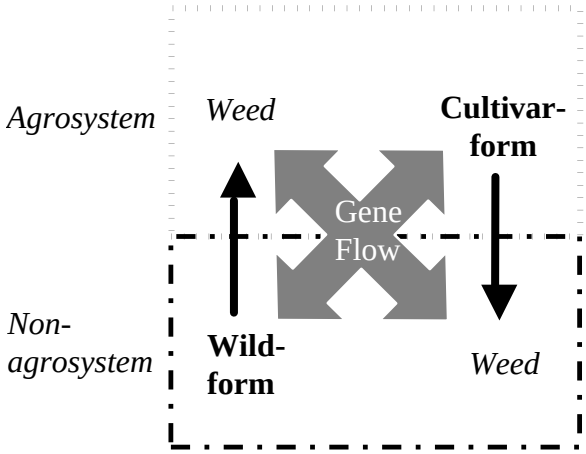

Fig. 1: Seed gene flow (small black arrows) in a wild-weed-cultivar system. Weeds are plants in the wrong place, here, either as cultivars outside agricultural areas or wild plants in cultivated ground. Weeds may also evolve due to pollen mediated gene flow (large gray arrows). Wild and cultivar forms of a single species can be protected as a plant genetic resource in one place and be eradicated as weed in another (figure taken from Bartsch and Schmitz 2002).

Biosafety research cannot solve every open and basic question of general ecology (Kareiva et al. 1997). Following the best possible pragmatic use of the case–by–case and step–by–step approach, a well-designed monitoring program is necessary along with commercialisation. This monitoring must prove, on a larger scale, the prognostic assumptions made by previous biosafety research and assessment (Marvier et al. 1999). We know for sure that containment strategies do not work properly, and provide no justification to avoid monitoring (Sukopp and Sukopp 1993, Saeglitz et al. 2000). Monitoring must be flexible enough to recognise unforeseeable phenomena such as pleiotropic effects. Currently, we have no evidence that transgenic plants systematically express more pleiotropic effects than plants from classical breeding programs (Bartsch and Schuphan 2002).

Biosafety evaluated case-by-case and step-by-step: The sugar beet example

Beets have been cultivated for more than 2000 years in the eastern Mediterranean region. *Beta vulgaris* comprises to an extraordinary variable group, in which cultivated and wild forms are often difficult to distinguish (Bartsch and Ellstrand 1999). This is mainly due to the extensive use of sea beet (*B. vulgaris* ssp. *maritima* ARCANG.) gene resources in conventional breeding programs. Sea beet is largely a coastal taxon, with a wide distribution

from the Cape Verde and Canary Islands in the west, northward along Europe's Atlantic coast to the North and Baltic Seas. It also extends eastward to the Mediterranean region into Asia with occurances in Asia Minor, the central and outer Asiatic steppes, and in desert areas as far as western India. Sea beet varies from self-compatible annuals to self-incompatible, iteroparous perennials with a life span of one to more than eight years. Cultivated *B. vulgaris*, including Swiss chard, red garden beet and sugar beet, are biennial. The latter is partially self-incompatible due to the extensive use of male sterility genes in sugar beet breeding. All cultivated and wild subspecies of *B. vulgaris* are mostly wind-pollinated, although some insect pollination has been noted.

Conventional sugar beet (*B. vulgaris* ssp. *vulgaris*) has been cultivated for 200 years. This cultivar has not shown any unwanted ecological effects despite the introduction and spread of this European species to the New World (Bartsch and Ellstrand 1999). The only realistic way of assessing the environmental effect of transgenic beets is a comparison with classically bred cultivars. Transgenic attributes are genetically dominant in heterozygotes and, like conventional genes, passed on to wild beet populations.

In our biosafety studies, the transgenic beets expressed tolerance to rhizomania caused by *Necrotic yellow vein virus* (BNYVV), a disease which has spread through the sugar beet fields of Europe, California, Japan, and China. Rhizomania is transmitted via the soil fungus *Polymyxa betae* KESKIN (Cooper and Asher 1988). The disease leads to decreased sugar beet yields and a loss of up to 30% sugar content (Guinchedi et al. 1987). Once infested, the disease persists up to decades despite the eradication of all susceptible host plants. Tolerance against BNYVV may be of ecological advantage. This advantage becomes apparent in the ecological performance where parameters are measured in different life stages of beet including first year's vegetative growth, hibernation, and second year's bolting performance and seed formation. Tolerant genotypes are infected to a lesser degree, but still act as a potential host for the replication of the virus. We wanted to know whether transgenically mediated virus tolerance has a superior ecological effect on naturally virus tolerant beet genotypes, especially wild beet hybrids. We have more than nine years of experience in biosafety field-testing of transgenic sugar beet. In particular, we compared the ecological performance of rhizomania resistant genotypes under various environmental conditions with regard to parameters such as competitiveness, winter hardiness and seed production.

Germination of beet seedlings

Since young beets are confronted first with the soil borne rhizomania disease, germination and vitality of young seedlings were measured under both infestation and non-infestation conditions. We found no difference in seedling performance even under virus infestation (Bartsch et al. 1996).

Competitiveness

We tested the competitive performance of beet against *Chenopodium album*, a common weed in sugar beet fields and young fallow. Field experiments carried out between 1993 and 1999 demonstrated that transgenic beets grew better than virus-susceptible beets only when the virus was present. The difference between susceptible and resistant beets declined as more competing weeds were placed nearby. No differences were observed if the virus was absent (Bartsch and Brand 1998, Pohl-Orf et al. 2000).

Winter hardiness and seed production

The biennial sugar beet needs to survive cold winter temperatures in order to produce offspring. Winter hardiness is an important ecological factor for the geographical distribution of cultivated and wild beet in Europe. The natural distribution range is limited to mild areas at the seacoast in the Northern hemisphere. Some of our experiments focused on over-wintering of transgenic and non-transgenic sugar beet at different locations in Europe representing the range from mild to cold winters of the years 1994-1999. We found no survival differences even under virus infestation conditions (Pohl-Orf et al. 1999, and Figure 2A). Among the three plant genotypes, no significant differences were found at any given virus infestation level, neither in hibernation rate, nor in biomass production of bolters resulting from the surviving beets, nor in seed production (Figure 2B and 2C) with the only exception of better bolter biomass performance of the beet cultivar under virus infestation. There was a clear effect of the field location on hibernation rate. The plant survival rate was significantly lower at the virus-free site in comparison to the infestation site, which was most likely due to the colder winter at the virus-free site (winter cold sum: – virus / + virus: -27 ?C / -17 ?C). The sugar beet cultivar showed a significantly weaker hibernation rate at the virus free site but had a better bolter biomass production under virus infestation. Otherwise there were no differences between the two wild beet hybrids. No effect was found on seed biomass production (Figure 2).

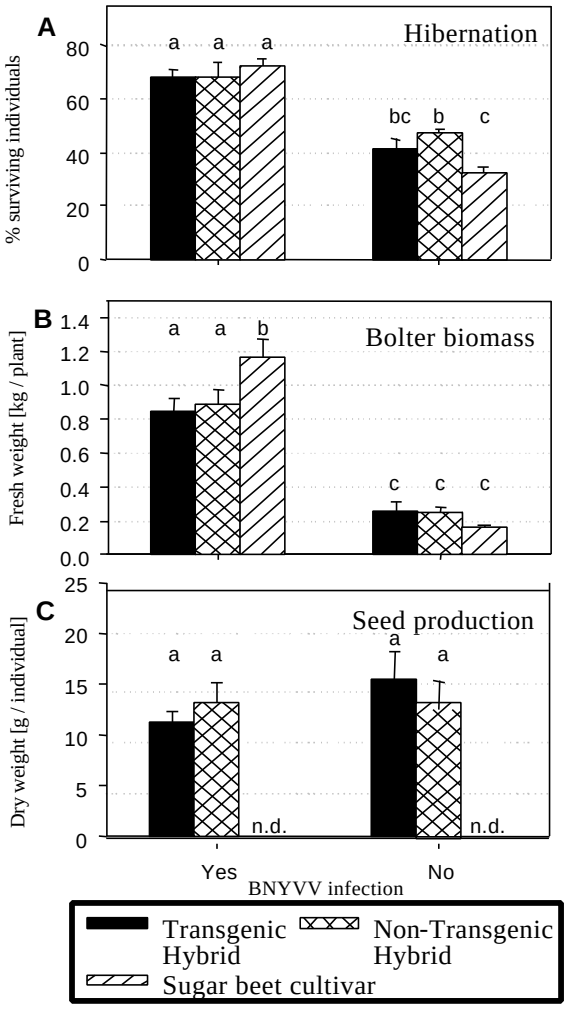

Fig. 2: Ecological parameters (hibernation, bolter biomass and seed production) of two wild beet hybrids and a sugar beet cultivar grown with or without virus infection. Mean levels of characters with the same indicator letter are not significantly different by 2-way ANOVA / Tukey-test (SEM = Standard Error of the Mean, n.d. = not detected)

Due to the potential for hybridisation between cultivated and wild beets, it is important to know whether transgenic virus tolerance can also increase the fitness of wild beet populations. The fact that we found no difference between transgenic and non-transgenic hybrids was most likely due to the genetically wild beet background of the hybrids. Natural virus tolerance was

inherited from wild beet to all three tested genotypes: The transgenic and isogenic control genotype by our hybridisation with wild beet, and the virus-tolerant cultivar by recent hybridisation with wild beet and subsequent back-cross-breeding to a high-performance cultivar. BNYVV tolerance has been widely found in wild beets (Whitney 1989), although no selection pressure from this disease is observable. An ecological examination of potential virus distribution in sea beet habitats has shown the absence of virus caused by soil conditions unfavourable for virus infection (Bartsch and Brand 1998). The general lower hibernation rate at the virus free site was most probably due to harder winter temperatures. Survival rates agree with the known correlation between survival and winter cold sum (Pohl-Orf et al. 1999). In many cases we found no costs of resistance – a result similar to Snow et al. (1999). The only exception observed was an experiment carried out 2001, in which the isogenic control performed better than the transgenes in the absence of virus infection (Figure 3).

Development of weediness due to early bolting

Weeds are simply plants in the wrong place, either in agricultural or nature conservation areas. Interestingly, the same species can be protected as a plant genetic resource in one country and eradicated as a weed in another. Seed bolters pose problems to mechanical harvest machinery and cause reduced yields. In sugar beet fields, they are therefore regarded as weeds. Early bolting and seed production in the first vegetation period is an important attribute for the ecological distribution of beet. At the end of its life cycle, beet seeds may have a higher chance to survive freezing temperatures in the Northern hemisphere. In addition, the development of an annual habit is also important for the weediness of beet in disturbed habitats such as agricultural fields (Figure 1).

Since there is no barrier to crossings between wild and cultivated forms of *B. vulgaris* (Bartsch and Pohl-Orf 1996), spontaneous hybridisation in sympatric wild and cultivated beets is common in some sugar beet seed production areas (Boudry et al. 1993, Bartsch et al. 1999, Viard et al. 2002). Hybrids between sugar beet and wild sea beet (e.g. F1s of *Beta v.* ssp. *vulgaris* x ssp. *maritima*) tend to start bolting and flowering in the first vegetation period, since the first year flowering habit is partly genetically dominant over the first year vegetative habit in beet. These hybrids are commonly found as weed beet flowering within sugar beet and cause serious yield losses in Europe (Hornsey and Arnold 1979, Mücher et al. 2000, Soukup et al. 2002).

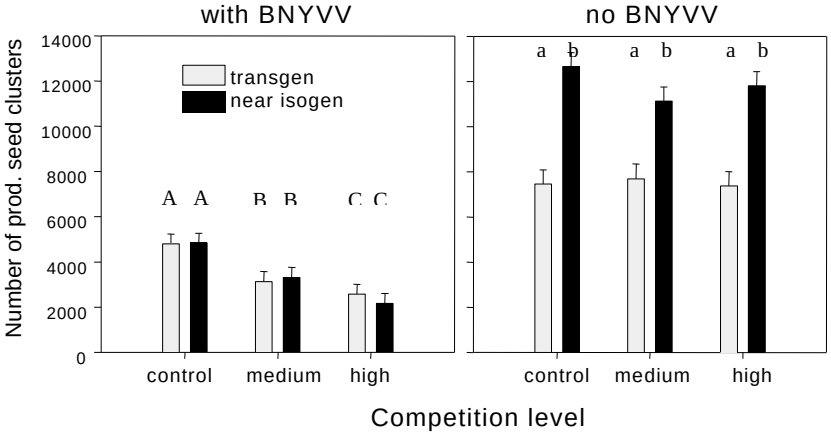

Fig. 3: Competitiveness in terms of seed productivity of two weed beet hybrids grown with or without virus infection. Mean levels of characters with the same indicator letter are not significantly different by 2-way ANOVA / Tukey-test (SEM = Standard Error of the Mean). The experiment was carried out 2001 with the methodology published by Pohl-Orf et al. (2000). Other geological and climatic conditions at the virus infested site in comparison to the virus free test site may have caused additional differences in plant performance.

In summary, the unwanted annual habit can evolve in two ways: random introgression of genetically dominant genes from wild beet (see next Section) or selective re-evolution towards wild characters (genetic draw-back). The latter phenomenon was targeted by one of our field experiments. We found that the transgenic genotype had a much "safer" performance due to its higher resistance to early pre-bolting than the isogenic control (Bartsch et al. 2001). Since the physiological background is still unknown, this pleiotropic effect should be carefully considered and cannot be related to transformation events *per se*.

Sexual reproduction

Transgenic attributes are subject to natural reproduction and gene flow to all sexually compatible relatives. One pre-requisite is sympatric growth of cultivars and their hybridisation partners. There are only a few plants that can cross with sugar beet: Swiss chard, Fodder beet, Table beet and wild *Beta* species belonging to the Section Vulgaris. No difference was found in the hybridisation ability of transgenic, in comparison to non-transgenic controls (Bartsch and Pohl-Orf 1996, Dietz-Pfeilstetter and Kirchner 1998). The resulting hybrids between sugar beet and relatives were also the subject of experiments investigating biosafety in terms of germination, competitiveness, winter hardiness, seed production (Pohl-Orf et al. 2000, see Fig. 2). We found no special transgenic effect.

Consequences of gene flow and pathogens to coastal wild beet populations

There is no evidence that the studied rhizomania disease plays any ecological role in non-agricultural areas, since the virus cannot be found in sea beet populations (Bartsch et al. 1996). We conclude that ecological implications due to the introduction and spread of virus-resistant transgenic hybrids will be minimal in this special case. However, long term monitoring by collecting basic data on geographic distribution and genetic diversity of wild plant populations is absolutely necessary for the detection of any effect (Table 1).

Table 1: Population size of representative sea beet populations in north-western Italy. This area is of special concern since gene flow from sugar beet seed production affects wild beet populations (Bartsch and Schmidt 1997). In most cases, individual numbers >90 are estimations, missing data are marked as "?"

	Year	1994	1995	1996	1997	1998	1999	2000	2001	Sum
	Location									
1	Cervia Saline	0	?	?	200	400	30	8	6	644
2	Boccasette	70	87	51	20	21	5	14	100	368
3	Scanarello	5	4	0	6	3	4	6	25	53
4	Porto Levante (sea dike)	5	70	1	1	0	0	30	35	142
5	Porto Levante (harbor)	3	3	6	13	32	23	25	21	126
6	Albarella (harbor)	2	27	6	6	1	6	8	2	58
7	Albarella (near P.Levante)	18	?	2	2	12	?	4	12	50
8	Albarella (yachting club)	5	5	29	49	53	?	150	100	319
9	Albarella (inland dike)	100	100	28	61	83	5	55	107	541
10	Chioggia	100	30	100	17	200	?	?	46	495
11	Pellestrina	23	30	12	12	0	?	0	88	165
12	Porto di Malamoco	70	?	8	18	20	?	20	54	190
13	Fusia	60	89	10	124	203	?	?	123	609
14	Cimitero San Michele	5	?	601	46	200	?	90	170	1112
15	San Erasmo (sea dike)	?	?	20	38	12	?	?	200	270
16	San Erasmo (inland field)	?	?	?	3000	400	?	?	3000	6400
17	Torcello	?	?	71	600	1450	?	101	3000	5222
18	Punta Sabbioni	10	1	1	0	0	0	?	0	12
19	Bilione (Valpelina)	?	?	1200	500	60	?	?	27	1789
20	Ausa Corno	18	4	20	12	0	2	?	66	122
21	Grado (sea dike)	50	200	100	51	65	?	5	49	522
Approx. sum over locations		*546*	*652*	*2269*	*4778*	*3215*	*?*	*?*	*7232*	

Wild sea beet habitats are naturally and man-made disturbed areas. Many populations have rarely more than 100 individuals. Since year-to-year monitoring data demonstrate no clear picture on population establishment and spread, plant genetic data reveal the evolutionary history best. For this reason we studied the ecological impact of a century of gene flow from traditionally bred cultivated beets to wild sea beet populations of north-eastern Italy (Bartsch et al. 1999b).

We found substantial evidence for gene flow from cultivar to wild beet accessions (Table 2). We demonstrated that gene flow from crop to wild relative does not necessarily result in decreasing genetic diversity of the wild plant, although the cultivated beets are less diverse and outnumbered the wild relatives by the factor 10,000 to 1. These data support the view that gene flow alone should not be regarded as an adverse environmental effect of transgenic plants.

Table 2: Gene flow from sugar beet to wild beets: Range of isozyme *Mdh2-1* (common sugar beet allele) and *Aco1-2* (common Swiss chard/red beet allele) frequency and rate of accession occurrence (RO) per group. Accessions with null alleles are excluded.

Variety/Wild location	***Mdh2-1***	**RO (%)**	***Aco1-2***	**RO (%)**
Sugar beet	0.067 – 1.000	100	0.018 – 0.278	57
Swiss chard	–	0	0.344 – 0.971	100
Red beet	0.063 – 0.063	14	0.500 – 1.000	100
Sea beet (outside seed prod. areas)	0.017 – 0.278	21	0.014 – 0.675	53
Sea beet (inside seed prod. area)	0.013 – 0.583	65	0.111 – 1.000	100

Gene flow from cultivars to weed beet in California

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) hybridisation 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 the 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.

A hot spot for gene flow is the Imperial Valley in Southern California. Here, *B. macrocarpa* grows as a weed in fields of sugar beet, which is grown as a winter culture. Due to moderately cold winter temperatures, vernalisation of the biennial plants is a common phenomenon ("bolting"). A 1998 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 what is typical of this area, which is probably due to an extraordinary cool winter in 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 of 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, introgression in this

area was 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* (Figure 4). This gene flow has led to a de facto increase of genetic diversity in the *B. macrocarpa* weed.

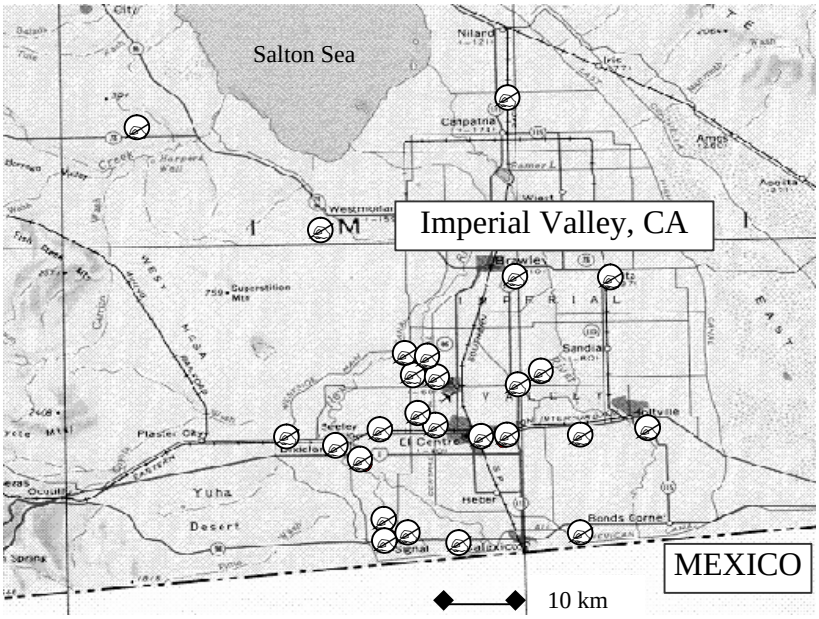

Fig. 4: Gene flow from cultivar to weed in California's genus *Beta*: Circles indicate number of individuals of a *B. macrocarpa* population with gene introgression from *B. vulgaris*. Total number of populations: 20; Sample size 20 individuals per population.

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 to or higher than the tendencies of current cultivars planted in this area. This is why an increased tendency of beets to bolt in this area and the impact of an engineered trait escaping into wild populations would have to be assessed. Isozyme alleles specific to *B. vulgaris* were found in 2% of Californian *B. macrocarpa* individuals, leading to a higher genetic diversity of these accessions in comparison to European accessions (Table 3).

One question remains: Can Californian wild beet be regarded as a plant genetic resource? If wild beet in this area is commonly considered to be a weed (according to Figure 1) population genetically monitoring endpoints are redundant (Figure 4). In Europe however, this genetic monitoring will play an important role even in agricultural areas, since nature conservation includes rare plants in agricultural systems.

Table 3: Genetic diversity data of **Californian wild beet** in comparison to other *Beta* groups. Number of populations examined (n), proportion of polymorphic loci (A*P*), the mean number of alleles among all loci (*A*) and among polymorphic loci (A_P), estimated heterozygosity (*H*), and total number of alleles found within a population group (*U*). Highest values within *B. vulgaris* are underlined.

	A	*AP*	*P*	*H*	*U*
B. vulgaris USA/Europe (n_{all} = 47)	2.92	3.08	0.923	0.330	36
Sugar beet USA/Europe (n = 16)	2.23	2.42	0.923	0.343	29
Swiss chard USA (n = 4)	1.85	2.30	0.769	0.248	24
Red beet USA (n = 5)	2.15	2.60	0.769	0.250	27
Sea beet Europe(n = 13)	2.69	2.83	0.923	0.304	35
Sea beet California (n = 9)	**2.38**	**2.64**	**0.846**	**0.284**	**30**
***B. macrocarpa* California (n = 9)**	**2.31**	**2.89**	**0.682**	**0.125**	**28**
B. macrocarpa Europe (n = 4)	1.62	2.13	0.615	0.145	13

Monitoring scope and the role of baselines

Monitoring is used for any post-commercialisation measure that provides data on the fate or effects of transgenes in the environment. Monitoring requires baseline data of the evolution of a given (eco-) system structure and system process (Figure 5). Indirect and direct methods are both helpful for detecting the possible impact of transgenes or their products. Environmental monitoring of agricultural crops and crop production practices is generally needed, not because of any specific, identified risk, but to enhance our ability to develop more sustainable food production practices (Bartsch and Schmitz 2002).

Monitoring of transgenes is conducted to achieve four specific objectives: i) confirm compliance with regulatory requirements, ii) collect information necessary for controlling and managing potentially adverse environmental situations or systems, iii) assess environmental quality, and iv) detect "unexpected" and potentially damaging effects (Suter 1993). Monitoring may be recommended to reduce uncertainty remaining from risk assessment, confirm conclusions with additional data, or provide informational feedback on system status or condition. Monitoring is not a substitute for biosafety research or risk assessment. Rather, it is integrated with research and risk assessment to ensure that ecological systems and processes of value are being protected. Deciding for monitoring is ideally based on scientific information provided in the risk assessment or some other scientific rationale saying that a risk is possible. If a conclusion of minimal risk is drawn from scientific data, monitoring should not be *required* in order to concentrate limited resources on more significant areas.

Nickson and Head (1999) have divided monitoring of GMP's into two basic approaches: general and specific. General Monitoring, which is also referred to as surveillance in the new EU directive 18-2001, is not necessarily

based on any specific hypothesis of risk. It can be accomplished using expertise and infrastructure already present in agricultural systems and within conservation efforts. By gaining familiarity and experience with GMPs through general monitoring, one can conduct "range-finding" and possibly better define the nature of a perceived risk and benefit. Specific monitoring must be based on scientific hypothesis. Science-based monitoring means the use of a protocol with specific interpretable endpoints. Eventually, information from general monitoring can be refined through the development of specific monitoring protocols designed to determine what, if any, correlations exist between practices, technologies, activities, etc. used in agriculture and the overall condition of the system (Nickson and Head 1999).

In the case of beet, any monitoring should focus on the evolution of transgenic wild beet populations in comparison to baseline data. Two monitoring models can be discussed on how the endpoints can be measured: 1) “Prior-After” crop commercialisation against today’s baseline and 2) “Parallel” to crop commercialisation against transgene free reference areas/populations. Model 2 has the advantage of taking dynamic changes into account, e.g. point III and IV as baseline (Figure 5). Model 1 is superior if gene flow is so strong that unaffected areas/populations will not be found and natural variation has to be assessed at the point I and II baseline (Figure 5). In every case we need a baseline on how to detect any deviation from natural variation of certain endpoint parameters (Table 4).

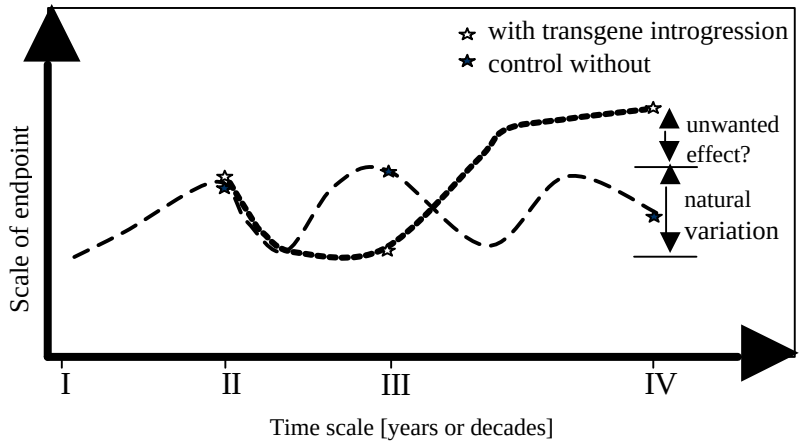

Fig. 5: Monitoring model: Critical points for measuring the potential impact of transgene escape into a wild plant population: I. before transgene release, II. at first commercial transgene release, III. and IV. representative time after transgene release. Suggestions for endpoint parameter are listed in Table 4.

Table 4: Suggestions for endpoint parameter at a time scale. Shaded cells represent areas where data are available for the Genus *Beta* (see section 2 of this paper). Abbreviations: T = population(s) with transgene introgression and C = population(s) without transgene introgression as control.

Time/stage Endpoint Parameter	I before trans-gene release	II first trans-gene release	III after trans-gene release	IV after trans-gene release	Remarks and special tasks
a. Number of individuals	C	C	C T	C T	population monitoring
b. Geographic range	C	C	C T	C T	estimation of spread
c. Genetic diversity	C	C	C T	C T	population monitoring
d. Level of gene flow	C	C T	C T	C T	cause–effect analyses
e. Fitness		C T		C T	mostly experi-mental

Causal analytic approaches need to compare transgenic gene flow with conventional gene flow effects. Finally, the additional effect of any transgene - if detected – needs a broader than just a scientific assessment to find out whether this effect is unwanted or acceptable. The monitoring will be – in the beet case – a combination of experimental biosafety research, ecological field observation and population genetic analysis.

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COMMERCIALIZATION OF TRANSGENIC PAPAYA: WEIGHING BENEFITS AND POTENTIAL RISKS

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Abstract

Papaya is Hawaii's second most important fruit crop, behind the pineapple. In 1992, papaya ringspot virus (PRSV) was discovered in Puna Hawaii, where 95% of Hawaii's papaya were being grown. By late 1994, PRSV was widespread in Puna and the papaya industry was facing severe crop loss. In 1998, transgenic papaya cultivars expressing the coat protein gene of papaya ringspot virus (PRSV) were commercialized in Hawaii and saved the industry from devastation by PRSV. This article briefly traces the development, deregulation, commercialization, and impact of the transgenic papaya on Hawaii's papaya industry. It also mentions recent work that is aimed at transferring the transgenic papaya technology to developing countries.

Keywords: Papaya, virus resistance, transgenic plants, papaya ringspot virus, PRSV

Introduction

The development of virus-resistant transgenic plants through the expression of viral genes has been successfully applied to numerous crops and viruses. However, the only virus-resistant crops that have been commercialized in the US are papaya, squash, and potato. Transgenic potato is no longer planted commercially. Transgenic squash that are resistant to zucchini yellow mosaic virus and watermelon mosaic virus 2 were commercialized in 1994, with subsequent releases of transgenic squash that have the additional resistance to cucumber mosaic virus (Tricoli et al. 1995). These squash can be freely grown throughout the US but plantings are more concentrated in the Southeastern states, where these viruses are most common. Transgenic papaya that are resistant to papaya ringspot virus (PRSV) were commercialized in 1998. The transgenic papaya are sold in the US, but license agreements only allow it to be grown in the state of Hawaii (Gonsalves 1998).

The deregulation and subsequent commercialization of squash and papaya occurred during the period when risk assessment studies were only beginning and when the debate over the use of genetically modified organisms (GMO) had not yet reached the high point that it is at today. This brief communication focuses on the circumstances under which the transgenic papaya was deregulated and commercialized, its impact on the papaya

industry, and our efforts to develop and transfer transgenic virus-resistant papaya to other countries.

Development of transgenic papaya coincides with papaya crisis in Hawaii

Papaya and PRSV have been in Hawaii for many years. Papaya has been grown in Hawaii for over a century, and PRSV was first reported in the 1940s on Oahu Island, where Hawaii's fledgling papaya industry was located. By the 1950s PRSV had severely limited commercial papaya production on Oahu Island, which provided impetus for the industry to relocate on Hawaii Island in the district of Puna. The papaya industry expanded rapidly in Puna due to several factors, mainly the absence of PRSV, availability of land, lots of rainfall and sunshine, and the development of the 'Kapoho' solo variety which was uniquely adapted to the environment and volcanic rock conditions of Puna. However, the threat of PRSV still remained, since the virus was in backyard papaya grown in the town of Hilo, which was 19 miles away from papaya orchards in Puna. Recognizing this threat the Hawaii state department of agriculture employed a small task force that regularly rogued trees in Hilo to keep the virus suppressed, surveyed papaya in Puna for PRSV, and did not allow the transport of papaya seedlings into Puna. Undoubtedly, these practices helped keep the virus out of Puna.

Nevertheless, research to control PRSV in Hawaii was started in 1978 (Gonsalves 1998). In 1983, a mild nitrous acid mutant was isolated and shown to be effective in controlling PRSV on Oahu Island, especially with the papaya selection solo line 8 (Mau, Gonsalves, and Bautista 1989). However, the mild strain did not become commercial because it caused too severe symptoms on the cultivar 'Sunrise' and it was not needed in Puna since the area was free of PRSV. The approach to control PRSV by developing transgenic papaya was started in 1986 with the isolation, cloning, and sequencing of the coat protein gene of the mild nitrous acid mutant of PRSV that was isolated in 1983 (Gonsalves 1998). The team consisted of molecular biologist Jerry Slightom of Upjohn Company, horticulturist Richard Manshardt of University of Hawaii at Manoa, tissue culturist Maureen Fitch, who was a graduate student of Richard Manshardt, and me, a plant virologist. Biolistic transformation of somatic embryos was initiated in 1987 with the Hawaiian cultivars 'Kapoho' and 'Sunset'. Effective transformation and regeneration of 'Kapoho' proved elusive, but an R0 clone, designated '55-1', of 'Sunset' was shown to be resistant to a PRSV strain from Hawaii in greenhouse inoculations that were done in 1991 (Fitch et al. 1992).

The year 1992 was pivotal, because of this biotechnology breakthrough in papaya and the discovery of PRSV in Puna. In April 1992, a small field trial using clonally propagated R0 plants of line 55-1 along with susceptible transformed and nontransformed papaya plants was started at the University

of Hawaii Waimanalo experiment station on Oahu Island. The rapid initiation of the field trial would prove crucial and timely, because PRSV was discovered in commercial papaya plantings in May 1992. By December 1992, all of the control plants in the field trial were infected while line 55-1 showed excellent resistance (Lius et al. 1997). In the mean time, massive efforts were immediately made to suppress the spread of PRSV in Puna. However, by late 1994 PRSV was widespread and the Hawaii papaya industry was facing a major crisis.

The 1992 field trial was timely in showing resistance and also proved indispensable for obtaining commercial quality transgenic cultivars. 'UH SunUp' (SunUp) is transgenic 'Sunset' (line 55-1) that is homozygous for the coat protein gene. Genetic analysis showed that SunUp resistance was conferred by single insert of the coat protein gene. 'UH Rainbow' (Rainbow) cultivar is an F1 hybrid of SunUp and Kapoho. Rainbow was developed to create a yellow flesh cultivar that had characteristics of 'Kapoho' (Manshardt 1998).

Deregulation and commercialization of transgenic papaya

The papaya crisis spurred us to test the resistance of the papaya in Puna under field conditions. Steve Ferreira of the University of Hawaii joined the team and led the deployment of the field trial which was established in October 1995. The field trial also had several other purposes, including to validate on a large scale the results of the 1992 trial, to evaluate performance of the 'Rainbow' (and to a lesser extent 'SunUp') under simulated commercial conditions, to allow growers to evaluate the performance of the new cultivars, and to serve as resource base in our upcoming efforts to deregulate the transgenic papaya. The field trial gave us very positive results on the resistance, yield, horticultural and packing quality, the potential grower acceptance of the cultivars, and served as a data base for our petition to deregulate the transgenic papaya (Ferreira et al. 2002). It should be mentioned that all of these efforts, including the deregulation process, were carried out only by the investigators, without help from companies.

Deregulation by APHIS (Animal Plant Health Inspection Service). A petition to deregulate the transgenic papaya was submitted to APHIS in early 1996 and approved in November 1996 (Gonsalves 1998). Some of the concerns that we addressed were:

Will the transgene flow from *Carica papaya* to other *Carica* species? Since previous works had shown that pollination between *C. papaya* and other *Carica* species does not occur naturally, we did not do experimental work to test for gene flow between *C. papaya* and other *Carica* species. Furthermore, other *Carica* species are not found in the wild in Hawaii.

Will the virus resistance characteristic of the transgenic papaya make it become a weed? Our argument was that Hawaii had kept records on the weeds of the state and papaya was not regarded as a weed. This information

was gathered even before PRSV was discovered in the 1940s. Again, experiments were not done to test answer this question.

Will transencapsidation and recombination result in abnormal transmission or the occurrence of recombinants that might harm the environment? Papaya is affected by only a handful of viruses, and PRSV is the most important one. In Hawaii, PRSV is the dominant virus that infects papaya. Tomato spotted wilt virus was reported to infect papaya on Kauai Island in the 1960s, but has not been reported to occur in commercial fields since then (Gonsalves and Trujillo 1986). Papaya leaf distortion mosaic virus is a potyvirus that causes similar symptoms as PRSV on papaya, but it is not related to PRSV (Maoka et al. 1996). It occurs primarily in Okinawa and has not been reported in Hawaii. These circumstances suggested that transencapsidation of other viruses by coat protein produced by transgenic papaya, and recombination between viruses with the transgene were not likely to occur under Hawaii conditions. Again, we were not required to do experiments to answer the questions as to whether transencapsidation or recombination might occur.

Deregulation by EPA (Environmental Protection Agency). EPA regards the coat protein produced by transgenic plants as a pesticide. Our petition to exempt the transgenic papaya from tolerance levels of coat protein produced in the plants was approved in August 1997 (Gonsalves 1998). Our experimental data showed that leaves of transgenic plants contained much lower levels of coat protein than nontransgenic plants that are infected with PRSV. Furthermore, we could not detect the coat protein in fruit of transgenic plants by using the enzyme-linked immunosorbent assay test.

Consultation with FDA (Food and Drug Administration). The consultation with FDA was completed in September 1997 (Gonsalves 1998). Papaya fruit from nontransgenic and transgenic trees were compared for their vitamins, brix and mineral content. Our data showed that the levels of these compounds were statistically similar. We did test the levels of benzyl isothiocyanate in transgenic and nontransgenic fruit, since this potentially toxic compound had been reported to be in very low levels in green fruit of nontransgenic papaya. The levels of this compound in transgenic papaya were virtually nondetectable, similar to those from nontransgenic papaya.

Licenses to commercialize transgenic papaya. The papaya administrative committee (PAC) was put in charge of obtaining the proper licenses to commercialize the transgenic papaya. PAC is composed of Hawaii papaya farmers that are under a USDA marketing order. The PAC assesses growers a set fee per pound of papaya that is sold, and the money is used for marketing and research that benefit the Hawaii papaya growers. The licenses were obtained in April 1998 (Gonsalves 1998). A term of the license was that the transgenic Rainbow and SunUp could only be grown in Hawaii but fruit could be sold in the US and in other countries that approve the importation and sale of these transgenic papaya. The SunUp and Rainbow papaya made

their commercial debut in May 1998, about seven years after line 55-1 was shown to be resistant under greenhouse conditions.

Impact of transgenic papaya on Hawaiian papaya industry.

Reclamation of infected fields in Puna began soon after papaya seeds were released to growers in May 1998. The transgenic papaya has shown excellent resistance to PRSV in Puna and Oahu Island. Numerous isolates of PRSV have been collected throughout Hawaii and none of them have been able to break the field resistance of Rainbow or SunUp. Table 1 traces the annual production of papaya in Puna and the state of Hawaii from 1992 to the present time. The impact of the transgenic papaya in increasing papaya production is obvious. Production levels increased starting in 2000, 18 months after seeds were distributed, and total production was nearly up to 1992 levels by 2001.

The transgenic papaya is also being commercially grown on Oahu Island. As mentioned earlier, commercial production on Oahu Island was largely abandoned in the 1950s, due to PRSV. Currently, SunUp, Rainbow, and other hybrid papaya derived from Rainbow are grown on Oahu Island. Maui Island grows papaya in backyards, and has small scale production of large fruited cultivars that are used for processing. PRSV was recently discovered on Maui. In the past, this discovery would have created a massive effort to eradicate the virus. The availability of the transgenic papaya has lessened the need for eradication measures. Crosses of Rainbow or SunUp with large fruited nontransgenic papaya and the use of micropropagation techniques should allow growers to have virus-resistant large fruited varieties in a short time.

Table 1: Papaya production in Hawaii during period of PRSV infection in Puna and following the release of PRSV-resistant transgenic papaya.

	Pounds of Fresh Papaya Sold	
Year	Total	Puna
1992	55,800	53,010[1]
1993	58,200	55,290
1994	56,200	55,525
1995	41,900	39,215
1996	37,800	34,195
1997	35,700	27,810
1998	35,600	26,750[2]
1999	39,400	25,610
2000	50,250	33,950
2001		40,000

[1] PRSV discovered in Puna

[2] Start transgenic papaya seeds distribution

Papaya as a model for biotechnology transfer to lesser developed countries.

Since papaya is widely grown throughout the tropical and subtropical lowlands, a number of countries are interested in utilizing the transgenic technology to control PRSV. We have worked with Brazil, Venezuela, Jamaica, and Thailand in developing virus-resistant transgenic papaya. The basic approach has been for a scientist or graduate student to come to my laboratory (formerly at Cornell University) with the specific focus of developing transgenic papaya using the coat protein gene of the PRSV isolate from their country, and to do it in a timely manner. We have successfully developed virus-resistant papaya for these countries and some lines are in advanced stages of field testing in Thailand and Jamaica.

These projects have had several common denominators. Although the technology to develop the transgenic papaya has proceeded in a timely manner, the introduction and deregulation has been far more challenging. The GMO controversy has obviously contributed to making the latter steps challenging.

Very recently, we have started a program, funded by USAID, to use transgenic papaya as a means for helping relieve the vitamin deficiency problems of people in Bangladesh. In Bangladesh, much of the papaya is grown in backyards, and consumed daily as a vegetable, if possible. However, PRSV limits the production of backyard papaya, which in turn deprives villagers of an excellent source of vitamins. Our goal is to develop virus-resistant transgenic papaya and make their seeds readily available for villagers to grow papaya without effects of virus infection. This would provide villagers a good source of vitamins as well as a delicious fruit. Our previous experiences suggest that the technical development of transgenic papaya will be straightforward. The more difficult parts will be the deregulation process and on dealing with the concerns over GMOs.

Summary comments

Arguably, the virus-resistant transgenic papaya that was developed for Hawaii is a case where the benefits of a biotechnological product clearly outweigh the potential risks. We hope to use the transgenic papaya in lesser developed countries to improve the health of people in villages especially. Numerous reports suggest that the technology for developing transgenic crops that are resistant to viruses is well established. In a broader sense, it is reasonable to think that similar effects could be shown for other crops where viruses limit their production. The main obstacles are regulations and assessment of the potential risks of these particular transgenic crops. However, efforts should be made to over come these obstacles because viruses cause significant economic damage to crops worldwide.

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Bacillus thuringiensis

INSECT RESISTANCE TO *BACILLUS THURINGIENSIS* TOXINS

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Abstract

Bacillus thuringiensis genes coding for insecticidal proteins (*cry* genes or *Bt* genes) have been transferred to agronomically important crops (such as corn and cotton) and this has conferred to these crops (Bt-crops) resistance to their most important insect pests. Although no case of field resistance to *Bt*-crops has been reported so far, the potential of insects to evolve resistance to insecticides is well known, and it is considered to be one of the threats posed to genetically modified insecticidal plants. Many insect pests have evolved, under laboratory conditions, resistance to *B. thuringiensis* formulated products. However, the diamondback moth (*Plutella xylostella*) is the only insect species that has evolved resistance to *B. thuringiensis* in the field, and this has even occurred several times independently. We have been working for over 10 years with the diamondback moth as a model system to test the potential of insects to become resistant to Cry toxins and we have paid especial interest in the mechanisms conferring resistance to multiple toxins.

Key words: Insect resistance, Cry proteins, *Bt*-crops, *Plutella xylostella*, diamondback moth.

Introduction

Bacillus thuringiensis as a source of insecticidal proteins

There is a long history of the use of *Bacillus thuringiensis* in plant protection and disease vector control. The insecticidal properties of this bacterium are due to the presence of a parasporal crystal that is produced at the time of sporulation. This crystal is composed of the so called "crystal proteins" ("Cry proteins") or "ICPs" (from "insecticidal crystal proteins"). In the crystal, these proteins are in the protoxin form which, upon partial protease digestion in the midgut of the insects, is converted into the active form of the toxin (referred to as "Cry toxin" or "*Bt-toxin*"). The Cry proteins are encoded by the *cry* genes, which are carried by plasmids of very large size (although in a few cases *cry* genes have been found in the chromosomal DNA). Several books have been published recently on basic and applied aspects of *B. thuringiensis* (Charles et al. 2000, Glare and O'Callaghan 2000, Caballero and Ferré 2001).

The genetic variability of *B. thuringiensis* must not be underestimated. Tens of thousands of *B. thuringiensis* strains have been isolated and are

currently maintained in private or public collections. These strains are classified into serovarieties according to their flagellar antigens (examples of serovars are *kurstaki*, *aizawai*, *israelensis*, etc.). Strains belonging to the same serovariety normally differ in their *cry* gene content, and therefore, in their insect activity spectrum. So far, more than 100 different *cry* genes have been cloned and characterized (Crickmore 2002), though just a handful of Cry toxins are active against any given pest.

Bt-crops

Some *cry* genes have been transferred to agronomically important crops, and this has conferred to these crops (*Bt*-crops) resistance to their most important insect pests. In 2001, around 7.7 million hectares were planted to *Bt*-corn and 4.3 million hectares to *Bt*-cotton (the insect resistance trait either alone or in combination with herbicide resistance). The largest proportion of these crops is currently planted in the U.S.A., but the area planted to *Bt*-cotton is anticipated to increase dramatically and very rapidly with the acceptance of this technology by China (which actually tripled in the last year the area devoted to this resistant crop), India and other developing countries for which cotton plays an important role in their economies (James, 2002).

The genes introduced into these crops have been those coding for *Bt-toxin*s highly active against the most important pest or pests of the crop. Thus, *Bt*-corn has been protected against *Ostrinia nubilalis* (European corn borer) using either one of the following genes: *cry1Ab*, *cry1Ac*, *cry9C*, or *cry1F*. By far, most of the *Bt*-corn currently planted is *cry1Ab* corn (Agbios 2002). Another type of *Bt*-corn that obtained registration contains the *cry3Bb1* gene and is protected against *Diabrotica spp.* (corn rootworms).

All *Bt*-cotton planted commercially is *cry1Ac* cotton. This gene has been chosen because Cry1Ac is highly active against *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), *Helicoverpa armigera* (Old World bollworm), and reasonably effective against *Helicoverpa zea* (cotton bollworm). The second generation of *Bt*-cotton, to be commercialised soon, combines the *cry1Ac* and *cry2Ab* genes in the same plant. The main reason to combine two *Bt-toxin*s is for resistant management purposes but, in addition, it will also increase the level of protection against those pests less susceptible to Cry1Ac.

Bt-potatoes protected against *Leptinotarsa decemlineata* (Colorado potato beetle) have also been planted commercially, and contain the *cry3A* gene.

Managing insect resistance to *Bt-toxin*s

Potential of insects to evolve resistance to Bt-toxins

Although no case of field resistance to *Bt*-crops has been reported so far, the potential of insects to evolve resistance to insecticides is well known, and it is considered to be one of the main threats posed to genetically modified

insecticidal plants. The first case of high resistance to *B. thuringiensis* was reported in 1985, in *Plodia interpunctella* (Indianmeal moth), after selection in the laboratory for 15 generations of insects collected from grain bins (McGaughey 1985). Selection of insect populations under laboratory conditions have shown that many insect pests can become resistant to *B. thuringiensis* formulated products and/or their toxins (Ferré and Van Rie, 2002). Among these pests are *O. nubilalis*, *H. virescens*, *H. armigera*, *P. gossypiella*, and *L. decemlineata*, for which *Bt*-crops have already been registered and planted at commercial scale. So far, the diamondback moth (*Plutella xylostella*) is the only insect species that has evolved resistance to *B. thuringiensis* commercial formulations in the field, and this has even occurred several times independently (Heckel et al. 2003, Ferré and Van Rie 2002).

Strategies for resistance management in Bt-crops

There seems to be a general consensus regarding the strategies to be used to preserve the use of *B. thuringiensis* technology in plant protection (Mellon and Rissler 1998). The most favoured ones are:

1) Ultrahigh expression of the *Bt*-transgene. This will make ineffective any mechanism conferring low to moderate levels of resistance to the insect. If the resistance level is not high enough as to make the plant resistant to individuals with two copies of the resistance allele (resistant homozygotes), the ultrahigh expression is intended to ensure that at least all heterozygotes are killed.

2) Temporal rotation of cultivars expressing different *Bt*-transgenes. This is based on the long ago adopted practice, in conventional agriculture, of crop rotation. Insects (and their resistant offspring) that escape the effect of the *Bt-toxin* of the first planted cultivar will be killed by the different *Bt-toxin* carried by the second cultivar. Similarly, insects resistant to the second cultivar will be killed when this is replaced by the first cultivar.

3) Expression, in the same plant, of more than one *Bt*-transgene ("pyramided plants"). The rationale is that if the chances of finding an insect resistant to one particular *Bt*-toxin are low, the chances of an insect to be resistant to two *Bt*-toxins simultaneously are negligible.

4) Use of refuges with non-transformed plants. This strategy will only work in those cases where resistance is recessive. The refuge will permit a certain fraction of the population to escape selection. These susceptible insects

will mate with the homozygous resistant insects that have survived from the exposure to *Bt*-plants. The heterozygous offspring will be susceptible and will die when feeding on *Bt*-plants.

5) Application of spatial mosaics of cultivars expressing different *Bt*-transgenes. The rationale is that those insects that are resistant to one of the *Bt*-toxins and survived exposure to the *Bt*-plant producing this *Bt*-toxin, when reaching sexual maturity, will lay part of its offspring in plants producing a different *Bt*-toxin. This offspring will die, since it will not be resistant to the second *Bt*-toxin. This is a way to lower the fitness of the resistant insects and, at the same time, to slow down the rate at which the resistance allele would otherwise increase in the population.

The high-dose/refuge strategy

The most favoured strategy for managing resistance to *Bt*-crops combines two of the above mentioned strategies: ultrahigh expression of the *Bt*-transgene and the use of refuges. The United States Environmental Protection Agency (EPA) and the United States Department of Agriculture (USDA) strongly recommend the use of refuges for those *Bt*-crops planted in extensive areas, such as *Bt*-corn and *Bt*-cotton (US EPA 1999, US EPA 2001).

The aim of the high-dose/refuge strategy is to preserve part of the insect population to ensure the survival of enough susceptible insects to increase chances of they mating with the resistant insects that survived exposure to *Bt*-plants. If the susceptible individuals outnumber the resistant survivors, and the refuges are at an appropriate distance from all *Bt*-plants, practically all resistant individuals will mate with the susceptible ones. If resistance is recessive, the resulting offspring will be heterozygous for the resistance allele and will die upon exposure to the *Bt*-plants. As a consequence, the requirements for this strategy to work are (Andow 2002):

1) Resistance has to be recessive.
2) The toxin concentration in plants has to be high enough to kill all heterozygotes.
3) Mating between resistant and susceptible insects must be at random.
4) The initial frequency of the resistance allele must be low.

Since most cases of resistance follow a mode of inheritance that is neither completely recessive nor completely dominant, adjusting the expression of the transgene to high enough levels can convert an incompletely dominant

resistance to an effective complete recessive resistance (Bourguet et al. 2000), readily killing all or most part of the heterozygotes (Figure 1).

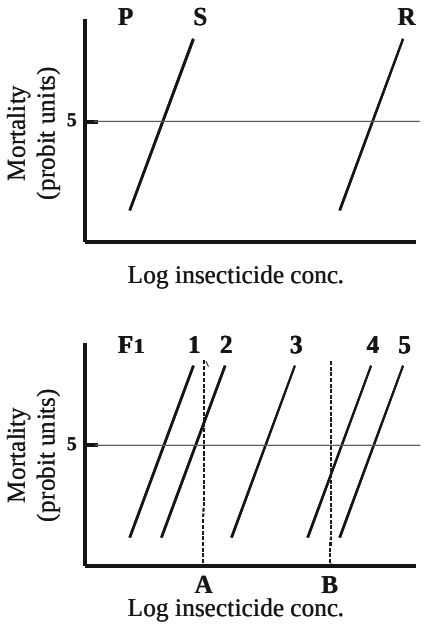

Fig. 1: Representation of ideal concentration-mortality responses to *Bt*-toxins. P: Parental strains, susceptible (S) and resistant (R); F_1: Offspring from the S x R mating with different probit curves depending on the inheritance of resistance being either completely recessive (1), incompletely recessive (2), codominant (3), incompletely dominant (4), or completely dominant (5).

In the scheme shown in Figure 1, some cases follow a mode of inheritance of resistance that deviates from complete recessivity (those labeled 2, 3, 4, and 5). In those cases where the concentration-mortality line is relatively close to that of susceptible homozygotes (cases 2 and 3) the refuge strategy could succeed or fail depending on the concentration of *Bt*-toxin in the plant. If plants produce a concentration of *Bt*-toxin equivalent to the one labeled as A in the figure, then the refuge strategy would be of little or no use. However, if plants can be used that produce a concentration of the toxin equivalent to the one labeled B, then the refuge strategy could succeed because the incompletely recessive inheritance according to the concentration-mortality curves (cases 2 and 3) would behave as being completely recessive at the test concentration.

The distribution of the refuge areas is also important because they have to ensure that any resistant insect that would survive has the chance to meet and mate with susceptible insects from the refuges.

Another aspect to consider in this strategy is the initial frequency of the resistance allele. It must be low enough so that the frequency of resistant homozygotes is low, otherwise the chance that two resistant individuals could mate becomes significant.

The strategy of using two toxins

The most interesting alternatives to the high-dose/refuge strategy are those that combine the use of two *Bt*-toxins effective against the same pest. These include the temporal rotation of cultivars with different *Bt*-toxins, application of spatial mosaics of cultivars with different *Bt*-toxins, and using cultivars producing two different *Bt*-toxins (Frutos et al. 1999). All these three strategies are based on the fact that, if resistance to one toxin is rare, the chance of finding individuals simultaneously resistant to two toxins is extremely low (for example, if resistant insects to any single toxin are found to occur at a frequency of 10^{-6}, then the expected frequency of "double" resistant insects is 10^{-12}).

The most important requirement for the success of any of these strategies is the lack of cross-resistance between toxins. Cross-resistance is when resistance to one insecticide is acquired by selection with a different insecticide. In our case, cross-resistance is when insects become resistant to one *Bt*-toxin by exposing them to a different *Bt*-toxin. This phenomenon occurs when the insecticides share a common step in their mode of action and this step is the one modified in resistant insects.

Similarly to the high-dose/refuge strategy, the strategy of using a combination of two toxins also requires that the initial frequency of resistance alleles is low, otherwise "single" resistant insects would not be rare and, as a consequence, the chance of finding "double" resistant insects would no longer be negligible.

A third requirement is also important in those cases where the two toxins are presented to the insects non-simultaneously, either in spatial mosaics or in temporal rotations. In these cases resistance to both toxins would build up gradually unless a fitness cost is associated to resistance. If insects that eventually become resistant to toxin A have a lower fitness than susceptible insects when not exposed to this toxin (either when feeding on plants expressing toxin B or on plants that fail to express the transgene), then the frequency of the allele conferring resistance to toxin A will tend to decrease. This will permit reversion of resistance (to toxin A) and will counteract the effect of the positive selection exerted by plants expressing toxin A. The same situation applies when considering resistance to the second toxin (toxin B in this example).

Genetic diversity of *B. thuringiensis* resistance: the diamondback moth as a model system

Evolution of resistance to Bt-toxins in the diamondback moth

The diamondback moth, *P. xylostella*, has been the only insect so far to evolve resistance to *B. thuringiensis* in open field populations. Since the first report in 1990 (Tabashnik et al. 1990), resistance has appeared several times in different parts of the globe. Thus, resistance has been reported in Hawaii, the Philippines, Japan, Florida, China, Malaysia, Pennsylvania, Central America, Thailand, and South Carolina (listed by chronological order) (Ferré and Van Rie 2002). In all cases resistance appeared in populations that had been exposed to *B. thuringiensis* treatments. It is likely that if it wouldn't have been for the diamondback moth, resistance management plans for *Bt*-crops wouldn't have been pursued so actively.

The diamondback moth provides us with the unique opportunity to study mechanisms of resistance that have been selected in the field, in opposition to those studies with populations that have been obtained by selection in the laboratory. Laboratory selection experiments normally start with a rather limited sample size and the type of resistance obtained (if they succeed) is not normally representative of the mechanisms that would be selected in the field. Artificial selection tends to select for minor genes, which are normally additive in their mode of inheritance. However, field selection, with populations with practically an infinite number of individuals, tends to select for major genes that normally follow a Mendelian type of inheritance.

Genetic and biochemical studies with the diamondback moth have allowed us to answer questions relevant to the potential of insects to evolve field resistance, such as: how common is cross-resistance among *Bt*-toxins and which toxins does it generally involve? Which is the most common mechanism of resistance? How is resistance generally inherited? And, which is the genetic relationship among resistance genes?

Mode of action of Bt-toxins: a mechanisms of resistance

The study of the mode of action of *B. thuringiensis* is of great interest to understand how *Bt*-toxins work and how their genes can be manipulated to produce more active toxins or toxins active against a wider spectrum of pests. Although the main features in the mode of action are known, complete understanding of some aspects is still lacking (Schnepf et al. 1998). However, there is general consensus on the following main steps:

1) Solubilisation of the crystal.
2) Proteolytic activation of the protoxin.
3) Diffusion of the activated toxin through the peritrophic membrane.

4) Binding of the toxin to specific sites in the epithelial membrane of the midgut.

5) Insertion into the membrane and formation of pores.

6) Cell lysis with disruption of the midgut epithelium.

The first step is obviously not applicable to *Bt*-plants. Regarding the second step, though *Bt*-transgenes introduced into plants have been modified to just code for the part of the *Bt*-protein that constitutes the activated toxin (except in few cases where wild type genes have been inserted into chloroplasts), the protein synthesised by the plant may still undergo additional proteolytic modification inside the gut of the insect. The rest of the steps apply to both *Bt*-plants and conventional *B. thuringiensis*-based insecticides. It is still not clear how the toxin inserts into the membrane and how the pore is formed.

Most *Bt*-toxins share a high degree of sequence similarity, especially in some conserved domains, and X-ray crystallography has shown that Cry proteins have a similar three-dimensional structure (Cyt proteins, another type of *Bt*-toxins, are completely different from the Cry proteins) (Morse et al. 2001). Therefore, most steps in the mode of action of *Bt*-toxins are assumed to function in a similar way irrespectively of the Cry protein being considered. However, it has long been known that different toxins may recognize different binding sites in the midgut epithelium, and this step is considered to be the most specific one in the mode of action of these toxins. The presence of binding sites is a necessary requirement (though not sufficient by itself) for a *Bt*-protein to be toxic to a determined insect.

In principle, any step of the mode of action is susceptible to being altered in such a way as to disrupt the toxic pathway of the toxin. However it does not seem realistic to expect that all steps are as easily susceptible to alterations without imposing a heavy load on the insect. For example, changing the midgut pH (to decrease crystal solubility) or the mesh of the peritrophic membrane would have a drastic impact on food digestion. Studies aimed at characterising the mechanism of resistance have shown that, in most cases where resistance attains high levels, resistance is due to an alteration of the binding site. In these cases, the resistant insects continue to be susceptible to other toxins that bind to different binding sites. Just in two cases proteolytic activation has shown to contribute, though partially, to the observed resistance in the diamondback moth (Liu et al. 2000, Sayyed et al. 2001).

Binding site model in the diamondback moth and its use at predicting cross-resistance

Our group has been working for over 10 years with the diamondback moth as a model system to test the potential of insects to become resistant to *Bt*-toxins. Besides the interest to determine the contribution of the alteration

of binding sites in resistance, we have paid especial attention to the mechanisms conferring resistance to multiple toxins.

Binding studies are performed using a labeled *Bt*-toxin (mostly with ^{125}I or biotin) and either tissue sections of the midgut, isolated membrane proteins, or brush-border membrane vesicles (BBMV). The most informative binding analyses are those that use ^{125}I-labeled toxins and BBMV since they allow calculation of binding parameters and they also seem to reflect more closely the way binding of *Bt*-toxins takes place *in vivo*. By competing the labeled toxin with the same unlabeled toxin (homologous competition) for binding to the BBMV, the binding affinity (K_d) and the concentration of binding sites (R_t) can be calculated. By competing the labeled toxin with different unlabeled toxins (heterologous competition) one can obtain information on whether different toxins bind to the same binding site. If this is the case, then an alteration in the common binding site could confer resistance to different toxins.

Using seven different toxins and this type of competition experiments, an integrative binding site model has been proposed for the diamondback moth (Ballester et al. 1999, Ferré and Van Rie 2002) (Figure 2). The model proposes the occurrence of four different binding sites for these seven toxins. There are three binding sites recognised by just one type of toxin and another binding site shared by five toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, and Cry1J). Cry1Aa binds to two different binding sites. Cry1B and Cry1C each recognise one binding site not shared with other toxins. Cry1Ab and Cry1Ac just bind to the common site. Cry1F and Cry1J also bind to this site, but since they were not tested with label, the possibility of binding to other additional sites has not been excluded.

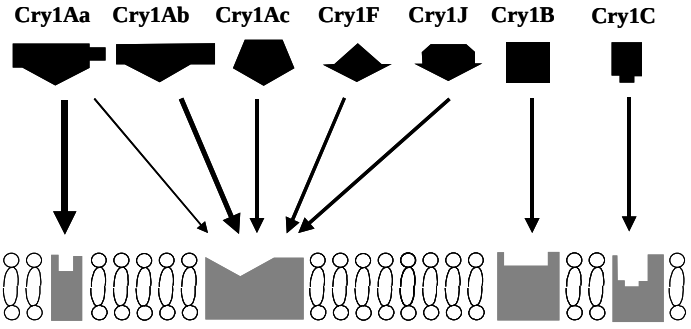

Fig. 2: Proposed model for binding of Bt-toxins to the brush-border membrane of epithelial cells in *P. xylostella*. The width of the arrows represents the relative binding affinity.

The predictive value of this model regarding cross-resistance is obvious. A major modification of the common binding site could affect binding of the five toxins and, consequently, this would confer resistance against at least

four of them (the possible exception being Cry1Aa, which still would bind to the non-shared site; it has been speculated that binding to this site might not be effective in producing toxicity). In addition, a minor alteration of the binding site could also confer resistance to just one or a few toxins, by affecting binding of these without affecting binding of the others. Evidence exists that Cry1Ab and Cry1Ac bind to different determinants in the binding site, although both toxins compete for binding in all insects tested. In *H. virescens*, binding of Cry1Ac to the 170 kDa membrane protein aminopeptidase N was inhibited by N-acetylgalactosamine, whereas binding of Cry1Ab to this same protein was not (Luo et al. 1997). Therefore, sharing a binding site indicates potential for development of cross-resistance by the mechanism of binding site alteration, though it must be kept in mind that there are examples of cross-resistance for which binding to BBMV was not altered (Gould et al. 1992).

Characterisation of resistance to Bt-toxins in 8 populations of diamondback moth

In collaboration with other research groups, we had the opportunity to study 8 populations of diamondback moth that had evolved resistance in the field (Ferré and Van Rie 2002). Two of these populations were from the Philippines, two from Hawaii, two from Malaysia, and two from eastern United States (Pennsylvania and South Carolina) (Table 1).

Table 1: Resistance to *Bt*-toxins in selected diamondback colonies.

Name of colony	Place of collection	Resistance pattern
BL	Philippines	Cry1Ab
PHI	Philippines	Cry1Ab (later to Cry1Aa and Cry1Ac as well)
NO-QA	Hawaii	Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, Cry1J
NO-95C	Hawaii	Cry1C, Cry1Ab, Cry1Ac, Cry1F
SERD3	Malaysia	Dipel[?] (which contains Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa) and Florbac[?] (contains Cry1C and Cry1D in addition to Cry1A toxins)
1AcSEL-MEL	Malaysia	Cry1Ab, Cry1Ac
PEN	Pennsylvania	Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, Cry1J
Cry1C-Sel	South Carolina	Cry1C

The two colonies from the Philippines (BL and PHI) were collected from the same area but separated three years apart. BL was highly resistant to Cry1Ab, but not to Dipel®, a commercial formulation containing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa. PHI was also found to be highly resistant to Cry1Ab, but not to any other toxin tested, including the structurally related Cry1Aa and Cry1Ac, and Cry1F and Cry1J. This colony, upon selection in the laboratory with a chimeric protoxin Cry1Ac/Cry1Ab evolved resistance to Cry1Aa and Cry1Ac.

NO-QA and PEN had the same resistant phenotype. Both had been selected with Cry1A toxins and developed resistance to these toxins and cross-resistance to Cry1F and Cry1J.

More limited studies were carried out with the two Malaysian colonies. SERD3 was just tested with commercial products and was resistant to Dipel® (a formulation based on *B. thuringiensis* var. *kurstaki*) and Florbac® (a formulation based on *B. thuringiensis* var. *aizawai*). 1Ac-SEL-MEL was selected with Cry1Ac in the laboratory and attained high levels of resistance to this toxin and also high levels of cross-resistance to Cry1Ab.

Two diamondback moth colonies have been selected with Cry1C and have become resistant to this toxin (NO-95C and Cry1C-Sel). In both cases cross-resistance to Cry1A toxins was originally present. In the Cry1C-Sel colony, single-pair crosses followed by selection of those families susceptible to Cry1Ac succeeded in eliminating the cross-resistance to Cry1A toxins (Heckel et al. 2003).

Mechanisms of resistance to Bt-toxins in the diamondback moth

Studies with the above colonies have shown that Cry1A resistance is due, at least in part, to a reduction of binding (Ferré and Van Rie 2002). Specifically, Cry1A-resistant insects fall into two categories depending on the type of binding site alteration. These have been called Type I and Type II, depending on whether just Cry1Ab binding is affected or if the alteration affects binding of other Cry1A toxins, respectively (Figure 3).

These two types of binding site alteration explain, reasonably well, the resistance phenotypes of most colonies tested. For example, the highly specific original resistance of PHI (and probably also that of BL) would be accounted for a minor modification of the binding site just affecting determinants used by Cry1Ab. However, in the case of NO-QA, PEN and 1AcSEL-MEL, a more drastic alteration of the binding site would prevent the three Cry1A toxins to bind the common site, and it would presumably prevent binding of Cry1F and Cry1J as well (at least in the two former colonies, for which cross-resistant to these two toxins has been shown).

It is interesting to note that in the two cases of resistance to Cry1C no major alteration of the binding of this toxin was observed. Although the resistance ratio in the NO-95C was relatively low (19-fold for the activated

toxin and 48-fold for the protoxin), the resistance ratio in Cry1C-Sel was extremely high (63,100-fold). Impaired proteolytic activation must also play a role in conferring resistance in the NO-95C colony, since the resistance ratio is significantly higher when using protoxin than when using toxin.

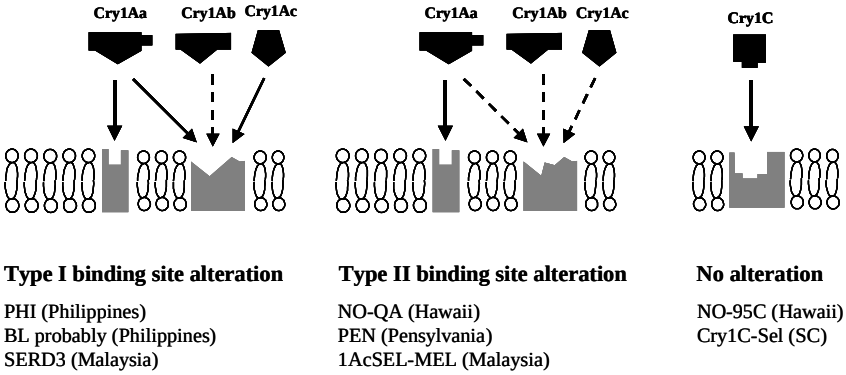

Fig. 3: Proposed model for binding of *Bt*-toxins to the brush-border membrane of epithelial cells in resistant insects of *P. xylostella*. Broken arrows indicate decrease (or complete loss) of binding affinity.

Genetics of resistance to Bt-toxins in the diamondback moth

Resistance to Cry1A toxins was generally incompletely recessive and due to an autosomal gene (in BL, NO-QA, PEN, and 1Ac-SEL-MEL). However, the PHI colony showed incompletely recessive inheritance for Cry1Ab, codominance for Cry1Ac, and incomplete dominance for Cry1Aa. Cry1C resistance was incompletely dominant in NO-95C and partially recessive in Cry1C-Sel, but in both cases resistance behaved as completely recessive if high enough Cry1C concentration was used (Ferré and Van Rie, 2002).

The only reported experiment carried out so far to test for allelism among *B. thuringiensis* resistant colonies is the one that involved crossing of the NO-QA, PEN and PHI colonies (Tabashnik et al. 1997). Only Cry1Ab resistance was tested, since Cry1Aa and Cry1Ac resistance was not recessive in the PHI colony. The results indicated that the mutations conferring resistance to Cry1Ab were allelic in all three colonies. Since the phenotype of PHI differed from that of the other two colonies (in terms of binding site alteration, and resistance and cross-resistance pattern), the mutation in the resistance gene carried by PHI must be different from the mutation in the other two colonies (which in turn could be the same or different in the two colonies).

Simultaneous resistance to Cry1C and Cry1A toxins in the Cry1C-selected colonies was shown to segregate independently in both colonies,

NO-95C and Cry1C-Sel. This indicates that resistance to these two toxins is due to different genes occurring on different chromosomes.

Conclusions

Studies on the diamondback moth and other insect species have shown that resistance to *Bt*-toxins is very likely to evolve in populations repeatedly exposed to these toxins. From the various strategies to retard or prevent resistance, the high-dose/refuge strategy is the one most recommended, but a limiting requirement is that resistance must be recessive. Alternative strategies involve the combined use of two *Bt*-toxins for which cross-resistance is unlikely. Appropriate implementation of these strategies should safeguard the future of *B. thuringiensis* for insect control.

Binding analysis using ^{125}I-labeled toxins and BBMV can be of predictive value for cross-resistance. Binding site alteration is the most common mechanism or resistance to Cry1A toxins, and it may confer, in some cases, cross-resistance to Cry1F and Cry1J.

Resistance to Cry1A toxins is generally incompletely recessive and due to an autosomal gene. Resistance to Cry1C may be incompletely dominant, but behaves as completely recessive if a high enough concentration of Cry1C is used. This fulfils the most important requirement of the high-dose/refuge strategy. Resistance to Cry1A and Cry1C segregate independently indicating that the genes conferring resistance to these toxins control different mechanisms of action. Similarly to the case of Cry1A and Cry1C, the use of two toxins for which resistance depends on genes acting on different mechanisms of resistance fulfils the key requirement of the strategies that make use of the combination of two toxins. In the only case where resistant colonies have been crossed among each other, the mutations responsible for resistance to Cry1Ab were allelic. If this is the rule, rather than the exception, molecular diagnostic tools, once they become available, will be of great help, since a single DNA probe could be used to detect mutations conferring resistance in different insect populations.

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BT-CORN: IMPACT ON NON-TARGETS AND ADJUSTING TO LOCAL IPM SYSTEMS

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Abstract

Field trials with *Bt*-corn (DK 440 BTY- transformation event MON 810) and with its isogenic line (DK 440) were established in Hungary to measure the effect of *Bt* corn on selected components of the corn-herbivores-predators system. Experimental fields were arranged in a randomised complete block design with 6 replications based on medium sized plots. All plots were planted to the same transgenic variety and its isoline. The sampling scheme was designed to collect herbivores and predators in corn stands to determine their presence, the abundance of main non-target species, and to estimate the impact of *Bt* corn on arthropod assemblages from 2001 to 2003. Sampling the first year (2001) allowed us to draft a trophic relation table for the arthropod assemblage, to determine dominance structure, and to select certain taxa for studies of trophic interactions. Second year (2002) sampling was continued for selected taxa, and additional methods were used to compare data for the two types of corn.) Larval infestation by European corn borer (ECB), the target species, was determined in corn stalks. The present paper gives details on the insect assemblage structure, on dominant herbivores, predators in *Bt* and isogenic corn, on the effect of *Bt* toxin on secondary pest *Heliothis armigera*, and identifies one potential test organism at the top predator level.

Keywords: *Bt* and isogenic corn, insect assemblage, ECB, *H. armigera*, spiders

Introduction

Corn is a traditional and key crop in Europe, and is produced for food, animal feed or seed. Corn fields cover a total of 14.1 million hectares in Europe (FAO Statistical Databases 2001). Farming and pest management practices in corn vary by region (traditions, production aims, soil and climatic conditions, etc.). In addition to this, the region itself determines on which pests and beneficial integrated pest management (IPM) strategies and practices should be based. Corn growing and pest management practices have a large impact on the presence of natural biological resources in a given region. Corn fields as habitats support a wide range of arthropod species, as

was pointed out in faunistical studies in Hungary (Mészáros et al. 1984). In light traps operated on two large scale corn fields in Hungary, Kiss and Mészáros (1989) detected 236 Macrolepidoptera species, of which 10% may feed on corn, while a majority of the species may feed on herbaceous undergrowth in and around the corn field. IOBC WPRS Guidelines for integrated production of arable crops (Boller et al. 1997) give several objectives (promoting and maintenance of high biological diversity in the agro-ecosystems concerned, priority of natural regulating mechanisms, minimum occurrence of pesticide residues) and the need of case-by-case studies when decision will be taken on use of genetically modified organisms (GMOs). Thus, introduction of new pest management strategies and new methods in IPM (like *Bt* corn) needs careful monitoring, adaptation and impact assessment within local agro-ecosystems.

Cry1Ab-expressing *Bt*-corn, which is toxic to European corn borer (ECB), *Ostrinia nubilalis* Hbn, is one of the transgenic crops that could potentially be grown by farmers in Europe. Important issues in growing transgenics are their impact on non-target organisms and potential for causing resistance or tolerance to develop in the targeted species. These could be the consequences of replacing non-*Bt*-corn with *Bt*-corn if the corn production system is not managed properly.

Several studies are already available in Europe dealing with the impact of *Bt*-corn (mainly Cry1Ab toxin) on non-target arthropods. In most cases (Pilcher et al. 1997, Orr and Landis 1997, Lozzia 1999, Bourguet et al. 2002) no differences for several herbivore and predatory taxa in *Bt* and in isogenic corn were found. Farinós et al. (2001) in Spain also have not detected any detrimental effect on spiders, carabid and *Orius* species in corn (cv. Cimpa CB). In a recent study, Bourguet et al. (2002) did not find significant differences in species number of dominant aphids, syrphids, ladybirds, lacewings over the growing season in *Bt* and isogenic corn. However, the above authors raised the importance of the need for longer term field assessments (detection of possible cumulative effects over more seasons), threshold for detecting any effects and selection of suitable species for impact studies. Laboratory studies (Hilbeck et al. 1998, 1999; Dutton et al. 2002) pointed out the possible negative effects of *Bt*-toxin on *C. carnea* larvae through combined prey availability and the food chain. The widespread availability of the Cry1Ab toxin in corn plants all season long might affect a great number of linked organisms besides the target pest, in different ways. In order to make a broad assessment of such possible effects feasible, a 3 year field study was established in 2001 in Hungary using *Bt*-expressing corn (Cry1Ab), as part of the EU 5th framework project *Bt*-BioNoTa (Effects and mechanisms of *Bt*-transgenes on biodiversity of non-target insects: pollinators, herbivores and their natural enemies). The field study, aiming to assess the impact of *Bt*-corn on non-target insects through trophic interactions needs a long term evaluation. Selection of food chain elements is essential for assessing any effect of *Bt*-toxin, since herbivore insects with different feeding

mouthpart types (chewing, sucking) may uptake various amounts of toxin. Thus we aimed to survey and compare herbivore and predator insect assemblages in *Bt* and isogenic corn, to create a trophic relation table, and to select food chain elements (dominant herbivores and predators) to assess the impact of *Bt*-corn through that chain. Since we are in the second year only, the present paper will not deal with impact assessment through trophic relations yet. In order to assess the cumulative indirect effects of *Bt*-corn on non-target arthropod assemblages through trophic interactions, we have established a tetratrophic field study model as well. The potential test organism must meet several requirements: abundant in corn fields, present over the whole growing season, generalist predator without considerable prey preference or aversion, and not sensitive to disturbance, or can be studied without disturbance. In addition to these, the test organism should have an easy-to-follow reproduction process, since the most sensitive indicator parameters are presumably associated with reproduction.

Materials and methods

Field trials with *Bt*-corn (DK 440 BTY- transformation event MON 810) and with its isogenic line (DK 440) were established in Hungary to measure the effect of *Bt*-corn on selected components of the tritrophic system (corn-herbivores-predators). Experimental fields were arranged in a randomised complete block design with 6 replications based on medium sized plots (30x30m). All plots were planted to the same transgenic variety and its isoline. Sampling of corn plants for the first year (2001) was designed to draft a food chain table of the corn insect assemblage, to determine dominance structure, and to select certain taxa for trophic interaction studies. Sampling methods for arthropods were as follows:

Randomly selected corn plants (different ones from one week to another) over the growing period of corn with removal of insects:
- *Helicoverpa armigera* Hbn larval numbers and damage on cobs were recorded,
- additionally, flea beetles (Chrysomelidae, Alticinae) on permanent corn plants were also recorded.

Insects on permanent corn plants were recorded but not removed to avoid disturbance of their development. However this method did not allow correct identification to species level (except in the case of *H. armigera*, or certain other typical pests) therefore additional sampling methods were used.

Randomly selected corn plants over the growing period of corn:
Sampling for aphids: 10 corn plants/plot/week.
Sampling for spiders (as for selected predatory taxa): 500 plants/plot/week.
Infestation of *Ostrinia nubilalis* (ECB) larvae was surveyed at the end of the growing season (early October) by checking 100 corn stalks/plot).

The second year (2002), sampling was continued for selected taxa, and an additional method was used for flea beetle and leafhopper sampling (visual

yellow sticky Pherocon AM trap Trece Inc. California, USA). Insect species collected were taken to the laboratory and stored in alcohol until determination. Herbivore insects were determined for taxa as follows: Thysanoptera (Thripidae, Phlaeothripidae), Heteroptera (Pentatomidae, Miridae), Homoptera (Delphacidae, Cicadellidae, Aphididae), Coleoptera (Nitidulidae, Chrysomelidae), Lepidoptera (Pyraustidae, Noctuidae), Diptera (Agromyzidae).

All predatory insects were collected weekly from 10 randomly selected corn plant on each corn plot. Collections and identification were made for taxa as follows: Coleoptera (Coccinellidae, Carabidae, Staphilinidae) Heteroptera (Nabidae, Anthocoridae), Thysanoptera (Aeolothripidae), Neuroptera (Chrysopidae, Hemerobiidae), Diptera (Syrphidae), and also for their potential prey - aphids, thrips, and the mite, *Tetranychus urticae*. Larval stages of predators were reared to adults in the laboratory. Individual numbers of selected herbivores and predators on *Bt* and isogenic corn were analysed by ANOVA.

After our exploratory study in previous years in corn, the spider species *Theridion impressum* L. Koch was selected for further investigations, and a low disturbance method was developed and tested to measure its reproductive parameters. *T. impressum* females, egg-sacs and webs were collected weekly from the end of June 2001 by individual plant search as described in sampling methods. The following parameters were counted: number of hatched spiderlings, and number of empty egg-shells, larval exuvia and dead eggs remaining in the egg-sac after hatching. Two spider-surveying methods were compared:

- High disturbance method: removal of females together with their guarded egg-sacs and webs. Parameter: spiderling count after hatching. Females were preserved in 60% isopropanol and identified under microscope examining both epigyne and vulva in each case.
- Low disturbance method: in situ female identification; removal of abandoned egg-sacs and webs only; parameter: egg-shell and exuvium count.

Our hypothesis was that the reproductive parameters resulted from the two methods are identical. In addition, webs of *T. impressum* were examined under microscope in order to analyse prey composition.

Results and discussion

Herbivores

A total of 41 herbivore insect species were recorded on *Bt* and on isogenic corn plots (on Pherocone AM traps and on corn plants) in 2001 and 2002. Difference of species number of insect assemblages in *Bt* (37 species) and isogenic corn plots (30 species) does not mean relevant variation since several species were identified on corn plants in low individual numbers (Table 1).

Table 1: Species composition of herbivore insect assemblages on *Bt* and isogenic corn plots (Sóskút, Hungary, 2001 and 2002)**

SPECIES/TAXA	*Bt*-corn	Isogenic corn
THYSANOPTERA		
Thripidae		
Chirothrips manicatus		X
Limothrips denticornis	X	X
Frankliniella tenuicornis	X	X
Odontothrips sp.		X
Phlaeothripidae		
Haplothrips aculeatus	X	X
Hoplothrips sp.	X	X
HETEROPTERA		
Pentatomidae		
Aelia sp.		X
Miridae		
Lygus rugulipennis	X	
Trigonotylus coelestialium	X	X
HOMOPTERA		
Auchenorrhyncha*		
Delphacidae		
Laodelphax striatellus	X	X
Cicadellidae		
Empoasca solani	X	X
Zyginidia pullula	X	X
Macrosteles laevis	X	X
Aphididae		
Rhopalosiphum padi	X	X
Rhopalosiphum insertum		X
Sitobion avenae	X	X
Metopolophium dirhodum	X	X
Schizaphis graminum	X	X
Diuraphis noxia	X	X
Hyalopterus pruni		X
COLEOPTERA		
Nitidulidae		
Meligethes aeneus	X	
Chrysomelidae		
Oulema lichenis		X
Oulema melanopus	X	X
Diabrotica virgifera virgifera	X	X
Phyllotreta atra	X	X
Phyllotreta nigripes	X	X
Phyllotreta undulata	X	X

Phyllotreta vittula	X	X
Phyllotreta nemorum		X
Phyllotreta cruciferae	X	X
Phyllotreta diademata		X
Longitarsus succineus		X
Longitarsus exoletus		X
Longitarsus sp.	X	X
Chaetocnema aridula	X	X
Chaetocnema concinna	X	X
Chaetocnema hortensis	X	
Chaetocnema tibialis	X	X
LEPIDOPTERA		
Pyraustidae		
Ostrinia nubilalis		X
Noctuidae		
Helicoverpa armigera	X	X
DIPTERA		
Agromyzidae spp. (larva and its leaf damage)	X	
TOTAL in *Bt* and in Isogenic corn	**30**	**37**
TOTAL in corn	**41**	

Remarks: X mark indicates the presence of species or taxa concerned
* A total of 44 species were detected, identification is still in progress
**Identification of certain taxa is in progress

Among herbivores, the following taxa were selected (abundant and important prey for predators or being a secondary pest).

Thysanoptera

Since Thysanoptera species feed on corn pollen and on tissue and serve as prey for several predators, we identified them to species level. Altogether 86 thrips individuals of 6 herbivore species were collected on *Bt* and 91 individuals on isogenic corn plants from 04 July to 03 October 2001. Thrips species were as follows: *Frankliniella tenuicornis, Haplothrips aculeatus, Limothrips denticornis, Chirothrips manicatus,* one unidentified *Hoplothrips* and one *Odontothrips* species. Some individuals of the *Odontothrips* species, *Hoplothrips* species and of *Chirothrips manicatus* were identified, the latter two in the isogenic corn stand only. *F. tenuicornis* and *H. aculeatus* were the dominant species, both on *Bt* and isogenic corn plants. The most individuals were collected in autumn (September). The individual number of non-dominant thrips species observed during the sampling period was very low. As regard to the dominant Thysanoptera density, there was no difference in their individual number on *Bt* and isogenic corn, as was found by Bourguet et al. (2002) for the same event, Mon 810.

Auchenorrhyncha

During the 2001 sampling, we observed high density of leafhoppers on upper leaves of corn. Therefore we initiated more intensive sampling (by Pherocon AM trapping) in 2002. An extremely species rich and abundant leafhopper assemblage was found in corn in 2002. For the first part of the 2002 sampling period, the total number of leafhopper species was over 44. Dominant species were *Empoasca solani*, *Laodelphax striatellus*, *Macrosteles laevis* and *Zyginidia pullula*. For leafhoppers on *Bt*-corn we have not found data in European field tests. Since leafhoppers may also be important preys of predators and are known as meso-cell feeders, their *Bt*-toxin ingestion and possible effect on predators through trophic relations should be studied.

Aphididae

Eight aphid species were found on corn plants from 01 June to 10 October 2001. Aphid species were as follows: *Rhopalosiphum padi, Rhopalosiphum insertum, Sitobion avenae, Metopolophium dirhodum, Schizaphis graminum, Diuraphis noxia, Hyalopterus pruni*. Some individuals of *D. noxia, R. insertum, H. pruni* were identified, the latter two in the isogenic corn stand only. *R. padi* was the dominant aphid species both on *Bt* and isogenic corn plants with high numbers in autumn, when the second peak of aphids on corn occurs under Hungarian conditions, (Figure 1). In France (Bourguet et al. 2002), *R. padi* was also among the first 3 aphids on corn, with no differences between *Bt* and isogenic corn. Significant difference was found (Kruskar-Wallis ANOVA, p=0,036) between the number of individuals of *R. padi*, with more individuals on *Bt*-corn plants compared to isogenic ones in 2001. If analysis of the data collected in 2002 confirms the results of 2001, an explanation for the above differences should be sought.

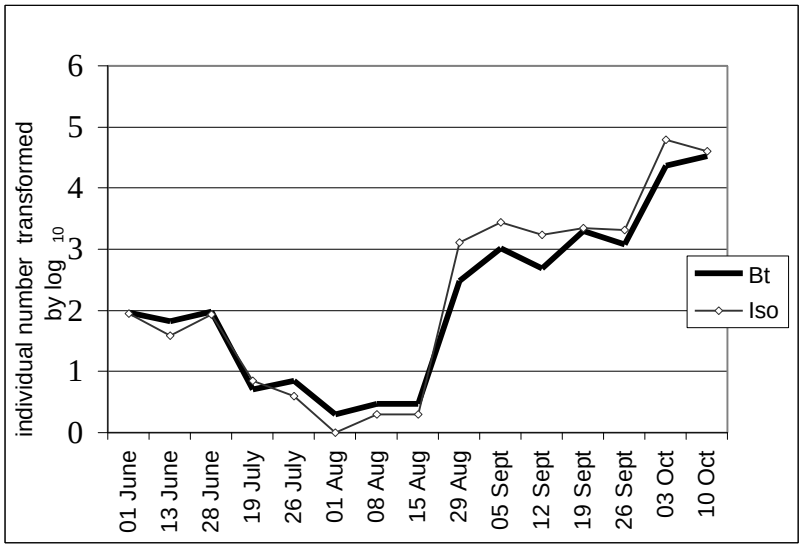

Fig. 1: Individual numbers of *Rhopalosiphum padi* on *Bt* and isogenic corn plants (2001, Sóskút, Hungary)

Chrysomelidae, Alticinae

In 2001 we observed feeding symptoms and a significant number of flea beetles and leafhoppers as herbivores in corn. These taxa therefore were selected for more detailed sampling through the use of Pherocon AM yellow sticky traps in 2002. In both years *Phyllotreta* species were dominant in corn. Flea beetles ingested the *Bt*-toxin by feeding on corn leaf tissue, silk and pollen. Therefore, these species as preys could be selected for assessing the impact of *Bt*-toxin on certain predators through trophic relations.

Lepidoptera, Pyraustidae and Noctuidae

Larvae of the target organism, *O. nubilalis* (European corn borer: ECB) were found only in the isogenic corn stalk, but the average infestation level, was moderate in 2001 (10.83%) and 2002 (13,17%) (Fig. 2). In *Bt*-corn stalks (100 plants/plot) we did not find ECB larvae, and thus were not able to evaluate parasitisation of ECB larvae.

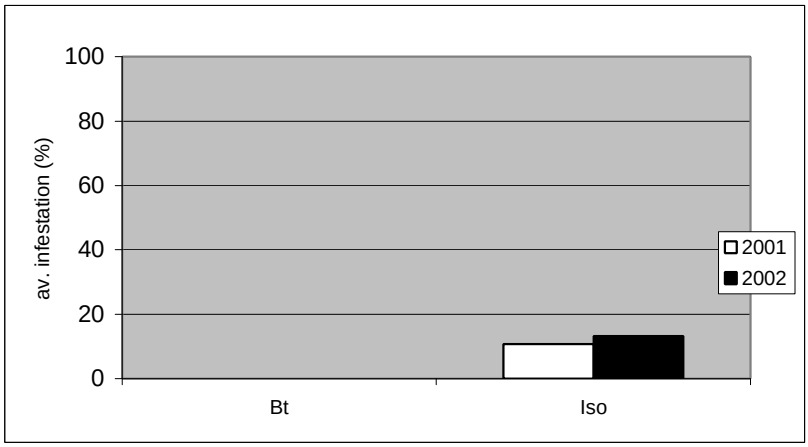

Fig. 2: Average infestation of ECB larvae in *Bt* and isogenic corn stalks (October 2001, 2002, Sóskút, Hungary, 100 plants, 6 reps)

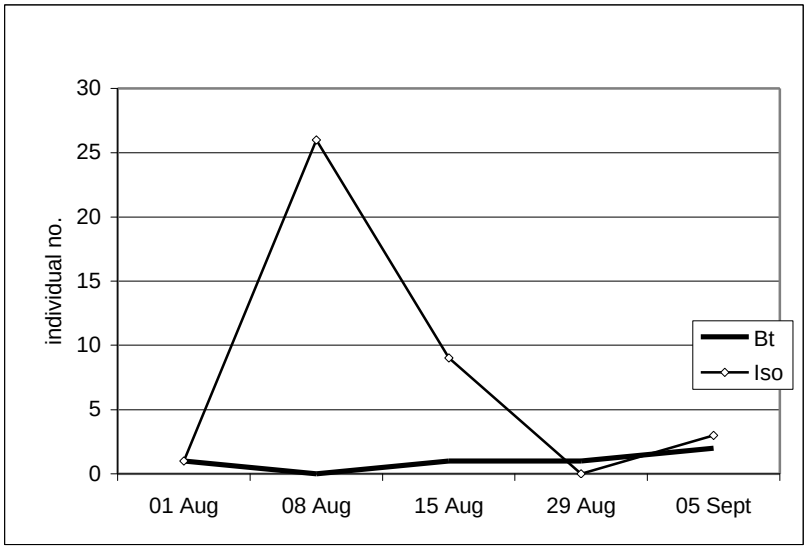

Fig. 3: Individual number of *H. armigera* larvae on *Bt* and isogenic corn plants (2001, Sóskút, Hungary, 60 plants)

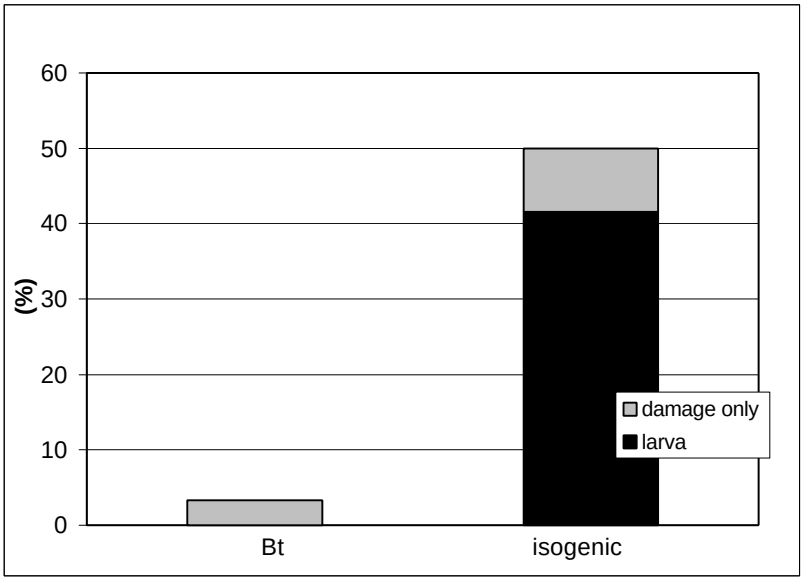

Fig. 4: Infestation of corn by *H. armigera* larvae and their feeding damage to corn (08. August, 2001, Sóskút, Hungary, 10 plants/plot, 6 reps.)

H. armigera is an economic corn pest in Hungary. Its larvae feed on silks, and seeds under husky leaves. Its damage contributes to increased infestation of kernels by *Fusarium* spp. The pest may have one (Figure 3) or two larval peaks on corn. For infestation of *H. armigera* larvae differences were found between the number of individuals on *Bt* and isogenic corn plants. Significantly higher number of individuals was found on the isogenic corn ($p < 0.000$) (Figure 4). Minor feeding symptoms of *H. armigera* larvae on corn silks and on the ears, and the presence of some larvae were observed on *Bt*-corn plants in 2001 and in 2002. This indicates that the Cry1Ab toxin, has a negative effect on *H. armigera* larvae.

Predators

The field survey and sampling of insect natural enemies were conducted from early June to early October in 2001 and 2002. A total of 32 predatory insect species were recorded from corn plants during the sampling period (Table 2). The whole predator assemblage was found on both corn plot types. The most abundant predatory species were among the coccinellids (*Propylea 14-punctata*, *Coccinella septempunctata*, *Stethorus punctillum*), thrips (*Aeolothrips intermedius*), anthocorids (*Orius niger*).

Table 2:. Species composition of above ground predatory insect assemblages on *Bt* and isogenic corn plots (Sóskút, Hungary, 2001 and 2002)*

SPECIES	*Bt*-corn	Isogenic-corn
DERMAPTERA		
Forficulidae		
Forficula auricularia		X
THYSANOPTERA		
Aeolothripidae		
Aeolothrips intermedius	X	X
HETEROPTERA		
Anthocoridae		
Orius niger	X	X
Orius majusculus	X	X
Orius minutus	X	X
Nabidae		
Nabis ferus	X	X
Nabis pseudoferus	X	X
Nabis punctatus	X	X
NEUROPTERA		
Chrysopidae		
Chrysoperla carnea s. lat.	X	X
Chrysoperla lucasina	X	X
Hemerobiidae		
Wesmaelius subnebulosus		X
Hemerobius humulinus	X	X
Hemerobius micans	X	
Micromus variegatus	X	X
Micromus angulatus	X	X
COLEOPTERA		
Carabidae		
Demetrias atricapillus		X

Staphylinidae		
Tachyporus hypnorum	X	X
Paederus riparius	X	X
Paederus littoralis	X	X
Coccinellidae		
Adalia bipunctata	X	X
Brumus quadripustulatus	X	X
Calvia (Anisocalvia) quatuordecimguttata	X	
Calvia decemguttata		X
Coccinella septempunctata	X	X
Coccinula quatuordecimpustulata	X	X
Hippodamia (Adonia) variegata	X	X
Hippodamia tredecimpunctata	X	X
Oenopia conglobata	X	
Propylea quatuordecimpunctata	X	X
Scymnus frontalis	X	X
Stethorus punctillum	X	X
DIPTERA		
Syrphidae		
Episyrphus balteatus	X	X
TOTAL in *Bt* and in Isogenic corn	**28**	**29**
TOTAL in corn	**32**	

*species identification for certain taxa is still in progress

In 2001 and 2002, almost the same numbers of predatory species were collected in *Bt* and isogenic corn. Within the most frequent predatory insect groups, no significant differences of species spectrum were detected between *Bt*- and isogenic-corn plants (Table 3). There were significant differences in total number of coccinellids and that of *Hippodamia variegata* sampled in *Bt* and isogenic corn in 2001. Syrphid larvae, and imagines of *Nabis* and *P. 14-punctata* were represented at a slightly higher abundance on isogenic control plots than on *Bt* ones in 2002. Further analysis of the data collected in 2001 and 2002 will clarify whether there is an effect of *Bt*-corn (and its prey availability) on coccinellid density and development.

Table 3: Dominance distribution (%) of some predatory insect groups within assemblages on *Bt*- and isogenic-corn plants during July and August (2001. Sóskút, Hungary)

Family of predatory insects	Dominance (%)	
	in *Bt*-corn	in isogenic corn
Aeolothripidae	17.9	15.8
Anthocoridae	52.5	52.2
Nabidae	7.6	8.3
Hemerobiidae	1.9	1.7
Chrysopidae	0.8	0.7
Coccinellidae	17.5	19.8
Staphilinidae	1.4	1.3
Syrphidae	0.4	0.2
Number of individuals	**1824 (100%)**	**2077 (100%)**

Tetratrophic interaction

No significant difference was found between high disturbance (removal of females, egg-sacs and webs; spiderling count) and low disturbance (in situ female identification; removal of abandoned egg-sacs and webs; egg-shell and exuvium count) methods (One-Way ANOVA; $p=0.83$). In situ female identification was correct. Population fitness parameters of *T. impressum* were significantly higher of those sampled in isogenic plots (One-Way ANOVA; $p<0.05$).

An extremely broad prey spectrum was found in the webs, consisted of aphids, leafhoppers, thrips, phytophagous and predatory bugs, click-beetles, flea-beetles, coccinellid larvae and adults, lacewing larvae and adults, syrphid (and other) flies, ants, vespid wasps, parasitoid wasps, honeybees, lepidopteran larvae and adults. These data supplemented with our field observations demonstrate the complexity of existing trophic relations in corn (Figure 5). A significantly higher number of aphid (mainly *Rhopalosiphum padi*) individuals were found in the webs of isogenic plots compared to transgenic ones, while occurrence of other orders did not show significant difference (One-Way ANOVA; $p<0.05$).

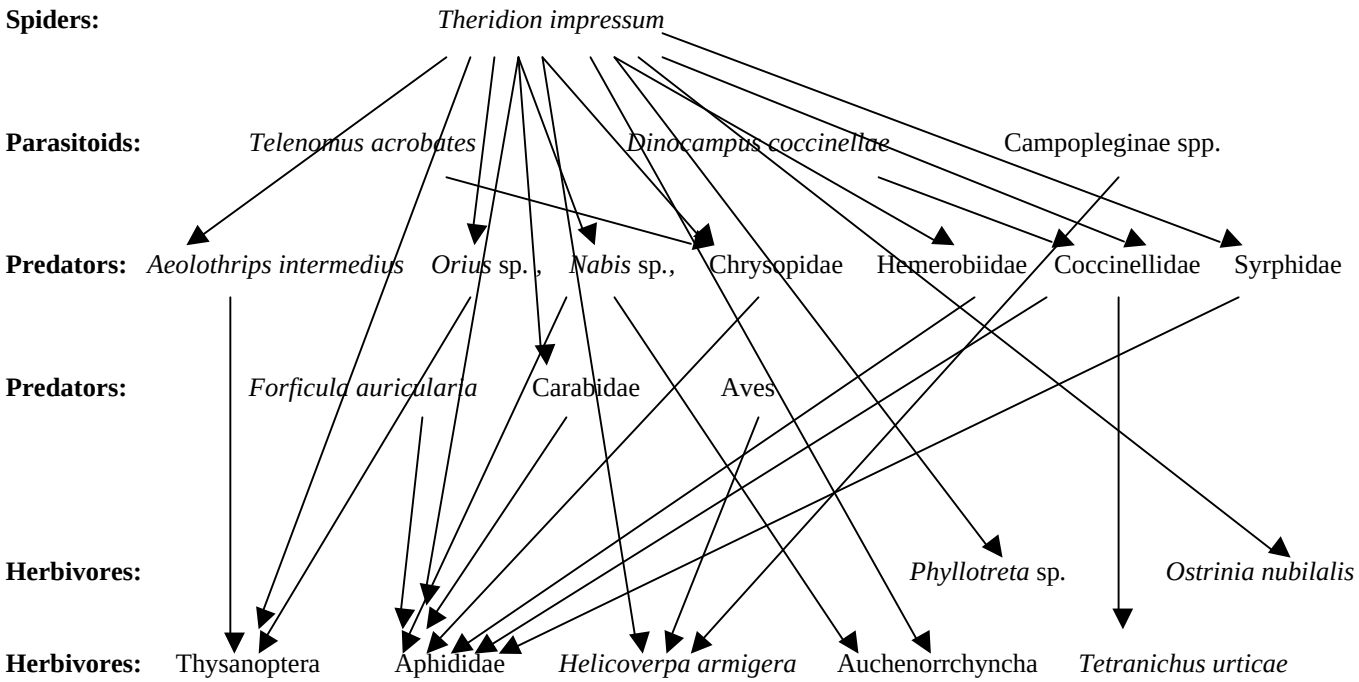

Fig. 5. Trophic relations within the arthropod assemblage sampled on *Bt* and isogenic corn plots (Sóskút, Hungary, 2001, 2002)

Conclusions

For field level assessment of the impact of *Bt*-corn on non-target arthropod assemblages, a long-term regionally adapted field trial is relevant. A region-based survey of non-target insects in corn demonstrates the importance of such herbivores (leafhoppers and flea beetles in our case) that are not (or at low density) present in other regions.

Besides the target pest (ECB, European Corn Borer), a regionally important secondary pest (*Helicoverpa armigera* in our case) was strongly affected by the toxin. Therefore, an insect resistance management plan should also be developed for the secondary pest. In a broader view, the impact of Cry1Ab toxin on any Lepidoptera larva can also be evaluated on regional bases since secondary pests or Lepidoptera species in corn ecosystem differ from region to region.

Based on our field trial, the Cry1Ab toxin expressed in *Bt*-corn (Mon810 event) did not cause any significant effect on the non-target herbivore insect species spectrum compared to that of the same isogenic line. Simple species numbers and species abundance values are important indicators, but impact of toxin on non-target insects including predators and parasitoids must be evaluated through trophic relations. According to our results, *Theridion impressum* is a relevant model species for tri- and tetratrophic studies. Prey composition and reproductive fitness can be measured by collecting only abandoned webs and egg-sacs. Following this low disturbance method additional data (e.g. spider phenology) can be gained.

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EFFECTS OF A *BACILLUS THURINGIENSIS* (*BT*) TRANSGENE ON THE FECUNDITY AND ABUNDANCE OF WEEDS: A CASE STUDY OF SUNFLOWER

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Abstract

A widely acknowledged risk associated with transgenic crops is the possibility that hybridization with wild relatives will cause fitness-related transgenes to persist in wild populations. If wild plants acquire transgenes coding for resistance to herbivory, disease, environmental stress, and/or commonly used herbicides, they could become more abundant in their natural habitats or invade previously unsuitable habitat. In addition, wild populations containing transgenes that provide resistance to herbivores may have additional effects on natural insect populations. However, little is known about whether these concerns are justified. For example, many weedy species are affected by herbivores, yet the impact of insect damage on population densities and invasiveness has rarely been examined. In order to determine if a transgene poses a risk to wild populations, or to the species with which the wild plant interacts, three questions must be addressed. These are: 1) are there genetic or geographic barriers to the escape of the transgene from the crop into wild populations? 2) is the transgene expected to increase in frequency in wild populations? and 3) what are the ecological consequences of escape? We are investigating these questions in the cultivated/wild sunflower system (*Helianthus annuus*). In answer to the first two questions we have found: few barriers to gene flow between the crop and wild population, and decreased lepidopteran herbivory and increased seed production in wild plants containing a *Bacillus thuringiensis* (*Bt)*-toxin gene specific to lepidopterans. Furthermore, no fitness costs were associated with the transgene when plant were grown in a greenhouse. In answer to the third question, preliminary analyses suggest that increased seed production in individual plants (caused by, for example, a *Bt* gene) could lead to an

increase in the size of wild populations. We are currently doing experimental and modeling work to determine if larger local populations will lead to larger or more persistent metapopulations.

Keywords: crop-wild hybrid, transgenic insect resistance, *Bacillus thuringiensis*, *Bt*, sunflower, *Helianthus annuus*, herbivory, fecundity, fitness cost

Introduction

Evaluating the ecological risks of transgenic crops has become increasingly important as more varieties are released, and as more acres are planted in these varieties. A widely acknowledged risk associated with transgenic crops is the possibility that hybridization with weedy relatives will cause fitness-related transgenes to persist in wild populations. Despite this understanding there exist almost no data appropriate for evaluating the ecological effects of the escape of transgenes from crops into wild populations. We argue here that assessment of the risks associated with the escape of transgenes must sequentially address three questions (Snow et al. 1999).

The first of these questions is: is the transgenic variety of the cultivated plant sexually compatible with wild relatives? Clearly, if cultivated and wild populations are not sexually compatible, there exists no risk of escape of a transgene, and risk assessment need go no further. However, many crop species, including rice, sorghum, canola (oilseed rape), sugarbeet, oats, squash, carrot, radish, strawberry, clover, sunflower, and others are known to hybridize with their wild relatives (Snow and Moran-Palma 1997, Zemetra et al. 1998). The fecundity of crop-wild hybrids is often found to be lower than the fecundity of purely wild types (e.g. Snow et al. 1998). However, fecundity in F_1s must be zero to prevent the transfer of beneficial alleles into wild populations.

If the crop can successfully hybridize with its wild relative, it is necessary to answer the second question: does the transgene confer a fitness benefit on the wild plant? If the transgene is of no benefit (or is always costly) to the wild plant, genetic drift (or purifying selection) will determine its fate in the wild population. For example, it may be that transgenes controlling traits important to harvesting the crop and shelf-life of the product, such as those associated with fruit-ripening, will be neutral or costly in wild populations. Consequently, these traits are not expected to increase in frequency, and may pose little ecological risk. By contrast, transgenes for characters like insect or pathogen resistance and drought tolerance may be beneficial to wild populations, and for this reason they may increase in frequency in wild populations by natural selection. Transgenes for herbicide tolerance are

unlikely to increase fitness in purely wild populations, but weedy populations containing these genes may be more difficult to control with herbicides. Very little is known about the fitness effects of transgenes in wild populations; studies of the fitness effects of transgenes in wild relatives have been performed for no commercially released transgenes.

Finally, if a transgene confers a fitness benefit on a wild relative it is necessary to answer a third question: what are the ecological consequences of the escape of the transgene into a wild population? Specifically, it is necessary to determine if the transgene alters interactions between the wild plant and its biotic and abiotic environment. A transgene that increases in frequency in wild plants does so, by definition, because it increases survival or fecundity, and one risk that has been discussed in the literature is the effect of this increased individual fitness on the population size, dynamics, and habitat use in the wild plant. In addition, transgenes that confer resistance to herbivores and pathogens will have effects on native species using the wild plant as a host. Clearly, these questions must be the crux of any ecological risk assessment. However, virtually no work has been done in these areas.

We have been addressing these three questions in using *Helianthus annuus*, which is the wild progenitor of cultivated sunflower, and a *Bt*-transgene that confers resistance to lepidopteran herbivores. Here we summarize the work we have done towards answering each of these questions.

Sunflower and its insect herbivores as a model system

Wild sunflower represents an excellent model system with which to address these questions. Wild *H. annuus* is a native, self-incompatible, annual plant that is widespread throughout much of the USA, reaching its greatest abundance in midwestern states (Heiser 1954), where most cultivated sunflower is grown (Schneiter 1997). Wild sunflower is a disturbance specialist, and populations are typically patchy and ephemeral, relying on the soil seed bank and long-distance dispersal for opportunities to become established in new areas. In the absence of tilling or other types of disturbance, population size declines. In agricultural areas, however, repeated tilling allows wild sunflower populations to persist for many years, especially along field margins.

Wild sunflower is host to many insect herbivores, and many of these species are also pests in the crop. Some herbivores have negative effects on fitness in wild populations (Pilson 2000), and they can also substantially reduce yield in some years and locations (Charlet 1997). The most damaging insect pests of cultivated sunflower are those that infest developing seed

heads (weevil, moth, and midge larvae) and those that transmit disease (e.g., stem weevils that transmit phoma black stem) (Schneiter 1997). In wild *H. annuus*, insect resistance is typically polygenic, and efforts to introgress strong resistance into the crop have been unsuccessful (Seiler 1992). For these reasons cultivated lines with transgenic resistance conferred by *Bt*-toxins are being developed by a number of seed companies, and several field trials have been approved (http://www.isb.vt.edu). Different *Bt*-toxins are specific to different groups of insects, including Lepidoptera, Coleoptera, and Diptera. Bt-induced resistance to Coleoptera was first field-tested in the US in 1996 and resistance to Lepidoptera was approved for field-testing in 1999, although none have been commercialized to date. Additional field trials have taken place in the Netherlands and Argentina (http://www.isb.vt.edu; http://siiap.sagyp.mecon.ar/http-hsi/english/conabia/liuk4.htm).
Broad-spectrum resistance involving multiple *Bt*-transgenes and other genes for insect resistance (e.g., Stewart 1999) may also be developed in the future.

Are wild and cultivated sunflower sexually compatible?

The process of crop-to-wild introgression has been well documented in sunflowers. Field experiments have shown that pollinators can transfer crop pollen to wild plants as far as 1,000 m away, with the frequency of hybrid seeds being greatest (up to 42%) at the crop margin (Arias and Rieseberg 1994, Whitton et al. 1997). Additional studies have shown that first generation wild-crop hybrids usually produce fewer seeds per plant than their wild counterparts, but the magnitude of this difference varies a great deal among plants, regions, and growing conditions (Snow et al. 1997, Morán Palma 1997, Snow et al. 1998). Under some field conditions, seed production of F_1 crop-wild hybrids is comparable to that of purely wild plants, and in several cases hybrids produce at least 50% as many seeds per plant as wild genotypes. Furthermore, selectively neutral crop markers have persisted for many generations in wild plants sampled in California, Kansas, North Dakota, and Canada (Whitton et al. 1997, Linder et al. 1998). These studies demonstrate that introgression of neutral or beneficial crop genes into wild gene pools can be an ongoing process wherever these taxa occur sympatrically. Moreover, cultivated sunflower, which is primarily planted in North and South Dakota, Nebraska, Kansas, and eastern Colorado, is nearly always sympatric with wild *H. annuus* (Schneiter 1997, Heiser 1954). Clearly, both genetic and geographic barriers to gene flow from crop to wild sunflower are minimial. Thus, the answer to the first question is yes, and further studies of pre-commercial transgenes in the wild genetic background are necessary.

What is the fitness effect of the *Bt*-transgene in the wild background?

Because it is clear that any transgene deployed in cultivated sunflower planted in North America will escape into wild populations, it is necessary to determine if the transgene(s) increases the fitness of wild plants. In the case of a *Bt*-transgene we need to know if plants carrying the gene are resistant to herbivory (by the species specifically targeted by the transgene), and further, whether this reduction in damage leads to an increase in fitness. We have addressed these questions for a *Bt*-transgene that is specific to Lepidoptera (Snow et al. unpublished data; Pilson, et al. unpublished data). Our study involved the *Bt*-protein Cry1Ac, which is toxic to many lepidopteran species but is not expected to affect other insect taxa (Estruch et al. 1997). Ingesting a very small amount of *Bt*-toxin (e.g., parts per billion) typically causes susceptible insects to stop feeding and die within a few days (Estruch et al. 1997), or move to a nontoxic host plant (Davis and Onstad 2000).

Determining the ecological effects of pre-commercial transgenes is inherently difficult due to biosafety and regulatory concerns. Uncaged plants must be exposed to natural levels of insect damage and cross-pollination, yet dispersal and persistence of the transgene(s) must be prevented. Our solution to this difficult problem was to use male sterile plants for the field experiments so that the possibility of transgene escape through pollen could eliminated.

To simulate the effects of introgression of a *Bt*-transgene from the crop, male-sterile wild plants from a population near the Cedar Point Biological Station in Nebraska were bred with transgenic cultivars to create BC_1 and BC_3 progeny that segregated for both the *Bt*-transgene (Bt+ or Bt-) and for male-sterility (male-sterile or male-fertile). However, to prevent the accidental escape of the transgene, we did not use Bt+/male-fertile plants in the field. BC_1 progeny were planted in the field in 1999 at the Cedar Point Biological Station in western Nebraska and in an agricultural field near Burlington, in eastern Colorado, and BC_3 progeny were planted in Nebraska in 2000. The effect of the transgene was examined by comparing insect damage and fecundity between *Bt*+/male-sterile and *Bt*-/male-sterile plants,. We also compared *Bt*-/male-sterile and *Bt*-/male-fertile plants to determine the effects of male-sterility on herbivory and seed production (some seed predators feed on pollen [Korman and Oseto 1989, Delisle et al. 1989] and might avoid male-sterile plants).

The *Bt*-transgene led to reduced lepidopteran damage at both field sites and in both years (Figure 1 for data from the Nebraska 1999 experiment). However, the *Bt*-toxin had no effect on amounts of damage caused by four

non-lepidopteran species (Figure 2 for Nebraska 1999, Colorado 1999 and Nebraska 2000 show similar patterns). Although some of the non-lepidopteran species are negatively affected by competition with lepidopterans (Paulsen and Pilson unpublished), they did not cause more damage on *Bt*-plants than on controls. As expected, damage by some herbivores was reduced on male-sterile plants relative to male-fertile plants (Figures 1 and 2). *Bt*+ plants produced an average of 55% and 23% more seeds per plant than *Bt*- plants in Nebraska in 1999 and 2000, and 14% more seeds per plant in Colorado in 1999 (Figure 3). The reduction in herbivory on male-sterile plants suggests that using male-sterile plants to test for *Bt* effects may underestimate the fecundity advantage associated with the transgene. Had we been able to use pollen-producing *Bt*-plants in the field, we might have documented more dramatic fecundity benefits of the transgene.

In any study of a single transformation event, it is not clear whether phenotypic effects (e.g., greater fecundity) are caused by the transgenic construct or by other mechanisms, such as position effects, pleiotropy, or close physical linkage with other crop genes. Thus, it is useful to determine whether effects associated with the *Bt*-transgene can occur in the absence of lepidopteran herbivores. We performed a greenhouse experiment using BC_1 plants to examine this possibility, while recognizing there are many biotic and abiotic differences between field and greenhouse conditions. The *Bt*-transgene had no effect on the number of inflorescences or seeds per plant in the greenhouse, regardless of whether the plants were grown under water-stressed, drought-stressed, or control conditions, and regardless of whether they were male-fertile or male-sterile (Snow et al. unpublished data; Figure 4). This suggests that the transgene was not associated with an inherent fitness cost or benefit. It would be preferable to employ a wider range of growing conditions and several transgenic events in this type of study, but our results suggest that the fecundity advantage of transgenic plants in the field was due to protection from lepidopteran herbivores.

This study shows that selection favoring an increase in the frequency of a *Bt*-transgene has the potential to be quite strong. If herbivores cause more damage to F_1 (Cummings et al. 1999) and BC_1 sunflowers than to wild genotypes, it is possible that the fecundity advantage associated with *Bt* would diminish with subsequent generations of backcrossing. On the other hand, because male-sterile plants had less damage from lepidopterans than those with pollen, we may have underestimated the fecundity advantage of *Bt* in this study. In addition, we have observed higher levels of lepidopteran damage on wild plants in other years (Pilson 2000, Pilson and Paulsen unpublished). Therefore, we expect that subsequent generations of *Bt* wild

plants would produce more seeds per plant than non-transgenic individuals in many locations and growing seasons. If so, the transgene is expected to spread quickly and kill susceptible, native lepidopterans that feed on wild sunflower.

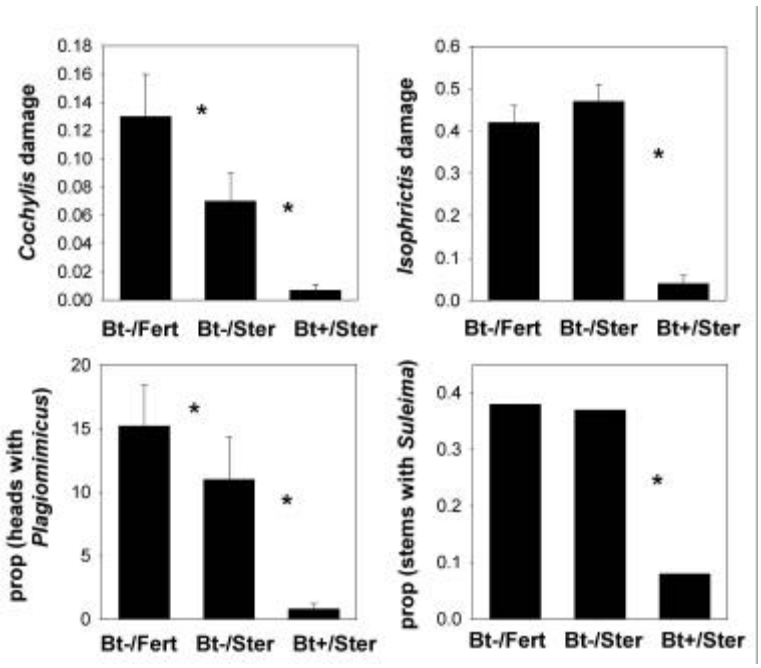

Fig. 1: Lepidopteran damage in Nebraska in 1999. *Cochylis hospes* (Chchylidae) and *Isophrictis similiella* (Gelechiidae) damage were categorized as 0, 1, or 2 (0, 1-30, or >30 seeds eaten) for each inflorescence, and the mean value over all inflorescences on each plant was analyzed by ANOVA. The proportion of heads on each plant attacked by *Plagiomimicus spumosum* (Noctuidae) was analyzed by ANOVA. The proportion of plants with stem damage by *Suleima helianthana* (Tortricidae) was analyzed by categorical ANOVA. In all analyses we made planned contrasts between *Bt*-/male-sterile and *Bt*-/male-fertile plants, and between *Bt*-/male-sterile and *Bt*+/male-sterile plants, and significant contrasts (at $p<0.05$) are indicated by asterisks.

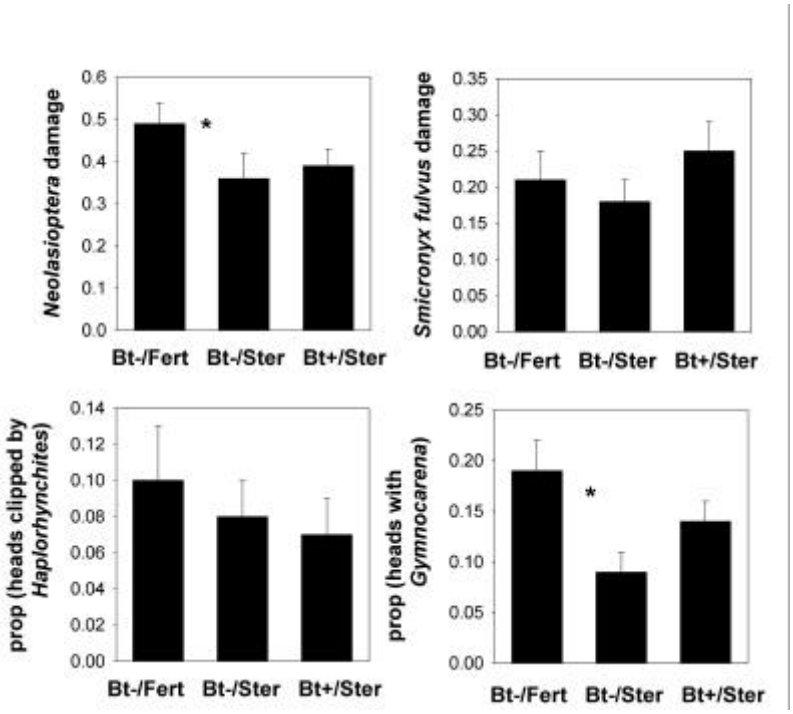

Fig. 2: Damage by non-lepidopteran herbivores in Nebraska in 1999. Damage by *Neolasioptera helianthi* (Cecidomyidae) and *Smicronyx fulvus* (Curculionidae) was quantified categorically (as described in Figure 1). These data, as well as data for *Haplorhynchites aeneus* (Curculionidae) and *Gymnocarena diffusa* (Tephritidae), were analyzed by ANOVA with planned contrasts (as described in Figure 1).

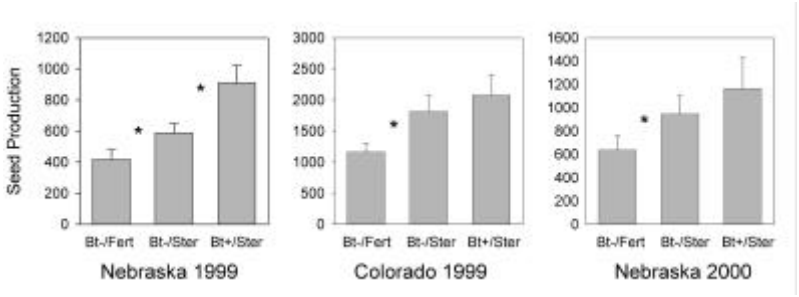

Fig. 3: Effects of the *Bt*-transgene and male sterility on seed production per plant in Nebraska and Colorado in 1999 and Nebraska in 2000. Untransformed means and 1 SE are shown; N = 58-60 in Nebraska in 1999 and 47-49 in Colorado in 1999 and Nebraska in 2000. Data were analyzed by ANOVA followed by planned contrasts as described in Figure 1. When both years and sites were combined in a single ANOVA the planned contrast between *Bt*+/male-sterile and *Bt*-/male-sterile plants was significant at $p<0.0206$. The selection coefficients favoring the *Bt*-transgene were 0.35 in Nebraska in 1999, 0.13 in Colorado in 1999, and 0.19 in Nebraska in 2000.

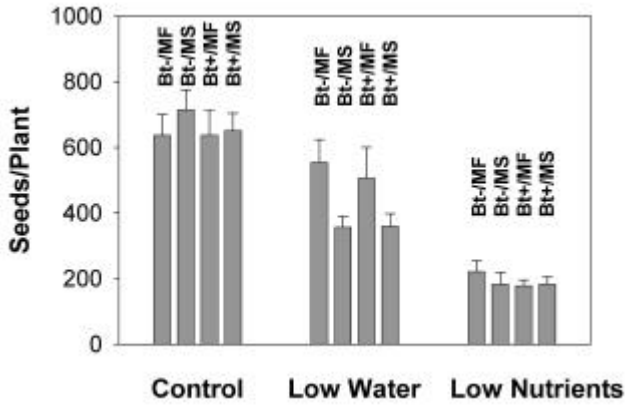

Fig. 4: Effects of the *Bt*-transgene and male sterility on seed production of plants grown under three conditions (Control, Low Water, Low Nutrients) in a greenhouse. Means and 1 SE are shown. The only significant effects in a three-way ANOVA were growing condition ($P<0.0001$) and the interaction between growing condition and male-fertility ($P<0.0042$) Sample sizes from left to right were Control: 19, 24, 16, and 27 plants per treatment; Low Water: 17, 27, 12, and 24; Low Nutrients: 14, 11, 14, and 12.What are the ecological consequences of the escape of a *Bt*-transgene into wild populations?

What are the ecological consequences of the escape of a *Bt*-transgene into wild populations?

For transgenes to have important ecological effects in wild populations, not only must they increase the fitness of wild plants, they must also alter interactions between the wild plant and its biotic and abiotic environment. Arguably, it is the potential ecological effects that should be the focus of attention in risk assessments. Although the escape of a transgene into a wild population, and its subsequent increase frequency by natural selection, are necessary, they are not sufficient to predict the environmental consequences of escape. Specifically, these processes are only important to the extent that they lead to the alteration of existing ecological interactions among species.

In ongoing work we are examining two types of ecological consequences of the escape of a *Bt*-transgene into wild populations. These are 1) the effect on the population dynamics of wild sunflower, and 2) the effect on the community of native herbivores that feeds on wild sunflower, and we present preliminary results from these studies here.

The effect of a *Bt*-transgene on the population dynamics of wild sunflower

We have shown that a *Bt*-transgene increases seed production in wild plants, and is therefore likely to increase in frequency in wild populations. However, increased seed production by individual plants will only lead to larger populations, or more populations, if wild sunflower is currently seed limited. Currently, little is known about processes controlling the population dynamics of wild sunflower. Thus, it is currently unclear what effect, if any, that a *Bt*-transgene will have on the dynamics of wild populations.

Surprisingly, there are few data with which to evaluate the importance of seed limitation on the population dynamics of any plant species. Silvertown and Franco (1993) and Silvertown et al. (1993) compared the sensitivities of demographic transitions in herbs and woody plants and found that population growth rates were more affected by changes in fecundity in semelparous species, suggesting that annual species, such as sunflower, are more likely than other plants to be seed limited. Louda and Potvin (1995) found that elimination of herbivores by application of insecticide increased not only individual fitness but also local population size in a native thistle. Crawley and Brown (1995) found that weedy roadway populations of oilseed rape were seed limited at the landscape scale. In contrast, Bergelson (1994) found that population size was not seed limited in experimental plantings of a

diminutive cress, *Arabidopsis thaliana*, a result that is likely due to insufficient open space and competition from surrounding vegetation.

To evaluate the effect of increased seed production on the population dynamics of wild sunflower we are using the following approach. First, in western Nebraska (in 2000 and 2001) and eastern Kansas (in 2000) we established experimental populations with varying amounts of seed production, and we are examining the effect of seed production in one year on population size and seed production in the following years. Second, we are using these experimental populations to derive parameters for spatially explicit metapopulation models. We will use these models to make predictions about the effect of seed production on metapopulation dynamics at our two study sites. And third, we will test our metapopulation models by comparison with empirical observation of metapopulation dynamics in both western Nebraska and eastern Kansas. Our two study sites are in very different environments (historically mixed short-grass prairie in western Nebraska, and tall grass prairie in Kansas), and we expect that the dynamics will be different in these habitats as well. This multi-year research program is in its early stages, so here we only present data documenting the effect of seed production in one year on population size the following year.

In 2000 we established 48 experimental populations at each of our study sites (at the Cedar Point Biological Station, near Ogallala, in western Nebraska, and at the Kansas Ecological Reserves, near Lawrence, in eastern Kansas); here we will only discuss preliminary data from the Nebraska populations. Each experimental population consisted of either 16 (n=32) or 21 (n=16) experimental plants. In each population the experimental plants were surrounded by either 45 or 40 plants that were not allowed to disperse seed, and which served to maintain a similar competitive environment for all experimental plants. All of these plants were sown in a central (rototilled) 2m x 2m square (experimental plants in the central 1m x 1m) located in the center of a larger 10m x 10m square. Half of the experimental populations with 16 plants were sprayed with a broad-spectrum insecticide to reduce herbivory (and increase seed production). Because sunflower is a disturbance specialist, and requires a recent disturbance for germination, we rototilled a 2m x 4m strip in one cardinal direction from each of the 48 plots (i.e. 12 tilled north, 12 tilled east, 12 tilled south, and 12 tilled west). The 45 or 40 competitor plants were removed before dispersing any seeds, and experimental plants were allowed to disperse their seeds naturally. Due to a severe drought during the 2000 growing season many plants performed very poorly, and the treatments (number of experimental plants and spraying) had no effect on seed production. Nonetheless, seed production (estimated by the

number of heads dispersing seeds and an estimate of mean head area) varied by approximately two orders of magnitude (from ~200-20,000 seeds).

In 2001 we counted the number of seedlings emerging in each experimental population (in both the original square and in the tilled strip). (Presumably, seeds were dispersed in all directions. However, with few exceptions, seeds only germinated in the tilled squares and strips.) We also counted the number of seedlings that survived to reproductive age and the number of inflorescences produced by each plant that survived to reproductive age. We measured the inflorescence diameter of 10 randomly chosen inflorescences in each experimental population, and estimated seed production in 2001 by estimating the total inflorescence area produced by each population.

We used regression analysis (SAS 1989) to evaluate the effect of seed production in 2000 on the number of seedlings, number of reproductive plants, number of mature inflorescences, and the estimated number of seeds produced in 2001. Because the direction in which the strips were tilled had a significant effect on all of these response variables (probably due to a consistent prevailing wind direction during seed dispersal in 2000), we performed these analyses on residual values after the effect of direction had been removed in an ANOVA (PROC GLM in SAS). While it may seem redundant to evaluate the effect of seed production in 2000 on all of these population responses in 2001, it is only redundant to the extent that there are no density dependant processes affecting seed production. For example, it might be the case that many seeds germinate, but that intraspecific competition in high-density populations reduces the number of plants surviving to reproductive age to the same seen in an originally lower density population. Similarly, many plants might survive, but plants might produce fewer inflorescences in higher density populations.

Preliminary analyses suggest that increasing seed production in one year increases both population size and the number of seeds dispersed the following year (Figure 5). Moreover, the relationships between seed production in 2000 and number of seedlings, number of reproductive plants, number of inflorescences, and estimated head area in 2001 appear to be very similar, suggesting that density-dependant factors are not important in these populations. From these data we tentatively conclude that sunflower populations are seed-limited, and that increased seed production may lead to increased local population size. Additional analyses of these data, and of the Kansas and second set of Nebraska populations, are necessary before we can draw firm conclusions.

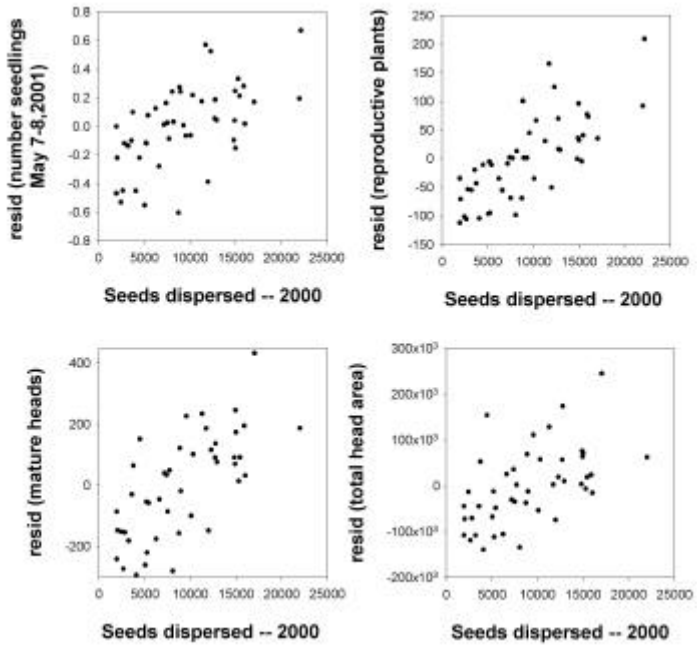

Fig. 5: The effect of the number of seeds dispersed in each of the 48 experimental populations in Nebraska in 2000 on seedlings, plants surviving to reproductive age, number of inflorescences with seeds, and total head area in 2001. Because tilling direction (see text) had a significant effect on these responses, data plotted are residual values after tilling direction was removed by ANOVA. Correlations coefficients (from upper left to lower right) are: 0.59, 0.75, 0.69, 0.54; all are significant at $p<0.0001$.

We are also using these experimental populations (and additional manipulations of these populations in later years) to derive parameters for our metapopulation models. Specifically, we are evaluating the effect of seed production on patterns of seed dispersal, the establishment and decay of the seed bank, and the longevity of our experimental populations (both with and without additional disturbance). These empirically derived parameters, measured on the scale of our 2m x 2m populations, will be used in a spatially-explicit metapopulation model. Metapopulation models were originally developed by Levins (1969), and in recent years have been a major focus of ecological inquiry (Hanski and Gilpin 1991, 1997; Tilman and Kareiva 1997). These models are often used in studies of organisms with

very distinct habitat patches (e.g. frogs in ponds, Sjogren Gulve 1994), but Antonovics et al. (1994) have illustrated how empirical metapopulation studies can be conducted with organisms that are patchily distributed but have broad habitat requirements. Our model will be written at the 2m scale, because that is the scale at which we are deriving our parameters, but it can be scaled up from 2m to 80m and beyond, enabling us to make predictions at larger spatial scales.

If the escape of a *Bt*-transgene into wild sunflower populations leads to an increase in the size or number of wild populations, there will likely be additional ecological consequences. For example, to the extent that sunflower increases in abundance, plant species that commonly co-occur with sunflower will probably be affected, and may decrease in relative frequency. These community level effects of the escape of a transgene are currently beyond the scope of our work.

The effect of a *Bt*-transgene on herbivore community structure

Clearly, if a *Bt*-transgene escapes into wild sunflower populations it will have a negative impact on the suite of susceptible native herbivores. Our data indicates that native herbivores will be killed by the *Bt*-protein (Figure 1), and as the frequency of a *Bt*-transgene increases in wild sunflower, the negative impact on these native herbivores will increase as well. In addition to the taxa shown to be affected in the present study, the wild sunflower at our study sites is also attacked by two other native lepidopterans (*Homoeosoma electellum, Eucosma womonana*), and these species are also likely to be negatively affected by a *Bt*-transgene. Moreover, these species are specialists, feeding on only *H. annuus,* or on only *H. annuus* and other species in the genus *Helianthus*, and for this reason they cannot easily find food on other host plants. Although these species may evolve resistance to *Bt*, at least initially the suite of native lepidopterans will be severely impacted by the escape of a lepidopteran-specific transgene into wild populations.

The effect of a lepidopteran-specific *Bt*-transgene on the non-lepidopteran members of the native herbivore community depends on existing interactions between these suites of herbivores. If the lepidopterans have negative competitive effects on any non-lepidopteran species, the release from competition provided by a *Bt*-transgene might allow these non-lepidopterans to increase in abundance. Experimental manipulations of damage by three of the lepidopterans (*Plagiomimicus, Homoeosoma,* and *Cochylis*) indicate that only *Plagiomimicus* competes with other herbivores. Damage by *Neolasioptera* (sunflower seed midge) and *Smicronyx fulvus* (red sunflower seed weevil) is lower in inflorescences with *Plagiomimicus* than in

inflorescences without this species (M. Paulsen and D. Pilson, unpublished data). However, because *Plagiomimicus* abundance is typically low, these interactions are probably only important every 5-10 years when *Plagiomimicus* reaches high abundance during population outbreaks (M. Paulsen, et al. unpublished data).

Summary

We have argued that assessing the ecological risks of the escape of transgenes into wild populations must sequentially address three questions. With respect to the question of cross-compatibility, we have found few barriers to gene flow between crop and wild sunflower, We also provided an unambiguous affirmative answer to the question of whether the transgene is positively selected in the wild. Wild plants containing a *Bt*-toxin gene specific to lepidopterans exhibited decreased lepidopteran herbivory and increased seed production. Thus, we expect that *Bt*-transgenes released in commercial sunflower will escape into and increase in frequency in wild populations (at least in locations and in years in which susceptible herbivores are abundant). Next it is necessary to evaluate the ecological consequences of transgenes in wild populations; however, virtually no work has been done in this area. Preliminary analyses suggest that increased seed production in individual plants (caused by, for example, a *Bt*-transgene) will lead to an increase in the size of wild populations. We are currently doing experimental and modeling work to determine if larger local populations will lead to larger or more persistent metapopulations.

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General concepts

DATA REQUIREMENTS FOR ECOLOGICAL RISK ASSESSMENT OF TRANSGENIC PLANTS

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Abstract

Current data on pre-release testing of non-target organisms for pre-registration purposes of transgenic plants are almost exclusively conducted according to standard ecotoxicological methodologies developed for registration tests of pesticides. For transgenic plants expressing insecticidal proteins from *Bacillus thuringiensis* (hereafter referred to as *Bt*-plants), the underlying additive two-part model that leads to the *Bt*-proteins being tested as an externally applied pesticide is being questioned and the deficiencies with the applied testing protocols are described. In conclusion, the current database regarding non-target effects of transgenic *Bt*-plants is only of limited use for risk assessment. In conclusion, currently applied methodologies leave great room for improvement and deliver data with limited conclusive value and scientific certainty to a risk assessor and regulator. New testing methodologies for transgenic plants must be developed that are designed for testing of a transgenic plant rather than a chemical only. The development of improved testing protocols and methodologies providing data for ecological risk assessment is the aim of a recent initiative by public sector scientists, called the 'GMO-Guidelines Project'. The GMO Guidelines Project was launched by a group of scientists, the Global Working Group on Transgenic Organisms in IPM and Biocontrol, under the patronage of the International Organization for Biological Control (IOBC). The Working Group brings together scientific experts on the environmental risks of transgenic organisms, and is open to all public sector scientists. The project aims to develop international, scientifically sound, conclusive and acceptable guidelines for assessing the environmental risks posed by GMOs.

Keywords: Ecotoxicology, testing guidelines, risk assessment, *Bacillus thuringiensis*, pesticide paradigm

Introduction

With the advent of applied gene technology, molecular biologists have been able to move gene constructs into and around the plant genome, introducing novel genes from unrelated species that are coupled with novel

regulatory sequences. This enables the plant, (or any other transgenic organism), to express novel compounds, including insecticidal substances that kill certain organisms that feed on these transgenic plants. For example, *Bt*-crop plants (corn, cotton, potato, and others) express a bacterial gene that produces an insecticidal protein. In the bacteria, these insecticidal proteins are produced in an inactive, crystalline form. Depending on the *Bt*-strain, different insectidical crystalline proteins are produced that exhibit different, typical shapes. This was used as a basis for a nomenclature wherein these proteins were called Cry-toxins (Crickmore et al. 1998). Aside of the promise to quickly achieve high levels of toxicity against pests and expression of transgene products, today's more educated and environmentally aware public demands a rigorous evaluation of their ecological risks from the beginning of the development of the technology.

Current transgenic plants synthesize their gene products over a long period of time, often over their entire lifetime. Should they escape and establish feral populations, or should the transgenes themselves move to other organisms, gene products could be expressed over many generations or indefinitely replicate and increase. This suggests that the ecological risks of transgenic organisms are similar to the ecological risks of a living, biological organism.

In this paper, firstly, the current risk assessment models and testing methodologies for evaluating environmental risks to non-target organisms from transgenic crop plants are examined. Secondly, the approach of an international initiative of public sector scientists will be presented that aims to develop improved testing methodologies based on ecological and scientifically sound principles.

Current Testing of Non-Target Risks Associated with Transgenic Plants - The Chemical Testing Paradigm

Chemical Testing Schemes

Increasing public concern about the continuing environmental degradation due to accidental or intentional releases of chemicals, including pesticides, over the last fifty years has played a large role in the enactment of legislation and policies for the pre-market regulation of chemicals. In 1977 and 1978 the Organization for Economic Cooperation and Development (OECD) launched a chemicals testing program (Forbes & Forbes 1994, Kitano 1992). One primary motivation behind the standardization and coordination of chemical testing was the desire to avoid trade barriers through harmonization of methodologies for assessing the potential hazard of chemicals. The objectives were: 1) to protect people and their environment from hazardous chemical substances; 2) to avoid trade barriers; 3) to reduce member countries' economical and administrative burden needed for the regulation of chemical substances; 4) to strengthen international information exchange for the regulation of chemical substances (Kitano 1992, Lee 1985

in Forbes & Forbes 1994). In 1981, the members of the OECD Chemicals Group formalized and published a set of 51 guidelines for testing of chemicals (OECD 1981). The number of guidelines had increased to 77 by 1991 and they continue to be reviewed and updated (Kitano 1992). They have been implemented with variations in the chemical and pesticide legislation of many countries throughout the world (Fent 1998).

Historically, ecotoxicological testing methodologies were first developed for aquatic systems when first environmental pollution problems occurred in water bodies, like DDT in rivers and lakes, surface water runoff contaminated with pesticides and fertilizers, or pesticides and their metabolites leaching into groundwater aquifers (Steinberg et al. 1995). Therefore, the primary route of exposure is thought of as being aqueous, and standard toxicity testing is routinely based on concentrations (e.g. lethal concentrations or doses LC/LD50 determination) in the external medium (e.g. water, soil, sediment, air) (Chapman 2002). The standard methodology for pre-registrational characterization of potential risks of chemicals, including pesticides, to non-target organisms is the tiered, hierarchical toxicology model using single indicator species (Forbes & Forbes 1994 (chap.2), Rand 1991). Hierarchical ecotoxicological testing schemes commence with simple, inexpensive range-finding tests using single species and proceed through a stepped sequence of tests which increase in complexity, sophistication, cost and often duration (Cairns 1981). An initial stage testing consists of acute screening tests on a few standard species. If the identified risk at a given stage is determined to be acceptable or if no effects are observed, no further tests are required. The goal of such hierarchical testing schemes is to limit the time and expense of the most thorough testing to those substances of greatest potential danger to the experimental exposure to single chemicals (Forbes & Forbes 1994; chap. 2). The result is the classification of their toxicological attributes, and recommendations for measures to be taken for their safe handling and use (safety equipment, waiting times before consumption, etc.).

Test species are chosen for their function as indicators, and also because they are widely available, easily cultured and genetically stable and uniform. A minimum first tier testing scheme for acute toxicity of chemicals would typically include three aquatic organisms from three different trophic levels, namely algae, daphnia (*Daphnia magna*) and fish (e.g. rainbow trout) (Table 1, OECD guidelines 201-203). When combining the information from these three tests, it is believed that “we can evaluate the effect of chemicals on aquatic environment, that is fish gives us the effect information on mortality and behavior, daphnia on reproduction and algae on growth” (Kitano 1992).

From the onset, this methodology was an imperfect compromise for characterizing non-target effects of chemicals and pesticides (Baird et al. 1996, Chapman 2002, Forbes and Forbes 1994). The above described acute toxicity tests can reveal a close relationship between intoxication and an observed reaction. But the data is of little use for revealing ecological

impacts (Chapman 2002, Kareiva 1996). For such a hierarchical scheme to be sufficiently protective, the criteria for proceeding or not proceeding from one step to the next must be scientifically sound. Because a negative result at stage 1 means no further testing is required, this is the level at which mistakes in assessments can have the most serious consequences (Forbes & Forbes 1994). Therefore, Cairns (1981) advocated the use of simultaneous, instead of hierarchical, testing because, in general, information derived from toxicity tests in earlier steps is not demonstrably correlated with responses obtained in the latter steps. He argued that the ability to predict sub-lethal effects, as well as effects at more complex levels of organization, from single-species tests has not been compellingly demonstrated.

Table 1: Common, standardized 1st tier chemical ecotoxicology test performed for pre-market regulatory purposes. (Fent 1998).

Organism	Type of test	Duration	OECD Guideline No.
Algae (*Chlorella vulgaris*, *Scenedesmus subspicatus*, *Ankistrodesmus bibraianus*)	Growth inhibition test	4 days	201
Waterfleas* (*Daphnia* spp.)	Acute immobilization Acute Toxicity	24 – 96 hours	202
Fish spp. (e.g. Rainbow trout)	Acute toxicity	24 – 96hrs.	203
Fish spp.*	Early life-stage toxicity	4 – 12 weeks	210
Compost worm* (*Eisenia foetida*)	Acute toxicity	7 – 14 days	207
Bobwhite quail* or mallard duck	Acute (dietary) Acute (gavage)	5 days treatment followed by 3 untreated observation 14 – 21 days	205
Honey bees*	Acute (oral) Acute (contact)	4 – 24 hrs	New (1998) 213 214

* common premarketing set of data for transgenic plants

Additionally, species selection relies on an indicator species approach that provides good data on toxicological response or bioaccumulation of heavy metals of a particular sensitive species. However, indicator species provide weak to no ecological information on important population, community and ecosystem responses (Cairns and Mount 1990). For example, collembola (springtails), and earthworm species play key roles in soil functioning and are vital indicators for soil ecotoxicology. Collembola species feed on fungal

mycelia growing on decaying plant material and are known to concentrate both metals (zinc, copper, cadmium) and organic pollutants (Cortet et al. 1999, Hunter et al. 1987). The immediate effects of pollutants are on individual organisms, by either direct toxicity or alteration of the environment, but the ecological significance or lack of it arises from the impact on species populations. The fact that a pollutant kills, say, half the individuals in a species population may be of little or no ecological significance, whereas a pollutant that kills no organisms, but delays development or decreases reproduction, may have a considerable ecological impact. Such effects can affect interaction between individuals of different species, eventually leading to changes in the structure of the community or disruption of coupled processes. *Folsomia candida*, a parthenogenetic species, is the most frequently used microarthropod used as toxicological bioindicator of pesticides and other environmental pollutants (Cortet et al. 1999 and references therein). However, most publications on this species indicate large differences between test results depending on strain, individual age and soil moisture content. Therefore, this species does not provide a consistent picture of any soil environment (Cortet et al. 1999). Earthworms can accumulate substantial amounts of heavy metals like lead, copper and cadmium. The earthworm species primarily used as toxicological bioindicator is *Eisenia foetida* (Cortet et al. 1999 and references therein). However, this species, the 'compost worm', likes high organic matter contents and rarely occurs in significant numbers in regular agricultural fields because organic matter levels are too low (Edwards and Lofty 1972, Topp 1981).

Application of the Chemical Testing Paradigm to Transgenic Plants

A transgenic plant is not a chemical

The current methodologies for non-target testing of transgenic plants for regulatory purposes draw heavily on the standardized protocols for chemical testing described above. Probably the most critical procedure is to divide the transgenic plant conceptually into (1) the unmodified crop and (2) the transgene and its product. This 'two-part model' allows the analyst to separately analyze the effect of the transgene product as a chemical isolated from its role in plant growth, development and metabolism (NRC 2002). To use the chemical testing paradigm, it is essential that experimental protocols isolate the chemical so that its effects can be evaluated. The two-part model attempts to accomplish this by comparing the transgenic plant with the unmodified crop and postulating substantial equivalence.

A key drawback is that the test methodology is not very relevant to transgenic plants simply because they are not chemicals. Firstly, based on the typical mode of action of synthetic pesticides, acute toxicity testing protocols provide a single large chemical dose at different concentrations combined

with short-term exposure. In contrast, transgenic plants release a continuous dose, sometimes high and sometimes low, calling for chronic rather than acute toxicity testing. Secondly, purified, microbially produced transgene products are used, or occasionally some highly processed plant-protein, and administered in an external media according to the common methodologies developed by the OECD and described above (Table 1). However, these products are rarely identical with what is produced in the plant. In Table 2, the currently available information on plant-expressed *Bt*-proteins in some common transgenic maize and cotton varieties is compiled. For lepidopteran active transgenic Cry1-maize events, transgene products are reported that range from 65 to 91 kDa sized fragments. The observation that sometimes more than one type of -toxin fragment is expressed is thought to be an indication that in-plant processing is occurring, further metabolizing the *Bt*-protein into variable sized fragments (Event 176: 36, 40 and 60 kDa fragments, TC1507 maize: 65 and 68 kDa fragments, Cry9C 55 and 68 kDa fragments; Table 2).

Mortality and other acute toxicity endpoints, while clear and measurable, cannot be used to extrapolate to effects across the lifetime of the organism or to cross-generational effects. Moreover, by relying on the typical standard indicator/toxicology testing species (Table 1), little can be inferred about effects on actual ecologically functioning populations, communities or ecosystems (Forbes and Forbes 1994). An ecological approach involving the testing of the well-characterized transgenic plant would be more appropriate for GMO non-target risk assessment.

Routes of exposure unrealistic

Bt-proteins in transgenic plants are an integral component of the plant. If the risks to transgenic plants are to be assessed, the plant must be involved in the testing. Ecotoxicity trials administer the test compound in a bi-trophic manner, e.g. the microbially produced, purified *Bt*-toxin is fed directly to a natural enemy while in nature they would be exposed to the protein primarily via their prey (Fig. 1). In some cases, the food used in current pre-release ecotoxicity trials for transgenic plants is highly unlikely to deliver the protein where it unfolds its effect, i.e. the gut, or to the most sensitive life stage, i.e. the larva (see EcoStrat Report 2000 for detailed discussion).

For example, *Bt*-coated mealmoth eggs are provided as test food to *Chrysoperla carnea* larvae (Sims 1995, EcoStrat Report 2000). However, these piercing-sucking insect drain the eggs of its fluid contents but cannot ingest the shells of the eggs outside of which the *Bt*-protein is located. Another example involves coccinellid testing species. Mostly adults are tested for example using a *Bt*-containing honey solution (Sims 1995). Firstly, adults are the least sensitive life stage for *Bt*-proteins. Secondly, many coccinellids are pretty strict aphid feeders which in turn are obligatory phloem feeders. In phloem however, no *Bt*-proteins could be detected to date

(Raps et al. 2001, Bernal et al. 2002). In summary, while *C. carnea* as polyphagous feeder is likely to be exposed to *Bt*-containing prey in the field, the test methodology does not provide the *Bt*-protein in a manner where the larvae actually ingest it. Information from such experiments can yield data that could lead to both a serious underestimation or overestimation of an effect. In any case, it provides data with insufficient accuracy and conclusive value to a regulator, which is undesirable. It is crucial that the plant and herbivore is presented to the non-target species in a way that mimics how the species would experience the plant or natural enemy in the field.

Experimental duration insufficient

The mode of action of *Bt*-toxins result in death of the susceptible target insect after 2-3 days. However, death often occurs later if the target insects feed on tissues expressing lower *Bt*-toxin concentrations (due to variable expression in transgenic plants), eat only small quantities or are simply a bit more robust than others. Bioassays with target insects for determination of *Bt*-toxin activity in transgenic plant tissue run typically for 4 or 5 days before final mortality values are determined. Therefore, any test routine for ecotoxicity effects on non-target organisms should at least allow for that amount of time, too. This is often not the case at present. Very few test routines exceeded 5 days with most being less than that and some lasting only hours (Table 2). For instance, water fleas (*Daphnia magna*) are often only tested for 48 hrs with static renewal of pollen, certain honeybee tests expose the organism to the *Bt*-protein for a few hours, and bobwhite quails are routinely fed with *Bt*-food for 5 days only with an additional 3 days of non-transgenic food (see Table 2, and EcoStrat Report 2000). Any such short-term testing routine would observe an acute effect only if the non-target organism were as susceptible as or more susceptible than the target insect itself. Any organism that is only slightly less susceptible than a target pest would escape detection. Considering the prolonged presence of *Bt*-proteins in the field during a crop production period, plus the long degradation of transgenic plant residues after harvest, and also considering the possible repeated and widespread *Bt*-crop production in some countries, using only short-term testing periods is insufficient for ecological risk assessment.

The Ecological Risk Assessment Approach

While it is acknowledged that information on toxicological effects of the transgene product expressed in transgenic plants on non-target organisms can be valuable for risk assessment, conclusions on ecological risks and benefits can only be made if ecologically relevant data is obtained. Testing protocols based on the chemical testing paradigm typically do not provide this information.

An improved testing procedure for risks assessment of transgenic plants must provide solutions to three essential problems: 1) species selection; a

number of relevant testing species must be selected from a wide range of species using clear, scientifically justifiable selection criteria, 2) end point identification; a clear, measurable endpoint informing about fitness effects on nontarget organisms must be identified that is relevant to the characteristics of a transgenic plant, and 3) testing methodology; the method must provide results that are relevant for application in the field and/or inform the decision-making process in order to trigger additional tests that would apply to the given field conditions or surveillance programs. Fundamental to the testing scheme is a good and appropriate characterization of the transgenic plant that provides information relevant for subsequent exposure analysis and species selection. We provide here only a bare outline of an ecological risk assessment approach.

Species selection

Criteria based on transparent, scientific principles must be developed to guide the selection process of test species. One approach is to select the species according to their ecological role and service function in the agro-ecosystem. This helps to avoid inappropriate conclusions associated with the indicator species approach, focussing the testing on critical processes and limiting the number of species that need to be tested. Some possible functional categories are: a) secondary pests, b) natural enemies, c) pollinators, d) decomposers, e) insects of cultural or economical significance.

Exposure Analysis

To further narrow the species list for testing, the potential exposure of the non-target species should be determined. This requires a good description of the transgenic plant. Those species with the highest potential exposure or the greatest expected sensitivity to the transgene product would remain candidates for testing. Non-target species can be affected by the novel compounds in GM plants via multiple pathways (Figure 1). Transgenic plant material and transgene products can affect non-target species directly via plant residue (above- or below-ground), senescent leaves, root exudates, seeds, pollen, floral and extra-floral nectaries, guttation fluids, and phloem sap. Any non-target organism feeding on the transgenic plant or parts of the plant can potentially come into contact with the transgene and its product. In addition, the transgene product might interact with existing plant compounds to affect non-target organisms. Transgenic plant material and transgene products can affect non-target species through another organism, such as a herbivore or honeydew from homoptera (aphids, scales, whiteflies). Non-target species could therefore be affected by a) unprocessed transgene products in the plant, plant secretions, herbivore, or herbivore excretions, b) metabolites of the transgene products, or c) interactions with other plant or herbivore compounds that alter plant or herbivore physiology (Figure 1). Indirect effects via altered plant physiology (including interaction effects with natural secondary compounds) are not detailed in Figure 1, but are

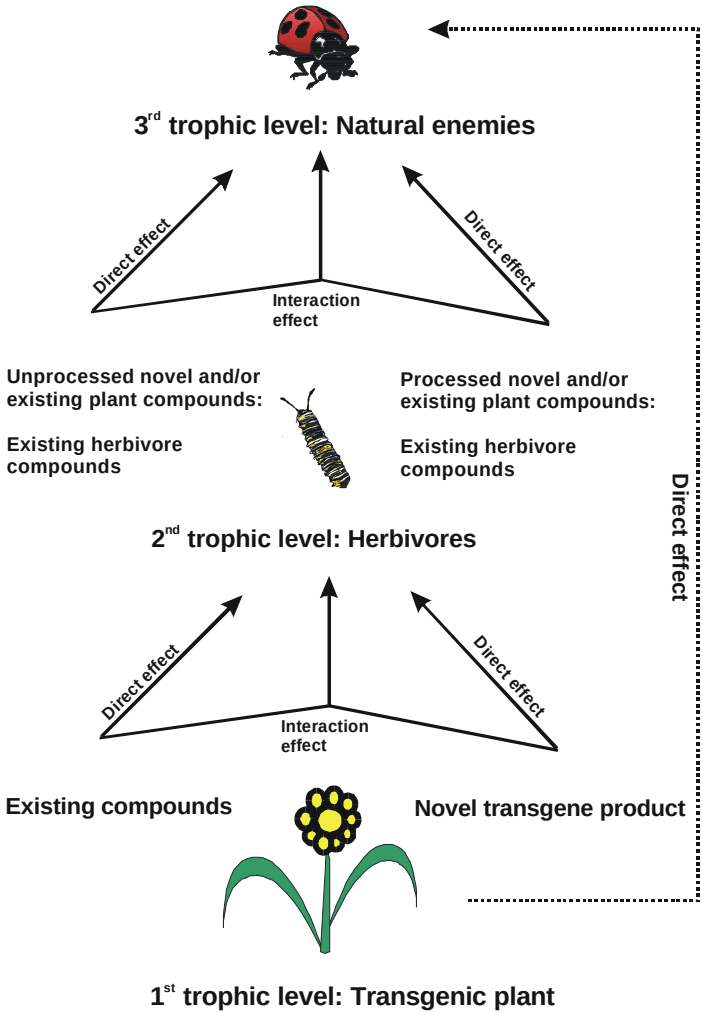

Fig. 1: Schematic food chain involving the transgenic plant (primary producer), herbivore (primary consumer) and natural enemy (secondary consumer).

subsumed within the effects of the whole plant or the herbivore in the diagram. The possible effects can be quite complex, making them difficult to measure. Using the whole transgenic plant allows testing the entire set of possible effects most efficiently.

For soil organisms there are three components to the exposure analysis, including exposure to: 1) 'free' *Bt*-proteins leaching into the soil from degrading plant material or any other plant part or plant secretion listed above. 2) 'bound' *Bt*-proteins adsorbed to surface-active particles, that can

maintain their insecticidal activity. 3) *Bt*-proteins 'in' degrading plant residue fed upon by soil organisms. Most research in the past has addressed components 1) and 2) with highly variable results. While some studies suggest a rather quick degradation of 'free' *Bt*-proteins in soils (Sims and Holden 1996) other data demonstrate that *Bt*-protein can last much longer in the soil than expected, probably because they are bound to surface-active particles like clay minerals or humic acids (Crecchio and Stotzky 1998, Tapp and Stotzky 1998, Koskella and Stotzky 1997). No consensus has emerged yet, and the question of potential accumulation of *Bt*-proteins in certain soil types with high surface-active particle content remains unanswered to date. Few studies so far have addressed component 3), and even less so in the field. Zwahlen et al. (2003) showed that *Bt*-protein in transgenic *Bt*-corn plant material declined in a bi-phasic mode, acting as a time-release mechanism for the *Bt*-protein. As long as degrading plant material was present, the *Bt*-protein could be detected throughout eight months over winter in the field of a temperate region (Switzerland). While 'free' *Bt*-protein might degrade quickly at constant high temperatures, this must be expected to be slower when sub-zero temperatures are reached. Certainly, degradation of the plant material is much slower, and release of the *Bt*-protein is therefore low over the winter months and picks up again as temperatures rise in the spring (Zwahlen 2003). However, decaying transgenic corn plant material containing measurable levels of *Bt*-protein was still present in June when the new corn crop is sown (Zwahlen 2003). Hence, any soil organism feeding on decaying plant material will be continuously exposed to the *Bt*-protein in its food, as well as from the soil. This calls for chronic long-term studies with selected non-target testing species.

Experimental endpoint and duration.

An appropriate experimental endpoint with ecological relevance is generational relative fitness or some relative fitness component. This is the relative survival and lifetime reproduction of the non-target species over an entire generation. Relative fitness can be difficult to measure because the non-target organism must be maintained under the experimental treatment over its entire lifetime. Hence, survival experiments should last at least through all of the immature stages of the non-target species, and, in addition, adult life stage parameters such as age-specific mortality and female fecundity and fertility should be recorded. In principle, the duration of the test should correspond both to the time the non-target species would be exposed to the GM plant and to the temporal expression of the transgene product in the plant. For example, if the transgene product is expressed constitutively throughout the entire growing season, such as occurs for all current *Bt*-crop plants, the non-target species should be exposed at least one entire generation. To obtain useful relative fitness estimates, true replications of the experiment should be carried out. The entire experiment should be repeated with new plants and insects over time. Replication and sample size

should be sufficient to detect differences between treatments of 25-30%. A statistical equivalence test provides good estimates for that and is recommended (Hoenig and Heisley 2001). In the analysis of several regulatory studies, Marvier (2002) found that no study allowed for a 90% chance to detect a 20% difference between treatments. Only 1 of 5 industry studies included a suficient number of replicates to have a 90% chance of detecting a 50% reduction in survival.

The GMO-Guidelines Project

In conclusion from the above, currently applied methodologies leave great room for improvement and deliver data with limited conclusive value and scientific certainty to a risk assessor and regulator. New testing methodologies for transgenic plants must be developed that produce conclusive data for pre-release environmental impact assessment in regulation. The development of improved testing protocols and methodologies providing data for ecological risk assessment is the aim of a recent initiative by public sector scientists, called the 'GMO-Guidelines Project'.

The GMO Guidelines Project was launched by a group of scientists, the Global Working Group on Transgenic Organisms in IPM and Biocontrol, under the patronage of the International Organization for Biological Control (IOBC). The Working Group brings together scientific experts on the environmental risks of transgenic organisms, and is open to all public sector scientists. The project is coordinated by a Steering Committee, which includes A. Hilbeck, D. Andow, N. Birch, B. B. Bong, D. M. F. Capalbo, G. P. Fitt, E. M. G. Fontes, K.L. Heong, J. Johnston, K. Nelson, E. O. Osir, A. Snow, J. Songa, and F.-H. Wan. The project aims to develop international, scientifically sound, conclusive and acceptable guidelines for assessing the environmental risks posed by GMOs. The work on the guidelines has been divided into five scientific sections:

Needs assessment / good agricultural practice

This section provides a framework for evaluating the need for the transgenic plant in specific crop production contexts. This includes providing an approach to evaluating projected changes in crop production practices, such as tillage systems or insecticide use. It will incorporate a precautionary approach to the issues as specified by the Biosafety Protocol and EU legislation.

Characterization of transgene construct and phenotype in the plant

This section specifies how a transgene should be described to enable evaluation of its stability and inheritance, and how the phenotypic effects of the transgene in the plant should be described; what, how, what plant parts,

and when product concentrations should be measured in transgenic plants to facilitate assessment and management of environmental effects.

Non-target and biodiversity impacts

This section specifies selection procedures to determine the non-target species or function/process that should be tested, and testing procedures for testing these species/functions/processes for the following categories of organisms; natural enemies, pollinators, soil organisms, species of conservation concern, species of cultural significance, non-target pests, and other non-target species. Routes of exposure need to be identified. The organisms that are exposed need to be determined through suspected causal chains of impact. Based on this information, protocols and methodologies for appropriate testing can then be developed. This section provides additional detail on the ecological risk assessment approach outlined above.

Gene flow and its effects

Gene flow is the route along which transgenes can spread genetically into populations of related species and geographically into other regions including protected areas of sensitive ecological value. Gene flow is considered a risk because of the great uncertainties associated with the possible consequences in the recipient ecosystems. Successful transgene flow will simultaneously affect both recipient plants and their associated organisms.

Pest resistance management

This section specifies procedures to determine the resistance risk of transgenic crops, and feasible management responses needed to reduce this risk. It will also consider approaches for developing a practical monitoring and response system to detect resistance and to adapt management appropriately.

The project includes a strong emphasis on scientific exchange and capacity building between developed and developing countries faced with the risk assessment of GMOs, in particular Kenya, Brazil and Vietnam. Kenya provided the first case-study to focus the development of the GMO Guidelines in a workshop in Nairobi this year. The draft guidelines will be published after each workshop and at the end of the project in 2004. Public sector scientists are invited to contribute to the guidelines by registering with the project on the website www.gmo-guidelines.info and specifying interest in one or more of the scientific sections, or by writing to the project secretariat at underwood@geobot.umnw.ethz.ch. Other interested people can subscribe to the mailing list of the project on the website, and will then be regularly informed of progress in the project.

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Table 2: Comparison of *Bt*-protein expression levels in various transgenic *Bt*-plants

Event-Crop (Company)	**Inserted gene**	**Size of the protein expressed in plant**	**Tissue-specific concentrations (? g/g fresh weight unless otherwise specified)**	**Reference**
Bt-Maize 176 (Syngenta)	Cry1Ab (synthetic) PEPC	65 kDa (Additionally, 3 immunoreactive proteins weighing ca. **60, 40 and 36 kDa** were detected in leaves but not pollen. It was suggested that these may represent breakdown products resulting from intrinsic proteolysis within the leaf tissue. – Source: Internet*)	Leaves (seedling): 0.6-1.16 Leaves (anthesis): 0.53-3.03 Leaves (maturity): 0.44-0.47 Leaves (senescence): 0.07-0.23 Pollen: 1.14-2.35 Roots: 0.008 Pith: < 0.008 Kernels: <0.005 Leaves (anthesis): 2.13-3.27 Pollen: ca. 2.4 (mean)	Internet* Fearing et al. 1997
Bt-11-Maize (Syngenta)	Cry1Ab (truncated, modified) CaMV35S (modulated by IVS6 intron)	N. i. Probably close to activated toxin (see below) 'Data show that the truncated Cry1Ab toxin could be extracted from corn leaf tissue and this purified material displays characteristics and activities similar to that produced in *E. coli* transformed to produce Cry1Ab. The purified tryptic core proteins from both plant and microbe were shown to be similar in molecular weight by SDS-Page, …' 65 kDa	Leaves (young and mature): 15-29 Kernels: 3.7-4.76 Pollen: no expression detected Others: n.i. Leaves: 1.55-10.53	Internet* Raps et al. 2001 Bt-Plant-Incorporated Protectants October 15, 2001 Biopesticides Registration Action Document (http://www.epa.gov/pesticides/biopesticides/othersdocs/bt_br_ad2/2%20id_health.pdf) Australian New Zealand Food Authority 2001

continues…

Mon810-Maize (Monsanto	Cry1Ab (truncated) CaMV35S (enhanced; modulated by HSP70 intron)	91 kDa	Leaves: 9.35 (mean across 6 locations) Leaves (range of samples from 1 location): 7.93-10.34 Seeds: 0.31 Seeds (range of samples from 1 location): 0.19-0.39 Pollen: 0.09	Internet*
DBT418-Maize (Dekalb)	Cry1Ac CaMV35S (two copies octopine synthase enhancer and introns)	66 kDa	Leaves: 459.6-1194.4 ng/g dry wgt Kernels: 36-42.8 ng/g dry wgt Roots: 58-125.4 ng/g dry wgt Stalks: 40.9-123.6 ng/g dry wgt Pollen: no expression detected Silk: no expression detected	Internet*
TC1507-Maize (Mycogen/ Pioneer)	Cry1Fa2 (synthetic, truncated) Ubiquitin ZM1(2) (plus intron)	65 kDa 65 and 68 kDa (… two bands were detected in leaf, pollen, whole plant and grain tissue. Protein 'doublets' typically occur during gel electrophoresis if terminal amino acid residues have been removed from the protein as a result (continue…)	Leaves: 110.9 ng/mg total protein Pollen: 0.32 ng/mg total protein Kernels: 89.8 ng/mg total protein Leaves (V9): 56.6-148.9 pg/?g total extractable protein Pollen: 113.4-168.2 pg/?g total extractable protein Stalk: 355.9-737.4 pg/?g total extractable protein Kernels: 71.2-114.8 pg/?g total	Internet* Company registration documents

		of the activity of proteases released during processing of the plant tissue for analysis. … Hence, it appears that the doublet resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.')	extractable protein	
CBH-351-Maize (Aventis)	Cry9c (truncated, N-, C-terminal) CaMV35S	No clear precise size info provided: 'the CBH-351 expressed Cry9C protein was similar to the trypsin-resistant core protein…' Further explanations about C- and N-terminal cleaving suggest that the activated toxin is expressed. 'Replacement of arginine by lysine at position 123 in the plant encoded protein which reduces the susceptibility of the protein to trypsin cleavage to a **non-toxic 55 kDa fragment**.' 68 kDa 'MRID NO indicates that the protein can be partially degraded to a **55 kDa** form…'	No data on internet available	Internet* Bucchini and Goldburg 2000
Mon863-Maize (Monsanto)	Cry3Bb (addition of alanine residue at position 2 of protein) CaMV35S with 4 repeats of an	n.i. 74 kDa (active form)	10-81?g/g fresh wgt. depending on tissue Leaves (young): 65-**93** Leaves (forage): 24-45	Internet* Company registration information

	activating sequence and rice actin intron		Root: 25-56 Pollen: 30-**93** Kernels: 49-86	
Mon531-Cotton (Monsanto)	Cry1Ac (truncated) CaMV35S (with a duplicated enh ncer region)	N. i.	Line 531: 1.56 and0.86 in leaves and seeds, respectively. Expression varied about 3-fold Leaves: 2.7-3.9 (damaged and undamaged) Leaves during bloom: 2.1-2.2 (damaged and undamaged) Boll: 1.9 – 2.3 (damaged and undamaged) Pollen: 0.6 Other publications available quantifying the Bt-toxin concentrations in various tissues and plant stages	Internet* Greenplate 1997 Adamczyk and Sumerford 2001 Greenplate 1999

* website: http://www.essentialbiosafety.com; n.i. = no information found/provided

CONCLUDING REMARKS ON THE ECOLOGICAL IMPACT OF GMO DISSEMINATION IN AGRO-ECOSYSTEMS

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Considering the international tension regarding genetically modified organisms (GMOs), it was particularly appropriate to gather and discuss the "Ecological impact of GMO dissemination in agro-ecosystems" in the remarkably tranquil environment, surrounded by fields and forests, of the small Austrian village, Grossrussbach. Instead of running the risk of being redundant by providing a summary of what was presented at the workshop – and which is presented in detail in the preceding papers- we thought it more useful to comment on a few more general points, and to attempt to go beyond the work described in individual papers.

We were struck, for instance, by the numerous instances in which GMO biosafety research plays an important role in impelling research on biodiversity and ecosystem structure. This is evident for instance in studies on microbial ecosystems. GM microbes were the first GMOs released into the environment, and attempting to assess not only their efficacy in the field as compared to controlled laboratory conditions, but also their impact on microbial communities, has reinforced our appreciation of the complexity of microbial ecosystems. In a similar fashion, studies on the impact of gene flow from crops to wild relatives have taken on a new importance as an essential part of assessment of the impact of the introduction of genetically modified crops. It is also quite probable that the need to assess the ecological impact of crops expressing a *Bt*-transgene has provided the impetus for the detailed studies of insect fauna in agro-ecosystems that have been carried out recently. Obviously, these areas of research did not start de novo in response to the need for GMO biosafety research. It is clear that in many cases the evaluation of the ecological impact of a transgene has been based on studies on the impact of naturally occurring genes conferring a similar phenotype. For instance, studies on GM herbicide-tolerant crops have clearly been built – appropriately!- on the general principles generated from studies on non-GM herbicide-tolerant plants, and the features controlling the appearance of herbicide-tolerant weeds.

In the popular imagination, previous work on herbicide-tolerant weeds, coupled with the development of herbicide-tolerant crops, has led to the perception that the latter could lead to the creation of "superweeds", i.e. more invasive herbicide-tolerant ones. This has been an area of intense study, but it is important to note that herbicide-tolerant weeds will only have a selective advantage if the herbicide is used. More recently, attention has begun to be focussed for the first time on traits that could confer an advantage through resistance to biological selective pressures, such as those exerted by pests or

pathogens. Although this could be –and was- predicted from experiments carried out under controlled conditions, it is of great interest to see that indeed GM traits conferring either insect-tolerance or virus-resistance could confer a considerable selective advantage to wild relatives that are sexually compatible with GM crops expressing such traits. What this may imply in terms of changes in weediness of the wild relatives needs to be examined carefully. This is an area that should see rapid expansion, since it will also be essential to address equivalent questions regarding the potential impact of the salt- or drought-tolerance traits currently being developed, and other complex biological characteristics currently being modified.

It is reassuring to note that in many areas GMO biosafety research has indeed led to better understanding of the potential impacts on the environment. An excellent example here is the remarkable convergence of the field studies of the impact of *Bt*-crops on insect communities. To date, the studies show only minor effects, which are in all probability much slighter than the use of insecticides as the alternative for insect control. Understanding the mechanisms underlying potential risks can also make it possible to reduce or eliminate the source of risk. This has been the case for instance with potential risks associated with heterologous encapsidation in plants expressing virus-resistance due to a viral coat protein gene.

The wide-scale commercial use of *Bt*-crops has also played an important role in what may come to be regarded as the first step in a major change in the management of agriculture. One of the concerns with *Bt*-crops is the probability of selection for *Bt*-resistance in the target insect(s). *Bt* resistance has been observed in areas where bacterial *Bt* has been overused, and thus the potential for this to occur when *Bt*-transgenic crops are deployed is obvious. What seems to be an additional positive consequence is that in all the developed countries where *Bt*-crops have been used commercially, this has been associated with rather complex plans, based on refuges of non-*Bt*-crop, for preventing or delaying the appearance of resistant insects. This is probably one of the first case where government agencies, seed producers, and farmers have cooperated to implement a management scheme of this scale and complexity. This may be just the first step in more general implementation of higher level agricultural management. For instance, current practice is for farmers to manage freely their crop rotations. But, there is now evidence that rotations that include several types of herbicide-tolerant rapeseed could create new problems, associated with regrowth of individuals with unexpected tolerances, outcrossing and seed mixture. Will the use of a multitude of herbicide-tolerance genes also require management at a regional scale? Another emerging question concerns deployment of *Bt*-crops in developing countries. Cotton is an important cash crop in many developing countries, and these countries would benefit enormously from the reduced use of insecticides that replacement of standard cotton varieties with *Bt*-cotton would allow. However, in many developing countries, such as China or India, where cotton is grown on small farms along with other crops,

resistance management based on fixed refugia of non-resistant cotton hardly seems feasible. Development of insect resistance management schemes that are both effective and appropriate for developing countries, so that they too can benefit from *Bt*-crops, is a major task for the coming years.

We would like to point out that those concerned by GMO biosafety research are faced by a recurrent question of strategy, or perhaps of philosophy. One way of visualizing it is to note that we can attempt to make a catalog as complete as possible of all the possible effects that use of a novel GMO could have, while recognizing that this will always be limited by the current state of our knowledge. However, this approach raises the question of strategy/philosophy: for each particular case, among the myriad questions that can be asked, which ones need to be examined, and with what level of detail? It hardly seems necessary to point out that this is the key point of decades of disagreement, particularly between the US and Europe.

Nonetheless, we feel that there is good reason to be optimistic about the future. Although GMO biosafety research is a field that has been chronically under-funded, the small research community involved has been able to provide answers to quite a few remarkably complex and difficult questions. Also, as large-scale deployment of certain GMOs continues, this will finally make it possible to have a clearer idea of the reality of their benefits. Although the ancestral dogma has been that evaluation of GMOs will be entirely based on science-based risk assessment, this view turns out to be rather utopian. This simple model for the decision-making process will in all likelihood be replaced by a more complex one, in which residual impacts are balanced against benefits (not only financial ones). The process will require more than just evaluation of potential impacts associated with a GMO, since it will be based on a comparison of the risks and benefits associated with the different possible decisions, including implementation or not of unconfined use of a GMO in the open environment.

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