To be or not to be biosafe

- An evaluation of transgenic phosphinothricin-tolerant oilseed rape (*Brassica napus* L.) -

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STELLINGEN

1.   Uitkruising van transgenen vanuit een kruisbevruchtend gewas, zoals koolzaad, kan niet uitgesloten worden.
     Dit proefschrift

2.   Integratie van een transgen op het C-genoom van koolzaad vermindert de uitkruising naar wilde verwanten, zoals *B. rapa* en *B. juncea*, aanzienlijk.
     Dit proefschrift

3.   Chloroplasttransformatie is dé manier om uitkruising van transgenen zoveel mogelijk te beperken.
     Dit proefschrift

4.   De zogenaamde 'transgene-centered approach', waarbij gegevens van het transgen, het pro-duct, substraten en degradatie producten verzameld worden, kan ertoe bijdragen dat de discussie tussen verschillende groeperingen over de biologische veiligheid van een bepaald transgen helderder wordt.
     Dit proefschrift

5.   De ontwikkeling van gewassen die tolerant zijn voor herbicides, zoals chlorsulfuron en bromoxynil, die door hun persistentie of toxische werking een nadelig effect hebben, is ongewenst.
     Dit proefschrift

6.   Bij het gebruik van genetisch gemodificeerde planten zijn tijdrovende selectieprocedures noodzakelijk voor een stabiele expressie van het (trans)gen in opéénvolgende generaties.
     Dit proefschrift

7.   Een transgen eiwit is niet hetzelfde als een genetisch gemodificeerd eiwit.

8.   De uitspraak van 'the late Rear Admiral Grace Hopper "It is better to ask forgiveness than it is to get permission" gaat niet op voor het in het milieu brengen van genetisch gemodifi-ceerde organismen.

9.   Misdaadbestrijding is vergelijkbaar met resistentieveredeling; de dief en het pathogeen zijn vaak slimmer.

10. Een plantenbiotechnoloog heeft zowel 'witte' als 'groene vingers'.

11. Biotechnologie bij planten maakt tegenwoordig van plantenveredeling vaker een wetenschap dan een kunst.

12. Dat het eerste gekloneerde zoogdier een schaap is, is niet toevallig, immers als er één schaap over de dam is ...

Stellingen behorende bij het proefschrift getiteld "To be or not to be biosafe -an evaluation of transgenic phosphinothricin-tolerant oilseed rape (*Brassica napus* L.)-", door P.L.J. Metz in het openbaar te verdedigen op woensdag 4 juni 1997 te Wageningen.

Contents

Outline

Within the next few years an increasing number of transgenic crops will be commercialized. Transgenic crops will most likely contribute significantly to future agriculture. However, developments in the patenting of genes, discussions on their biosafety, the release regulations, performance, food labelling and consumer attitude will influence the rate of their implementation.

Genetic modification techniques enable the introduction of genes of plants and other organisms into crops. The general attitude of competent authorities in the Western world is, prior to the release of transgenic crops into the environment, to assess their biosafety until enough experience with transgenic crops has been gained. This biosafety assessment is especially needed to answer questions about impact on the ecological system, if transgenes code for new traits like herbicide tolerance and fungal or frost resistance. A limited number of crop plants, among which transgenic oilseed rape (*Brassica napus* L.), is at the forefront of field releases and commercialization of transgenic crops. This was the reason that several years ago oilseed rape was chosen by the European Union as model plant for biosafety studies.

One of the most frequently used traits transferred to crop plants is herbicide tolerance. Phosphinothricin, also known as glufosinate, is a non-persistent, broad-spectrum, non-selective, pre-emergence herbicide having no margin for discrimination between crop and weed. *Bar* and *pat*, phosphinothricin-N-acetyltransferase encoding genes of the actinomycetes *Streptomyces viridochromogenes* and *S. hygroscopicus*, conferring tolerance to phosphinothricin, were successfully introduced using standard transformation techniques into many crops, among which oilseed rape. In addition to its use as agronomic trait other applications of (transgenic) phosphinothricin tolerance in plants are: as selection marker during transformation and as selective trait in combination with a gene causing nuclear male sterility (Mariani *et al.* 1989) to multiply the female line for hybrid seed production.

The scope of this thesis was to gain knowledge about and familiarity with the biosafety of transgenic phosphinothricin-tolerant oilseed rape by:

1) reviewing scientific data concerning oilseed rape, the herbicide phosphinothricin, the phosphinothricin tolerance genes, their protein products and their putative metabolites

2) investigating whether or not the phosphinothricin tolerance transgene could be transferred to intra-specific, inter-specific and inter-generic hybrids of oilseed rape and

3) monitoring the expression and fate of the transgene in these different genetic backgrounds and in successive generations of selfings and backcrosses.

In Chapter 1 general aspects are described, such as taxonomy, cytogenetics, sexual reproduction and aspects of the biosafety of oilseed rape. Additionally, the biosafety of transgenic herbicide-tolerant oilseed rape can be evaluated by reviewing biological, biochemical, ecological and toxicological data with respect to the transgene and its gene product. As an illustration of such a 'transgene-centered approach', the evaluation of the non-persistent phosphinothricin herbicide and the phosphinothricin tolerance transgene is presented in Chapter 2. The transfer to different hybrids and monitoring of the expression and fate of the herbicide tolerance transgene in different genetic backgrounds and successive sexual offsprings is described in Chapters 3, 4 and 5. In Chapter 6 concluding remarks are made and the implications of the results for the market release, seed multiplication and breeding programs involving phosphinothricin-tolerant oilseed rape have been evaluated. Furthermore, the application of transgenic herbicide-tolerant crop plants is discussed in a broader perspective. A distinction is made between tolerance to non-persistent and persistent herbicides and the concepts of 'biosafety in narrow sense' and 'biosafety in the broad sense' are introduced in their biosafety assessment.

Chapter 1

Aspects of the biosafety of transgenic oilseed rape
(*Brassica napus* L.)

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Summary

Oilseed rape (*Brassica napus* L.) is at the forefront in the field release of transgenic crops and their commercialization, which makes it a good model crop for biosafety studies. Reviewing its taxonomy, cytogenetics, reproductive system and possible hybridization with (wild) relatives showed that complete containment of transgenic oilseed rape is not possible. Most inter-specific and inter-generic combinations only produce hybrid plants after *in vitro* culture. However, among the oilseed Brassicas *B. rapa*, *B. juncea* and *B. napus* spontaneous hybridization has been observed in agricultural fields.

Introduction

Genetic modification of crop plants has resulted in plants resistant against pathogens or showing improved quality. Within the coming years it is expected that more transgenic crops will be commercialized and there is little doubt that transgenic plants will significantly contribute to agriculture in the future (Dale & Irwin 1995). Calgene's Laurate oilseed rape has now full clearance by the US-authorities for commercialization (APHIS/USDA 1994). However, developments in the patenting of genes, the release regulations, food labelling and consumer attitude will influence the implementation rate.

Genetically modified or transgenic plants are defined according to Stiekema & van Vloten-Doting (1991) as plants which genome accommodates novel sequences of DNA which are introduced by other procedures than sexual crossing. In spite of the fact that close similarities exist between the phenotypes of transgenic and non-transgenic crops, the application of transgenic plants can not simply be equalled to traditional breeding. Genetic modification allows the circumvention of the natural crossing-barriers between species established by evolutionary processes. This may have unforeseen consequences (Maessen 1997) and, therefore, prior to the release of these transgenic crops, their biosafety has to be assessed (Kapteijns 1993). This includes the assessment of aspects like gene dispersal and introgression of these genes into their wild relatives, via, subsequently, greenhouse experiments, small-scale field experiments followed by large-scale field trials (Van Raamsdonk & Schouten 1997). In this respect the Dutch government follows a "case by case" and "step by step" policy on the biosafety assessment of releases of genetically modified transgenic plants into the environment. As starting point the 'yes, provided that...' principle is handled, which means that it is allowed to produce and grow genetically modified plants, provided that no ecological and toxicological negative side effects occur. The OECD 'familiarity principle' (OECD 1993a) -biotechnology is acceptable if no additional negative aspects are involved compared to conventional methods- and the criterion of 'substantial equivalence' (OECD 1993b) -transgenic food is acceptable as long as it meets already accepted threshold values for toxic components- express the same policy in an international context.

In this review aspects concerning the biosafety of transgenic oilseed rape (*Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk.) will be discussed. From a biosafety point of view

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oilseed rape is interesting as it is a partially allogamous crop with an average outcrossing rate between 15 and 45% (Rakow & Woods 1987; Becker *et al.* 1992). Furthermore, transgenic oilseed rape will be at the forefront in the field release of transgenic crops and their commercialization (Dale 1993; Dale *et al.* 1993; OECD 1993c; APHIS/USDA 1994; Ward 1994). In a report published by the OECD (1993c) it is stated that by far the biggest part of the field trials involving transgenic crops is done with oilseed rape. Thus, oilseed rape is suitable as model plant for biosafety studies.

Biosafety of transgenic oilseed rape

In this chapter a short historical perspective is given to illustrate the general appearance of oilseed rape. Furthermore, the reproduction system and taxonomy and cytogenetics of oilseed rape will be discussed. These determine pollen spread and hybridization potential, respectively, which are important factors concerning biosafety of transgenic oilseed rape. Finally, the effect of the transgene involved which is an important factor in biosafety studies of transgenic crops in general is briefly mentioned.

History

Ancient Sanskrit writings in India from 2000 to 1500 B.C. are considered to be the earliest references to oilseed rape (Singh 1958; cited in Appleqvist 1972). The Mediterranean area is suggested to be the centre of origin of this species which has been cultivated for thousands of years in Asia and the Indian subcontinent (Renard *et al.* 1993). It is assumed that both oilseed rape and turnip (*B. campestris*, syn *B. rapa*) have been cultivated as oil crops in those European countries where olive trees and poppy were unknown (Schiemann 1932). In one of his reports Linnaeus (1745; cited in Appleqvist 1972) remarks the overgrow of barley and rye by rape, reducing the grain yields. He wrote "No herb can be more easily planted than this one, which hardly can be eradicated from the fields, and thus none could be planted to greater advantage for oil production".

The abundant growth in grain fields may have led to domestication of rape. In the 17th and 18th century, methods to suppress the weed flora were not or not often applied. Eventually, rape got the upper hand, outcompeted the major (grain) crops and having favourable properties for humans was harvested. In addition to this, crop plants were

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adapted to growing conditions of man-made habitats (De Wet & Harlan 1975). This way, new culture forms such as oilseed rape and turnip originated. These crops are called secondary crops (Zeven 1975; 1977), in contrast to primary crops like rye and barley.

Oilseed rape has been domesticated fairly late. A reason for this may be the presence of a thioglycoside which hampered the use of the seeds for human consumption, because it causes goitre (Johnston & Jones 1966). It is noteworthy that in Canada, presently one of the worlds largest oilseed rape producing countries, commercial growing of rape seed only started in 1942 (Ohlson 1972).

The history of oilseed rape shows that it is a rather generally occurring species which, providing climatic circumstances are suitable, spreads easily and can even become a threat to other crops as noted by Linnaeus. These are relevant characteristics in relation to biosafety.

Taxonomy and cytogenetics

The genus *Brassica* belongs to the family of *Cruciferae*. U (1935) designed a so-called triangle, clearly describing the genomic relationships of some *Brassiceae* (Fig. 1.1). The corners of this triangle are the three diploid species: *B. rapa* or turnip (AA, n=10), *B. nigra* or black mustard (BB, n=8) and *B. oleracea* or cabbage (CC, n=9). *B. napus* is an

B. rapa
(AA) 2n=20 ——— **B. napus**
(AACC) 2n=38 ——————— **B. oleracea**
(CC) 2n=18

Turnip
Chinese cabbage
Pak choi

Oilseed rape
Fodder rape
Swede

Cabbages; Kales
Cauliflower
Broccoli

B. juncea
(AABB) 2n=36
Brown mustard

B. carinata
(BBCC) 2n=34
Ethiopian mustard
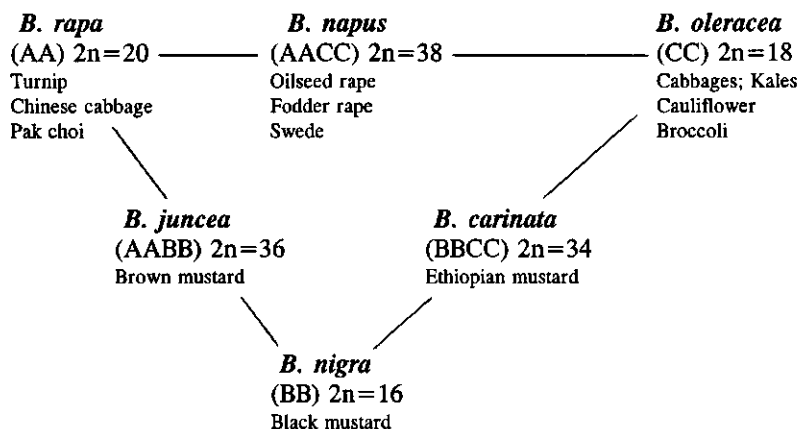
B. nigra
(BB) 2n=16
Black mustard

Figure 1.1 'U-triangle' representing the genomic relations among *Brassica* species (redrawn from U 1935).

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allotetraploid species derived from the hybridization of *B. rapa* and *B. oleracea* with the genome constitution AACC (2n=38). On the two other sides of the triangle *B. juncea* or Indian or brown mustard (AABB, 2n=36) and *B. carinata* or Abyssinian mustard (BBCC, 2n=34) are denoted. These allopolyploid species are, in contrast to the selfincompatible diploid species, self-fertile and show preferential chromosome pairing. They have probably arisen from natural hybridization in which 2n gametes were involved. The cytogenetic relationships between the diploid species were confirmed by nuclear DNA content (Verma & Rees 1974), DNA analysis (Erickson *et al.* 1983) and by genome specific chromosome markers (Hosaka *et al.* 1990). It is noteworthy that the A-genome of *B. rapa* is common to the commercial oilseed species: *B. juncea*, *B. napus* and *B. rapa*.

Based on the results of studies of the chromosome pairing in the pachytene of the meiosis of amphihaploid F_1's, the basic haploid chromosome number of the diploid *Brassica*'s is hypothesized to originate from that of an ancestor with n=6 (Röbbelen 1960). Based on nuclear Restriction Fragment Length Polymorphisms (RFLP's), Song *et al.* (1990) proposed a new hypothesis, according to which the most ancient group contains species with n=7. Duplication and triplication of certain chromosomes might have led to the current basic numbers. Mutual crosses of the three diploid species are still allowed in very low percentages (U 1935; Olsson 1960; Röbbelen 1966). Due to the independent evolution of these species their chromosome structure has altered so much that they are no longer homologous, but have become homoeologous, resulting in limited and hampered chromosome pairing.

Based on the two hypotheses about the origin of the three diploid species and the allotetraploids and on a study with genome-specific DNA markers (Hosaka *et al.* 1990), to a certain extent homoeologous chromosome pairing due to homologous chromosome parts might be expected. However, comparing established cultivars and resynthesized oilseed rape Lydiate *et al.* (1993) showed that only in the latter *B. rapa* chromosomes relatively frequently paired with *B. oleracea* chromosomes. Analysis with RFLP markers revealed 15% homoeologous recombination. This demonstrates the presence of controlled chromosome pairing in established *B. napus*. However, translocations which probably result of homoeologous recombination in the allotetraploid genome of oilseed rape, suggest that domesticated *B. napus* is unable to control chromosome pairing completely (Sharpe *et al.* 1995). Due to genetic linkage maps based on RFLP markers which have

been generated for *B. oleracea* (Slocum *et al.* 1990), *B. napus* (Landry *et al.* 1991; Lydiate *et al.* 1993; Parkin *et al.* 1995) and *B. rapa* (Chyi *et al.* 1992; Song *et al.* 1991) synteny studies are now in progress (Lydiate 1996).

Research comparing chloroplast-DNA of several *Brassica* species, among which *B. napus*, *B. rapa* and *B. oleracea*, indicates that probably a third unknown *Brassica* species, through introgression, is involved in the origin of oilseed rape (Palmer *et al.* 1983). The chloroplast-DNA from two of the three accessions of *B. napus* studied strongly differed from that of both parents, while the chloroplast-DNA of the third accession corresponded with that of *B. rapa*. These results suggest that *B. napus* has been arisen more than once in different ecological areas.

Hybridization of *B. napus* with species in other genera is also reported (McNaughton & Ross 1978; Kerlan *et al.* 1992; Lelivelt 1993; Scheffler & Dale 1994). However, using RAPD markers it was shown that *Raphanus sativus* and *Sinapis alba* were distinct from the *Brassica* taxa (Demeke *et al.* 1992). RAPD markers are similar to RFLP markers for estimating intra-specific genetic relationships, while estimating inter-specific genetic relationships RAPD markers may be less reliable than RFLP markers (Thormann *et al.* 1994). In a later section, the ability of *B. napus* to cross with different *Cruciferae* species and the possible ecological impact involved, are discussed in more detail.

Reproductive system

- Flower biology

The rapeseed flower consists of four half-spreading sepals, four diagonally standing petals almost twice as long as the sepals, six stamens, of which two shorter outer standing, and a superior ovary with two parietal placentas (Heywood *et al.* 1993). This flower structure is typical for *Cruciferae*. At the basis of the two shorter stamens oilseed rape flowers contain two functional nectaries, while at the basis of both pairs of long stamens two non-functional nectaries are located (Downey *et al.* 1980). The oilseed rape flowers are brightly yellow coloured and the presence of the nectaries, make them very attractive to bees. Studies with petal-less *B. rapa* mutants showed that, in this case, pollination was not reduced (Brunel *et al.* 1994). The floral arrangement of the *Brassiceae* is a corymbiform raceme. The relative position of the buds to the open flowers on a raceme makes it possible to distinguish between flowering plants of *B. napus* and *B. rapa* (Clapham *et al.*

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1958; Downey *et al.* 1980). In *B. napus* still closed buds overtop the opened flowers, while in *B. rapa* the opened flowers overtop the, compared to *B. napus* more compact, bud cluster.

Flowering begins at the lowest part of the raceme and from there upward. Both the onset of flowering and the duration of the flowering period vary and depend on weather conditions, particularly temperature. Flowers open very early in the morning and opening is completed at about 9 a.m. From 3 days before to 3 days after opening of the flowers the stigma is receptive (Mohammad 1935). Pollen from most of the oilseed rape cultivars can be stored for up to a year at low temperature (-20 °C) and desiccation over silica gel without any adverse effects on seed yield and embryo development (Brown & Dyer 1991). Dry pollen of oilseed rape measures about 20 x 40 μm (Wodehouse 1935).

- Pollination and fertilization
In contrast to both its parental species, in which a sporophytic incompatibility mechanism in the stigma prevents self-fertilization, oilseed rape is a predominantly self-pollinated crop with an average outcrossing rate between 15 and 45% (Rakow & Woods 1987; Becker *et al.* 1992). Environmental factors can greatly influence these outcrossing rates. Furthermore, among flowers at different positions on the plant the outcrossing rate varies from 11% at the top to 39% at the bottom of the plant (Becker *et al.* 1992).

Neither insect visits nor wind are a prerequisite for successful self-pollination of oilseed rape, although wind does stimulate this process. However, in greenhouse experiments, plants that were standing in an air flow produced more seed than plants that were not (Williams 1978; Williams *et al.* 1986) and under large-scale commercial production conditions under which insect pollination is of secondary importance, wind is the main pollinating agent (Downey *et al.* 1980; Timmons *et al.* 1995). For self-incompatible *B. rapa* both insect and wind pollination are important.

Because pollen is the main vector through which transgenes escape (Ellstrand & Marshall 1985; Den Nijs 1989) in the framework of an EU funded biosafety project field experiments were performed to study pollen dispersal of transgenic oilseed rape (De Greef 1990). It was found that the frequencies of transgene dispersal rapidly decrease with the distance to the source of the transgenes. At four meters the outcrossing frequency was already diminished to less than 1 in a 1000 (De Greef 1990), while Pauk *et al.*

(1995) observed less than 0.001% outcrossing at 1 m from the pollen source. Paul *et al.* (1995) also detected limited gene dispersal (0.012%) which frequency was strongly influenced by the immediately adjacent plants. The very limited transfer was found to be characteristic for pollen transfer by bees, but as oilseed rape is also wind-pollinated, the strong influence of immediately surrounding plants was not expected. Scheffler *et al.* (1993), who extensively studied pollen dispersal from transgenic oilseed rape, also found a sharp decline in outcrossing frequency of 0.02% at 12m. In addition, they did not find a directional effect due to wind or insect activity. However, evaluating the effectiveness of 200- and 400 m isolation distances for small- scale trials of transgenic oilseed rape, the frequency of hybrids detected at 400 m was ten times greater than estimated in the earlier study (Scheffler *et al.* 1993) for plants 47 m from the pollen donor source (Scheffler *et al.* 1995). A major difference in the two studies was the area of non-transgenic plants. In the second study donor and target plots were smaller and separated by greater distances. Therefore, bees may have been forced to forage in more than one plot regardless of the greater distance. If they could collect a full load of pollen and nectar in a small area, in and around the donor plot, they would not forage in more distant areas (Scheffler *et al.* 1995). Surrounding the transgenic plot with a trap or buffer crop of the same species that can release emigrating pollinators of transgenic pollen and provide a sufficient source of nectar and pollen so pollinators are not inclined to forage more distant sites was an effective strategy for reducing the escape of transgenic pollen (Morris *et al.* 1994; Scheffler *et al.* 1995). However, neither barren zones nor trap crops would guarantee total isolation.

Besides that of Scheffler *et al.* (1995), there are also other reports of large-distance pollen flow from *B. napus* at distances of 360 to 2000m (Downey *et al.* 1980; Downey & Bing 1990; Timmons *et al.* 1995; 1996). Exposing emasculated and subsequently self-pollinated plants to airborne pollen from an isolated field of another oilseed rape cultivar yielded 3.7% (5/135) inter-cultivar hybrids at 360 m (Timmons *et al.* 1996). Downey & Bing (1990) found 2.1, 1.1 and 0.6% outcrossing at respectively 46, 137 and 366 m from the pollen source. Discrepancies between distances of pollen flow may be due to differences in pollen donor plot size, which was 3 to 10 ha in the study of Timmons *et al.* (1996), while in the experiments of Scheffler *et al.* (1995) this was only 400 m². These results showed that care should be taken with predicting the performance of genetically

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modified oilseed rape under (semi)-standard agricultural conditions based on extrapolating information obtained from small-scale release experiments.

The above-mentioned outcrossing frequencies are based on pollen dispersal within populations. Ellstrand & Marshall (1985), however, concluded based on paternity analysis of radish populations that sometimes up to 20% contamination from adjacent populations and till 1000m occurred. These data led Klinger et al. (1991) as well as Ellstrand & Marshall (1985) to suggest that long-range transport of pollen cannot be ruled out. Thus, although reported outcrossing frequencies are low, gene dispersal can not be prevented. Therefore, precautions should be practised concerning predictions of pollen spread in general and oilseed rape pollen spread in particular as for this crop both insects and wind are vectors which act supplementarily. Especially when wind pollination is involved pollen spread can not be ruled out.

- Propagation and seed survival
Identified areas of concern associated with the release of genetically modified oilseed rape are twofold (Crawley et al. 1993). Genetically modified oilseed rape itself may become a weed and/or invade natural habitats or releasing genetically modified oilseed rape may enable sexual transfer of the inserted genes to neighbouring commercial or natural populations whose offspring may then become (more) weedy or invasive.

Significant differences were found in the distribution of weedy characteristics among weeds, 'normal plants' and crops (Baker 1965; Keeler 1989). For the average crop plant, such as oilseed rape, to become as 'weedy' as the average weed, it would need to acquire five weedy traits, which means the simultaneous acquisition of at least five gene substitutions. Therefore, it has been concluded that the probability of joint occurrence of new alleles producing significantly weedy plants from oilseed rape is extremely low (10^{-25}; Keeler 1989), provided pleiotropic effects giving stress tolerance are absent.

Oilseed rape propagates through seeds, which, when mature, disperse by pod shattering. One silique can contain ten to thirty seeds (Downey et al. 1980). After a stay for ten years in the soil still 10% of the seed is able to germinate (Cramer 1987). Crop rotation of oilseed rape and cereals is recommended as oilseed volunteers can then easier be controlled (Cramer 1987). Crawley et al. (1993) did not find a significant effect of depth of burial of rape seed on its survival, while seed survival of charlock (*Sinapis arvensis*

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L.), a weedy relative of oilseed rape, significantly increased with deeper burial.

In an extensive ecological study Crawley *et al.* (1993) also found significant differences in seed survival on burial between conventional and transgenic oilseed rape, where transgenic lines were less invasive and less persistent compared to non-transgenic lines. Although there was substantial variation in seed survival, neither plant growth and seed production between sites tested, experimental treatments performed, nor introduction of kanamycin resistance or herbicide tolerance through genetic modification does seem to increase the invasive potential of oilseed rape. Therefore, oilseed rape will become extinct in all experimental treatments and all habitats studied. In competition-free circumstances, however, a successful recruitment of oilseed rape from seed might be possible.

There are two types of oilseed rape, a winter and a summer type. In contrast to the latter, which does not require vernalization, the first type needs a period of cold before it will flower. Before winter, the plant forms a rosette and in the spring an elongated flower stem is formed and the plant starts flowering. Such vernalization requirement is a characteristic that contribute to the weedy nature of oilseed rape (Keeler 1989).

The transgenic trait will also influence the establishment of transgenic oilseed rape. Drought tolerance or disease resistance are expected to give a fitness advantage enhancing plant performance in natural habitats. Herbicide tolerance will only give a selective advantage if the herbicide is widely applied. In addition, also genetic drift, migration and mutation will influence this process (Evenhuis & Zadoks 1991; Van Raamsdonk 1995).

Hybridization

According to Hoffman (1990), Evenhuis & Zadoks (1991) and Darmency (1994) biosafety analyses of gene transfer have to deal with: a) emission, dispersal and deposition of pollen from transgenic plants, b) stable integration of the transgene in the host genome and introgression of the transgene into other (wild) species, c) stabilization and spread of the transgene in such species and d) ecological effects of the transgene in the new host population.

- Hybridization within Cruciferae

In the family of *Cruciferae* for several decades inter-specific and inter-generic crosses have been performed for different purposes. Since U (1935) gave a clear view of the

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relationships between different *Brassica* species, a lot of genetic analyses within the tribe of *Brassiceae* and within the family of *Cruciferae* were carried out for better understanding general genetic mechanisms (Yarnell 1956; Röbbelen 1960, 1966; Heyn 1977; Clauss 1978). McNaughton & Ross (1978) reviewed the possibilities for forage crop improvement through inter-specific and inter-generic hybridization. In this respect the development of new crops like x *Brassicoraphanus* (Oost 1984) or *Raphanobrassica*, resulting from sexual hybridization between *R. sativus* and *B. oleracea* or *B. rapa* (Karpechenko 1928; Dolstra 1982; Prakash & Tsunoda 1983) more specifically called Radicole (RRCC, McNaughton 1979) and Raparadish (AARR, Toxopeus 1985; Lange *et al.* 1989) should be mentioned.

- Hybridization of transgenic oilseed rape and related non-oilseed *Brassica*, *Sinapis* and *Raphanus* species

The success of hybridization between crops and wild relatives depends on the relationship between species involved. Dale (1994), extensively, described factors, which determine the likelihood of hybridization between crop plants and related species and their possible establishment in agricultural or natural habitats. In the framework of the EU-BAP (Biotechnology Action Program 1990) and EU-BRIDGE (Biotechnology Research for Innovation, Development and Growth in Europe 1992, 1993) projects, hybridization of transgenic, herbicide tolerant oilseed rape and several related species has been studied (Scheffler & Dale 1994). Lefol *et al.* (1991) obtained 2-3% *in vitro*-produced hybrids between transgenic oilseed rape and *B. adpressa.* Using male sterile oilseed rape, hybrids with *B. adpressa* and *Raphanus raphanistrum* were detected also in the field (Chèvre *et al.* 1992; Eber *et al.* 1994; Baranger *et al.* 1995). Such hybrids show normal female fertility. Male fertility is reported to be 13 and 35% for the hybrids with *B. adpressa* and *R. raphanistrum*, respectively (Eber *et al.* 1994). Pollen fertility varied from 1 to 30%.

Kerlan *et al.* (1992) described reciprocal crosses between herbicide-tolerant oilseed rape and five related species: *B. oleracea* L. var. *acephala*, *B. oleracea* L. var. *capitata*, *B. nigra* L. Koch, *B. adpressa* L., *Raphanus raphanistrum* and *Sinapis arvensis* L. The last three are commonly occurring in oilseed rape fields in France and were locally collected. All the inter-specific combinations tested were able to produce hybrid plants, but only when fertilized ovaries were established in *in vitro* culture. When rapeseed was

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used as female parent more hybrid plants were obtained. Probably, this can be explained by the higher chromosome number in oilseed rape, which was also found to influence hybridization capacity in other studies concerning reciprocal differences in yield of hybrid embryos (Mohapatra & Bajaj 1987; Quazi 1988). These observations show severe limitation in gene dispersal due to hybridization barriers. In contrast to the hybrids formed spontaneously, the *B. adpressa* and *R. raphanistrum* hybrids obtained by embryo rescue were mostly sterile (Eber *et al*. 1994). Also the other hybrids were male sterile or poorly fertile, except for two amphidiploid *B. napus* x *B. oleracea* plants, which showed a fertility comparable to oilseed rape. Such a reduced male fertility diminishes the possibility for gene dispersal.

Gene introgression after sexual hybridization depends on the percentage of chromosome pairing. The higher this percentage is, the higher the opportunity that a (trans)gene introgresses into the genome of the wild relative. Therefore, Kerlan *et al*. (1993) studied the meiotic behaviour of the hybrids between herbicide-tolerant oilseed rape and the five related species earlier mentioned together with the physical presence and expression of the Basta® tolerance, bar gene. Most of the 75 hybrids studied had a triploid structure (ACX). Comparing the percentage chromosome pairing in the hybrids with that of haploid oilseed rape allosyndesis between rapeseed AC genomes and the genomes of related species occurred. The presence of multivalent association in all hybrids also indicated the possibility for recombination. Also a good correlation between presence of the bar gene and herbicide tolerance, providing the T-DNA was inserted as a single locus was observed. If the T-DNA was present at three loci, two plants having the bar gene were nevertheless found to be Basta® susceptible. This might be explained by suppression of gene expression through a position effect (De Block *et al*. 1989) or through DNA methylation followed by gene inactivation (Matzke *et al*. 1989; Hobbs *et al*. 1990; Linn *et al*. 1990). Other explanations might be partial complementation caused by an insufficient transgene expression, co-suppression (Flipse 1995) or (anti)sense inhibition (Jorgensen 1990; Grierson *et al*. 1991; Mol *et al*. 1994; Flipse 1995).

Based on the study of the occurrence and the cytogenetical characterization of interspecific hybrids (Kerlan *et al*. 1992; 1993), the five related *Brassica* species were ranked by decreasing ecological impact: *B. oleracea*, *R. raphanistrum*, *B. adpressa*, *S. arvensis* and *B. nigra*. Their results showed that gene transfer would not occur to the weedy

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relatives *B. nigra* and *S. arvensis* due to natural cross barriers, which is in agreement to what is found by Downey & Bing (1990) and Bing *et al.* (1991; 1995). Between *B. rapa* and *B. nigra* gene transfer was shown to be possible, while gene transfer between *B. rapa* and *S. arvensis* was at the most difficult (Bing *et al.* 1996). Hybridization of oilseed rape and radish (*R. sativus*) was shown by Metz *et al.* (1995). However, this will not have an ecological impact. Although herbicide tolerance could be transferred from transgenic oilseed rape to the hybrid, hybridization was only possible under special laboratory conditions and, in addition, the hybrid plants were almost completely sterile.

- Hybridization among (transgenic) oilseed Brassicas (*B. napus*, *B. rapa* and *B. juncea*)
B. rapa, *B. napus* and *B. juncea* are commercially grown oilseed species. The last two accommodate the *B. rapa* AA genome.

- Hybridization of *B. juncea* and *B. napus*
B. juncea, cultivated in Asia, USA and Canada for oil and mustard production, is found as a weed or ruderal in Denmark and Sweden (Frello *et al.* 1995). In Southern Europe it is naturalized (Heywood & Akeroyd 1993).

Inter-specific hybrids of *B. napus* and *B. juncea* are easy to obtain in controlled crosses with *B. juncea* as female parent while spontaneous hybridization is also observed (Bing *et al.* 1991; Frello *et al.* 1995). On basis of RAPD analysis, a relatively high homology between the A-, B- and C-genome was found, making recombination between these chromosomes feasible (Quiros *et al.* 1991, 1994). Such introgression from oilseed rape into the genome of *B. juncea* has been reported (Frello *et al.* 1995), while hybridization between *B. juncea* and *B. rapa* has been reported too (Anand *et al.* 1985; Banga 1986). These hybridizations are not relevant for the Netherlands because *B. juncea* is not cultivated and very seldomly occurs in nature under dutch circumstances.

- Hybridization of *B. rapa* and *B. napus*
Turnip is an annual or biennial herb, cultivated on a modest scale. It is very frequently found on open waysides, disturbed ground and other unnatural habitats. (Sub)spontaneous populations are found in the wild, which might be regarded as wild relatives of oilseed rape. Many records of escapes of *B. napus* can be traced back as concerning *B. rapa*.

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De Vries *et al.* (1992) have made so-called botanical files for 42 species of cultivated plants grown in the Netherlands using a D_{pdf} code, consisting of 3 dispersal codes with 6 indices each. D_p gives an indication for gene dispersal by pollen, D_d for gene dispersal by seeds and diaspores and D_f for the frequency of the plants in the wild. The numerical code is a measure for the possible ecological effects of the cultivated plant on the wild flora of the Netherlands (Frietema De Vries 1996). Oilseed rape obtained a D_{pdf} code of 2.2.4, indicating that a medium ecological effect can be expected on the dutch flora (Frietema De Vries 1996). Turnip (*B. rapa*) obtained a D_{pdf} code of 5.5.4, which indicates the expectation of a substantial and wide spread ecological effect on the flora of the Netherlands. Under dutch circumstances oilseed rape and turnip flower simultaneously from April till August (Van der Meijden 1990) and hybridization of *B. rapa* and *B. napus* has been reported to occur occasionally (De Vries *et al.* 1992).

Reports on the crossability between oilseed rape and *B. rapa* are nevertheless controversial (Jørgensen & Andersen 1994). In breeding programs of oilseed rape, crosses with *B. rapa* were performed (Gowers 1982). Natural crosses between these species are thought to be either difficult and not likely to happen (Downey *et al.* 1980) or rather common in nature, which was exemplified by spontaneous hybridization in agricultural fields (Bing *et al.* 1991; Jørgensen & Andersen 1994). In Denmark, *B. rapa* is a common weed in cultivated areas, mostly in oilseed rape fields (Jørgensen & Andersen 1994). The hybrid plants identified, produced a small amount of viable seeds after open pollination, which indicated that these hybrids might survive the next generation.

The possibility for gene transfer from *B. napus* to *B. rapa* under natural circumstances will be less than observed under pollination conditions in field crossing blocks, because *B. rapa* flowers one to two weeks earlier than *B. napus* and because inter-specific hybridization is more successful when *B. rapa* is used as male parent (Downey & Rakow 1987; Bing *et al.* 1991; Bing 1991). It is in contradiction, however, to the results of Palmer (1962) who obtained after open pollinations with an excess of pollen, on turnip 88% hybrid plants and on oilseed rape only 11% hybrid plants.

Hybrids had quantitatively a good pollen production, but showed reduced fertility (Beversdorf *et al.* 1980; Bing 1991). After staining, viability of the pollen was found to be about 60% (MacKay 1977; McNaughton 1973b). The pollen fertility of the hybrid plants obtained from seeds harvested on *B. rapa* ranged from 21 to 86% in different

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experimental designs (Jørgensen & Andersen 1994). If plants were placed in a 1:1 mixture of *B. rapa* and oilseed rape, hybrids obtained from seeds harvested on oilseed rape plants had 41% (16-65%) pollen fertility. In contrast, Röbbelen (1966) observed in the cross *B. rapa* x *B. napus* complete sterility, which was suggested to be the result of aberrant embryo development.

Scheffler & Dale (1994) have reviewed the opportunities for hybridization between oilseed rape and related species. They also reported successful selfings and backcrosses of hybrids between turnip and oilseed rape. According to U (1935), McNaugton (1973b) and Beversdorf *et al.* (1980) most of such hybrids possessed 29 chromosomes. In the metaphase of the meioses 10 bivalents (the A genomes) and 9 univalents (the C genome) were observed (U 1935; MacKay 1973; McNaughton 1973b; Rousselle & Eber 1983). Apparently, the turnip chromosomes paired completely resulting in vital gametes.

Introgression is reported from *B. rapa* into *B. napus* (MacKay 1977; Goring *et al.* 1992). MacKay (1977) introgressed S-alleles from turnip and Goring *et al.* (1992) described the introgression of an S-locus glycoprotein CDNA. Because only oilseed rape with the desired cytoplasms was available, introgression of cold tolerance and black-rot resistance from *B. napus* into *B. rapa* (Pak choi) and *B. rapa* (Chinese cabbage) was accomplished (Guo *et al.* 1990; Heath *et al.* 1994).

We backcrossed hybrids of *B. rapa* and transgenic herbicide tolerant oilseed rape to *B. rapa* (Metz 1995), while Mikkelsen *et al.* (1996a) performed the reciprocal backcross. In the BC_1 and BC_2 generations herbicide tolerance was detected, which indicates that introgression of a transgene into *B. rapa* seems possible (Mikkelsen *et al.* 1996a; Metz *et al.*, Theor. Appl. Genet. accepted). These results show that if the natural conditions are as optimal as in the study of Mikkelsen *et al.* where inter-specific hybrids were grown in small plots together with *B. rapa* or as our experimental conditions where pollen is put in excess on the stigma of the receptor plant, a transgene can be transferred to *B. rapa*. This might confer a fitness advantage to *B. rapa* under selective conditions. However, it is difficult to conceive of a situation in which genetic modification for herbicide tolerance will influence the fitness of a plant in the absence of the herbicide (Gliddon 1994).

Introgression and gene transfer to *B. rapa* might be limited by introduction of a transgene into the C-genome of *B. napus*. It is expected that the transgene will probably be present in a lower that expected percentage of the plants after 2-3 generations of back-

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crossing with the wild relative *B. rapa*. However, in general, the occurrence of fertile, transgenic *B. rapa*-like plants after hybridization and two generations of backcrossing suggests possible gene dispersal from oilseed rape to its weedy relative *B. rapa*.

Impact of transgene features on biosafety of transgenic oilseed rape
It can be concluded that complete containment of transgenic oilseed rape is not possible. In the case of hybrids of *B. rapa* and oilseed rape, studies about the stability of transgene expression over generations and in different genetic backgrounds are relevant. Such studies can show the possible impact of transgene action and stability in these hybrids.

In general, there will be a shift from the question of possible transgene escape to the question of the ecological and toxicological impact of the introduced genes (Timmons *et al.* 1996). By order of the Dutch Ministries of Economic Affairs, and Housing, Spatial Planning and the Environment a series of literature reports was written about the 'transgene-centered' evaluation of genetically-engineered plants. The ecological and toxicological biosafety aspects of the phosphinothricin tolerance gene (Nap & Metz 1996) and the glyphosate tolerance gene (Nap *et al.* 1996) have been evaluated till now and more reports are in progress. Such a transgene-centered approach may prove the more useful in the near future (Metz & Nap, 1997).

Concluding remarks

It is expected that transgenic oilseed rape will be at the forefront of the commercialization of transgenic crops. Therefore, oilseed rape is a good model crop for biosafety studies. The taxonomy and cytogenetics of the family of *Cruciferae* give rise to ample possibilities for inter-specific and inter-generic hybridization, either with or without embryo-rescue techniques. Pollen is thought to be the main factor through which transgenes may spread. Vectors for pollination of oilseed rape are both insects and wind.

Monitoring pollen movement from (semi) commercial oilseed rape fields showed that extrapolating information obtained from small-scale release experiments must be done carefully. Although reported outcrossing frequencies were low, pollen spread and gene dispersal from transgenic oilseed rape to its (wild) relatives can not be prevented.

Studies on reciprocal crosses between transgenic oilseed rape and a number of related

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species showed that all inter-specific and inter-generic combinations tested produce hybrid plants, but in most cases only after elaborate *in vitro* culture. However, for some related species spontaneous hybridization has been reported under field conditions. Spontaneous hybridization among the oilseed Brassicas (*B. rapa*, *B. juncea* and *B. napus*) has been observed in agricultural fields. The occurrence of fertile, transgenic *B. rapa*-like plants after hybridization and two generations of backcrossing suggests gene dispersal from oilseed rape to its weedy relative *B. rapa* and introgression of oilseed rape genes in *B. rapa* is possible. Such gene dispersal and introgression might be limited by inserting the transgene in the C-genome. Studies about the stability of transgene expression over generations and in different genetic backgrounds can show the real impact in time of transgene action in hybrids of *B. rapa* and oilseed rape.

Because complete containment of transgenic oilseed rape is not possible, attention should now focus on the ecological and toxicological impact of the introduced genes. Such transgene-centered ecological and toxicological evaluation, irrespective of the genetically-engineered plant species may prove useful in the near future.

Chapter 2

A transgene-centered approach to the biosafety of transgenic phosphinothricin-tolerant plants

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Summary

The impact of the presence of the microbial *bar* and *pat* genes in plants is evaluated in a so-called 'transgene-centered approach' to biosafety. Conferring tolerance to the non-selective herbicide phosphinothricin (PPT; sold as Basta or Finale), the use of these genes could imply a considerable environmental gain compared to current-day herbicide cocktails. The ecological consequences with respect to weediness or spread of the transgenic PPT tolerance are concluded to be sufficiently minor, assuming responsible use of this trait in agronomy. The toxicological evaluation depends on whether or not the plant was actually spread with PPT. Consumption of the gene and/or gene product from unspread transgenic plant material will not have adverse effects. In case of PPT-sprayed material, however, PPT or derivatives might be present in food and feed. To date, the toxicological impact of such a putative exposure is not sufficiently clear. Premarket testing of and/or more familiarity with the trait seem required.

Introduction

With the introduction of transgenic plants, various aspects of the biosafety of such plants have been studied to evaluate their admission. Many 'transgene independent' studies concluded that transgenic plants should be evaluated on a case-by-case basis. Attention should be given to the ecological and toxicological impact of the introduced genes and gene products. These aspects are the motivation for the alternative, or 'transgene-centered' evaluation, in which all characteristics of a particular transgene are evaluated (Nap *et al.* 1992; Metz & Nap 1997). Such an approach helps to generalize outcomes irrespective of the plant species into which the transgenic trait is introduced. Furthermore, concentrating on the presence of gene *x* and the gene product X allows definite questions to be evaluated and may identify open issues more readily than general considerations. Biochemical, ecological and toxicological data of the gene, its product, substrates and degradation products will indicate if and if so which further data should be required for decision making. The approach may streamline the discussions about the ongoing commercialization of transgenic crops.

Here, the *bar* and *pat* genes whose gene products confer tolerance to the herbicide phosphinothricin (PPT) are reviewed as an illustration of the transgene-centered approach. The startpoint is: given current agricultural practice, what could be the consequences of agronomic crops tomorrow being transgenic PPT-tolerant.

Phosphinothricin and phosphinothricin tolerance

Phosphinothricin: properties and applications

PPT originates from the actinomycetes *Streptomyces viridochromogenes* and *S. hygroscopicus* (Bayer *et al.* 1972; Lea *et al.* 1984). Industrially it is synthesized as a DL-racemic mixture, of which only the naturally occurring L-PPT is herbicidal. PPT, or glufosinate, is sold under the brand names Basta®, Finale® and Radicale®. It is widely used as broad spectrum, pre-emergence herbicide and also for pre-harvest desiccation in potato, legumes and oilseed rape by application to the leaves. PPT has no margin for discrimination between crop and weed: it is a so-called non-selective herbicide (Botterman & Leemans 1988).

PPT interferes with amino acid synthesis by inhibition of glutamine synthetase. GS is the key enzyme in nitrogen metabolism that assimilates ammonia produced by nitrate reduction,

and recycles ammonia produced by processes such as photorespiration and deamination (Kishore & Shah 1988). As a structural analogue of the GS substrate glutamate, PPT inhibits GS irreversibly. This inhibition triggers ammonia accumulation to levels up to 100 fold higher than in control plants, resulting in cessation of photosynthesis and disruption of the chloroplast structure (Devine *et al.* 1993; Tachibana *et al.* 1986).

In common agricultural practice, two to four hours after application photosynthesis slows down and the plants will yellow and die in two to five days (Hoechst 1984). Over 40 monocotyledonous and more than 150 dicotyledonous weeds are sensitive to PPT (Hoechst 1984). Weeds generally require 0.6 - 2.0 kg/ha, but, for example, sicklepod (*Cassia obtusifolia*) requires 8.5 kg/ha, whereas green foxtail (*Setaria viridis*) is killed by 0.2 kg/ha. There is no example of absolute PPT resistance.

PPT tolerance

A successful strategy for obtaining PPT-tolerant crops has been based on the mechanism used by the PPT producing actinomycetes, which protect themselves against the autotoxic action by metabolizing the compound. They produce phosphinothricin-N-acetyltransferase (PAT) that acetylates the free NH_2 group of PPT, causing its inactivation. The PAT-encoding *bar* and *pat* genes were isolated from *S. hygroscopicus* and *S. viridochromogenes* Tü494, respectively (Murakami *et al.* 1986; Strauch *et al.* 1988). Both genes code for PAT proteins of 183 amino acids, which show 85% homology, variations of the genes being confined to the 5'-noncoding regions (Wohlleben *et al.* 1988).

For expression in plants, the PAT-encoding genes driven by plant promoters were successfully introduced in crops using standard transformation technology (De Block *et al.* 1987). Transgenic plants proved tolerant to 4-10 times the dose of PPT required to kill control plants. PAT levels of no more than 0.001% of the total soluble protein proved sufficient to confer tolerance at field dose applications of the herbicide (De Block *et al.* 1987). In large scale field trials, transgenic PAT-containing plants showed similar agronomic performance as controls (De Greef *et al.* 1989; Fredshavn *et al.* 1995).

Currently, there are three applications of transgenic PPT tolerance in the development and use of plant material: as selectable marker during genetic transformation; as agronomic character; and in hybrid seed production.

Biosafety issues

Transgenic PPT tolerance raises ecological and toxicological concerns. Transgenic PPT tolerance might transform a crop into an uncontrollable weed; it may spread from the crop to wild relatives or to other organisms, that as a result become uncontrollable; it might disturb ecological relationships of the crop in another way. The presence of the PPT tolerance gene or its gene product may directly or indirectly render the plant unsuitable for consumption or industrial processing. The use of PPT in transgenic crops may challenge consumers with the herbicide or its metabolites. Also, there may be any unexpected pleiotropic effects associated with the transgenic PPT tolerance.

Environmental impact of PPT and its metabolites
As chemical compound PPT is stable, but in the soil it is rapidly degraded to 3-methyl-phosphinico-propionic acid (MPP) by microbiological activity (Tebbe & Reber 1988). MPP is non-phytotoxic (Dröge *et al.* 1992) and has no herbicidal activity (Hoechst 1984). Traces of MPP were found for a short period of time. In metabolism studies no residues of the active compound could be detected in plants or in animal tissue, indicating there is a rapid secretion. Due to the high solubility in water, accumulation in the food chain will not occur (Lindhoud 1984).

Another important issue in the characteristics of transgenic PPT-tolerant plants is the relative environmental load of PPT. Generally, the environmental impact of PPT is considered to be less than the impact of the currently used cocktails of herbicides (Van Rijn *et al.* 1995). The use of PPT and PPT-tolerant crops would imply a considerable reduction in amounts of active ingredients of herbicides compared to current practice. PPT is considered to be safe for water and soil life and will not leach into groundwater in spring. It is considered less safe with respect to leaching into groundwater in autumn.

Weediness
The weediness of a PPT-tolerant plant largely depends on the interplay between the intrinsic characters of the plant, in combination with the specific habitat the plant lives in (Keeler 1989; Tiedje *et al.* 1989). The scenario relevant to biosafety is an enhancement of fitness. When selective PPT concentrations are found, such as in the field in which the PPT-tolerant

crop-of-interest is sprayed with PPT, a selective advantage will be created. Except perhaps occasionally in verges adjacent to production fields, it is unlikely that such selective conditions will be found elsewhere.

In the absence of spraying with PPT, PPT tolerance will not contribute to weediness *per se*. Investigating the competitiveness of transgenic PPT-tolerant oilseed rape under non-selective PPT conditions, no significant differences between transgenic and non-transgenic lines were observed. Inclusion of the more competitive *Sinapis alba* in the experimental design allowed to conclude that any change in competitiveness would not exceed the natural competition level of this reference species (Fredshavn *et al.* 1995).

No increase in invasive potential conveyed by the PPT tolerance was observed in a variety of habitats and under a range of climatic conditions in which there were no selective concentrations of PPT present (Crawley *et al.* 1993). Whenever there was any significant difference, transgenic lines were less invasive and less persistent than their non-transgenic counterparts. In the absence of selective conditions, there is no advantage for PPT-tolerant crops and there will be no increased weediness of these crops. The paper by Crawley *et al.* (1993) has been called a 'landmark paper in ecology' (Kareiva 1993), but also resulted in lots of discussions among ecologists and others about its scientific merits as well as about the experimental designs and the validity and generality of the conclusions drawn (Kareiva 1993; Miller *et al.* 1993; Miller *et al.* 1994; Crawley 1993; Crawley 1994; Williamson 1992). PPT-tolerant crops might be useful for the further development of ecological science, in combination with the application of molecular techniques (Williamson 1992). Such a 'molecular ecology' will yield valuable insights into the dynamics and plasticity of ecosystems and might contribute to the biosafety assessment of transgenic plants.

For the issue of biosafety, the experimental data available to date, however, seem sufficient to conclude that there is no likelihood for increased weediness of transgenic PPT-tolerant crops, irrespective of the application of PPT.

Spread of the transgene
The spread of the PPT tolerance transgene to wild relatives depends on a myriad of ecological situations, genetic factors and stochastic events (Tiedje *et al.* 1989). In view of current large-scale agriculture, it is prudent to assume that the PPT tolerance transgene will spread by cross-pollination in some conditions and at some locations. More important,

therefore, is the foreseeable effect of this spread. Outcrossing to a weedy wild relative next to a field may result in a PPT-tolerant weed that moves back into the field and can not be controlled anymore with PPT. Despite more than a decade of commercial use of PPT, no acquired PPT tolerance of weeds has been observed in the field. The introduction of transgenic PPT tolerance may result in a higher likelihood of the occurrence of acquired PPT tolerance in weeds. It is difficult to predict how fast and how total a putative loss of PPT as herbicide could be. Having accepted PPT as relatively benign herbicide, any impairment of the use of PPT due to transgenic tolerance could result in the need to use more environmentally unfriendly herbicides. This should be considered a negative development. For example, PPT-tolerant potato, legumes or oilseed rape will not allow the current post-emergence control with PPT. Therefore, it might be advisable to incorporate the use of PPT and transgenic PPT tolerance in a herbicide resistance management scheme.

A weedy wild relative will only be able to go out of control in case of selective PPT conditions outside agricultural fields. As argued above, in the absence of any selective pressure, it is unlikely that any wild relative will go out of control. Horizontal gene transfer to another organism requires a chain of events, each step having a little likelihood (Schlüter *et al.* 1995). The final outcome, irrespective of the time it will take to happen, will be an organism that is tolerant to PPT. The consequence of this tolerance will depend on the presence of selective conditions and, therefore, it is unlikely that such organism will go out of control. The use of PPT and PPT-tolerant crops in the production of hybrid seeds and as selection marker during transformation is fully biosafe. In these applications, PPT tolerance is either only applied as dominant selective marker under conditions for seed production or under controlled laboratory conditions.

Consumption
The introduction of the *bar* or *pat* transgene in crops, and subsequent use of PPT during crop cultivation, imply that three additional classes of molecules will or can be present: the transgene, the *bar* or *pat* gene, and it metabolites; the transgene product, PAT, and its metabolites; and the herbicide PPT and its metabolites. Each of these should be evaluated for undesirable effects in consumers. The large amount of DNA that passes the digestive tract daily indicates that DNA is not intrinsically toxic to human beings. DNA is efficiently degraded and no functional genes are assumed to remain present (Berkowitz 1990). In this

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respect, *bar* and *pat* DNA will not differ from any other DNA and will not pose any adverse effects. In the unlikely case that intestinal cells or micro-organisms acquire the *bar* or *pat* DNA it is comparable to putative horizontal gene transfer in ecosystems. The absence of any positive selective pressure for PAT-containing cells or organisms in the digestive tract of consumers will preclude any conceivable harm.

PAT and its metabolites

Undesirable effects due to presence of the PAT protein could result from enzymatic activity of PAT in either transgenic plant or digestive tract, the presence of PAT itself and/or the degradation products of PAT. PAT enzyme has a high substrate specificity for L-PPT (Dröge *et al.* 1992; Thompson *et al.* 1987). Glutamate and analogues are poor substrates, having affinities at least 500 times lower than PPT. The overall high substrate specificity suggests that enzymatic activity of PAT in the transgenic plant will not result in the establishment of pools of unfamiliar secondary metabolites. In the human digestive tract, no substrate is likely to be available and the gastric conditions preclude catalytic activity. The pH optimum for the enzyme is 7.5 and rapid thermo-inactivation is observed at temperatures exceeding 35 °C (Botterman *et al.* 1991; Walter *et al.* 1992). The gastric fluid has a pH of 2 to 4. PAT loses all enzymatic activity within one minute of exposure to gastric pH (Kok, pers. comm.). In addition, the required co-factor acetyl-CoA is not stable in such acidic conditions. Enzymatic activity of PAT in the human digestive tract can, therefore, be excluded.

Without enzymatic activity, the PAT protein molecule could prove toxic or allergenic upon consumption. Generally, proteins are non-toxic (Jones & Maryanski 1991). The OECD has summarized the criteria which do suspect allergenicity of a protein (FDA/EPA/USDA 1994): a relative abundance; glycosylation; resistance to proteolytic degradation and resistance to heat denaturation. The protein is not likely to exceed 0.1% of the total soluble protein content of the transgenic plant material. The protein has no glycosylation sites. Database comparisons with known protein sequences gave no hint of any allergenic or toxic potential of the PAT protein (Trinks 1995). The PAT protein has no homology to known toxic peptides and is rapidly and irreversibly degraded in the gastro-intestinal tract (Trinks 1995) with a halflife of 1-2 min (Kok, pers. comm.). All criteria indicate that no allergenicity or toxicity of the PAT protein or its degradation products are to be expected.

PPT and its metabolites

The use of PPT-tolerant crops will imply a shift from the current pre-emergence applications to post-emergence applications of PPT. The food and feed safety of transgenic PPT-tolerant plants will depend on the additional metabolites present. This, in turn, depends on whether or not and when the plant was sprayed with PPT prior to consumption. Without spraying with PPT, the additional metabolites that occur in transgenic PPT-tolerant crops are the *bar* and *pat* transgenes and the PAT enzyme. As shown in the previous paragraphs, these metabolites do not have any adverse effects. Transgenic PPT-tolerant plants are safe for consumption. All cases in which the PPT tolerance is only used as marker for transformation in the laboratory are covered in this scenario.

In the majority of cases, however, PPT-tolerant crops are likely to be used in combination with PPT. The additional metabolites present in transgenic PPT-tolerant plants upon PPT spraying are PPT itself, its metabolites and the metabolites formed through PAT activity. Commercial PPT is a racemic mixture of D- and L-PPT and requires the evaluation of both enantiomers. In plants with relatively high PAT amounts, L-PPT is quantitatively acetylated giving acetyl-PPT, while D-PPT remains stably present (Dröge *et al.* 1992; Dröge-Laser *et al.* 1994). If commercialized transgenic crops contain sufficient amounts of PAT protein to establish the quantitative acetylation of PPT, the metabolites acetyl-PPT and D-PPT need to be evaluated for consumption. Acetyl-PPT is a stable compound that may accumulate in the plant and some transport via the xylem into the fruits or seeds may occur (Dröge-Laser *et al.* 1994). Upon oral administration, acetyl-PPT, which is also formed in the gut of animals via the normal detoxification pathway (Trinks 1995), is excreted rapidly, the major amount via faeces and some via the urine. There is no deacetylation in the stomach that recreates PPT. Mammalian toxicity studies yielded LD_{50} values for oral and dermal administration larger than 2.8 g/kg body weight, indicating that acetyl-PPT is essentially non-toxic. Acetyl-PPT, therefore, poses no concern for consumption. The fate of acetyl-PPT upon food processing is unknown.

The toxicity of D-PPT has only been determined in combination with L-PPT. Although DL-PPT inhibits mammalian GS as well (Ebert *et al.* 1990), it is generally not or less toxic to mammals (LD_{50} 1.5 to 4 g/kg body weight) because of its rapid clearance by the kidneys (Kishore & Shah 1988). The commercial formulation of PPT, which includes DL-PPT and a wetting agent, must according to EU directive 83/467/EEC be classified as 'harmful' on

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the basis of the acute oral toxicity tests. It induced slight dermal toxicity and eye irritation and was slightly toxic following oral exposure to laboratory animals (Ebert *et al.* 1990). It is unclear whether these effects are due to the L-enantiomer only. No genotoxic, teratogenic or carcinogenic potential was observed (Ebert *et al.* 1990). There was no toxicity for bees, earthworms or soilmicro-organisms (Lindhoud 1984). A daily intake of 0.02 mg per kg body weight per day is proposed as acceptable (Ebert *et al.* 1990). It would seem highly unlikely that sprayed transgenic plants will ever accumulate such amounts of D-PPT. The fate of D-PPT upon food processing is unknown.

In plants with relatively low PAT activity, in addition to substantial amounts of L- and D-PPT and acetyl-PPT, the metabolites 4-methylphosphinico-2-oxo-butanoic acid (PPO), 4-methyl-phosphinico-2-hydroxy-butanoic acid (MHB) and MPP were observed (Dröge-Laser *et al.* 1994). Similar to non-transgenic PPT-sensitive plants, deamination of L-PPT results in PPO and subsequent decarboxylation yields MPP (Tebbe & Reber 1988). No further decarboxylation of MPP was detected (Dröge *et al.* 1992; Dröge-Laser *et al.* 1994). In plants, PPO can alternatively be reduced to MHB. In addition, 4-methyl-phosphinico-butyric acid (MB) was a PPT metabolite so far found only in monocots (Dröge-Laser *et al.* 1994). The possibility of species-specific PPT metabolites has therefore to be taken into account in the analyses of PPT metabolites in transgenic plants.

The presence of MHB and MPP in PPT-tolerant plants was dependent on the amount of PAT present, indicating a competition between the PPO-MPP/MHB and the PAT metabolic routes (Dröge-Laser *et al.* 1994). Both MHB and MPP were found to be final and stable products of the plant's metabolic pathways (Dröge *et al.* 1992; Dröge-Laser *et al.* 1994). Transport of these metabolites via the xylem to the upper regions of the plant was observed. No toxicological data concerning MHB and MPP or other putative metabolites are available. The putative accumulation and exposure to metabolites such as MPP or MHB deserves attention. It is currently insufficiently clear whether consumers are exposed to what levels of PPT and/or its metabolites. As long as there is not much familiarity with the trait, it would seem to be advisable to develop a protocol to evaluate the levels of PPT metabolites in PPT-tolerant plant food. This will indicate if, and if so which, further toxicological data are necessary.

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Pleiotropic effects

The presence of the *bar* or *pat* transgene or its product, or any of its metabolites, may in some unexpected way alter any of the manifold ecological relationships or toxicological characteristics of the crop. The same applies to any wild relative derived from outcrossing, or any organism derived from horizontal gene transfer or any product derived from it. For example, the tabtoxin resistance gene (*ttr*) from *Pseudomonas syringae* encodes an acetyltransferase, which inactivated tabtoxin but not bialaphos (Yoneyama & Anzai 1993). In case the PAT enzyme would inactivate the tabtoxin, *Pseudomonas* resistance could be a pleiotropic effect of PPT tolerance. Although there are no reports of PPT-tolerant plants tested for tabtoxin resistance, the high substrate specificity of PAT makes the occurrence of such a putative pleiotropic effect highly unlikely. In general, it is at the moment unclear whether pleiotropic effects do occur to the extent that any effect can be measured. And if any effect can be measured, it might be unclear whether such an effect has any relevance for the ecological relationships or toxicological characteristics of the crop. And if an effect has any relevance, it is unclear whether the outcome should be considered an adverse effect. The relatively minor and well documented changes brought about by the *bar* and *pat* transgenes indicate little need for concern. The dynamics and self-regulatory properties of ecosystems and consumers, in combination with the natural background of mutations, changes and nutritional versatility are likely to create sufficient 'noise' to allow the conclusion that pleiotropic effects will be of no or very minor importance.

The above evaluation of the transgenic PPT-tolerant phenotype by gathering various data of the transgene, its product, substrates and degradation products establishes a file of a particular transgene irrespective of the plant species into which the transgene is introduced. The availability of such a file is likely to make discussions about this particular gene among different groups more transparent and possibly more constructive. Ideally, for every individual transgene present in plants such a 'transgene-centered' file with specific data should in the future become publicly available.

Acknowledgements

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Chapter 3

Occasional loss of expression of phosphinothricin tolerance in sexual offspring of transgenic oilseed rape (*Brassica napus* L.)

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Summary

The commercial and economic value of genetically modified crops are determined by a predictable, consistent and stable transmission and phenotypic expression of the transgenes in successive generations. No gene inactivation is expected after selfings or crosses with non-transformed plants of homozygous transgenic oilseed rape plants if the phenotypic expression of the transgene in homozygous or hemizygous nature in such plants is stable.

The segregation ratios of phosphinothricin (PPT) tolerance in successive generations of selfings and mutual crosses of a few independent transgenic PPT-tolerant oilseed rape plants indicated a dominant, monogenic inheritance. In within-variety and between-variety crosses no transgene inactivation was observed. However, after selfings and backcrosses with non-transgenic oilseed rape infrequent loss of the expression of the PPT tolerance transgene was observed independent from its homozygous or hemizygous nature. Molecular analysis of PPT-susceptible plants showed that the loss of phenotypic expression was due to gene inactivation and not to the absence of the transgene. Besides methylation and co-suppression, somaclonal variation was mentioned as one of the mechanisms that might cause reduced or even loss of phenotypic expression of the transgene in later generations. The implications of this observation for seed multiplication of varieties and breeding activities with transgenic oilseed rape are discussed.

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Introduction

The ability to integrate (foreign) genes from all organisms into the nuclear genome of higher plants and to regenerate the transformants into fertile plants give plant breeders additional opportunities to modify and improve crop plants. Such transgenic crops are commercialized and transgenic plants most likely will significantly contribute to agriculture in the future (Dale & Irwin 1995). However, developments in the patenting of genes, the release regulations, food labelling and consumer attitude will influence the implementation rate. Besides these aspects, the commercial use of genetically modified plants will be determined by the level of expression and the stability of transgene expression in successive generations of seed multiplication of varieties or successive steps in a breeding program (Meyer 1995). Only when the transgenes are transmitted and expressed through subsequent generations in a predictable, consistent and stable manner, the transgenic crop will be of economic value (Conner & Christey 1994; Finnegan & McElroy 1994).

However, it is generally known that the level of transgene expression in transgenic plants varies between different transformants. For this reason, it is necessary to generate a large number of independent transformants and to screen them for those plants that have the appropriate expression level. For the potential of transgenic plants in agriculture the loss of transgene expression (Kilby *et al.* 1992; Meyer *et al.* 1992; Cherdshewasart *et al.* 1993), the non-expression of introduced traits following selection for a linked selectable marker gene (Heberle-Bors *et al.* 1988; Ottaviani *et al.* 1993) and non-Mendelian segregation of the transgenic phenotype in segregating populations (Deroles & Gardner 1988a; b; Mittelsten Scheid *et al.* 1991) are disturbing observations. The loss of expression in most cases was shown to be correlated with inactivation of the transgene rather than with the absence of it (Matzke *et al.* 1989; Mittelsten Scheid *et al.* 1991).

Theoretically, assuming the likelihood of inactivation for both alleles after selfing of transgenics to be equal, the frequency of gene inactivation in homozygous progeny is the square of that in hemizygous offspring. This correlation was observed by Müller *et al.* (1987) using kanamycin resistance in tobacco. However, in another study also in tobacco, the frequency of gene inactivation was approximately equal in homozygous and hemizygous offspring (Conner *et al.* submitted) suggesting that the inactivation of each allele did not occur independently. If the phenotypic expression of a transgene, when present in

homozygous and hemizygous nature is stable in transgenic plants, selfings of and crosses between these plants were not expected to give gene inactivation.

From a biosafety point of view these inactivation phenomena do not have a negative impact *per se* because the transgenes are no longer expressed. For economic use, breeding and seed multiplication of transgenic crops, reliable phenotypic expression of transgenes is a prerequisite. Therefore, it is appropriate to assess the extent in which the phenotypic expression of transgenes follows Mendelian segregation ratios (James *et al.* 1995). Studies on partial or complete transgene inactivation and elucidation of the underlying mechanisms are important when transgenic plants are to be used in applied plant breeding programs (Meyer 1995).

In this study, the transmission of the phosphinothricin tolerance was determined in successive generations of selfings, in mutual crosses of a number of independent transformants and in backcrosses of transformants with non-transformed plants. Harvesting of leaf material before spraying with the herbicide made it possible to perform a DNA analysis of PPT-sensitive plants. The results give an indication of the infrequent occurrence of inactivation of the phosphinothricin tolerance transgene.

Materials and methods

Plant material

Four independent phosphinothricin-tolerant oilseed rape (*B. napus*) transgenic R_1 populations of cv. Drakkar obtained after selfing of 4 transgenics, designated TP1, TP2, TP3 and TP4 (kindly provided by Dr. P. Rüdelsheim, Plant Genetic Systems, Ghent, Belgium) were used. These lines were transgenic for a T-DNA insertion locus conferring resistance to kanamycin and to the active ingredient of the herbicide Basta®/Radicale®, phosphinothricin (PPT). R_1 populations were expected to segregate 3 to 1 for PPT tolerance: sensitivity. Furthermore, transgenic PPT-tolerant *B. napus* cv. Westar plants, also kindly provided by Dr. P. Rüdelsheim, were used. This 'Westar T5' line was shown to be a single-copy transformant homozygous for the *bar* gene (Baranger *et al.* 1995). All transformants contained the *bar* gene (De Block *et al.* 1987; Thompson *et al.* 1987) from *Streptomyces hygroscopicus*. This encodes an acetyltransferase that inactivates PPT by acetylation of a free NH_2-group. As non-transgenic parent, *B. napus* cv. Drakkar was used.

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Experiments

Crosses that were made could be divided into two categories:

- within-variety transgenic oilseed rape

    - selfings and backcrosses with non-transgenic plants

    - between independent transformants

- between-variety transgenic oilseed rape.

A survey of the crosses made together with the aim of each cross is given in Table 3.1.

For crosses, female parents were emasculated 2 to 24 h prior to pollination. The emasculated pollinated flowers were bagged for three days. For the selfings, using closed flowers, this was a week to prevent uncontrolled cross-pollination. The experiments with transgenic oilseed rape were conducted according to Dutch regulations using a pollen cage placed in a greenhouse. Success of the crosses did not depend on the time of the day they were performed.

Table 3.1 Survey of the crosses made and the aim of the cross

* within-variety with *B. napus* 'Drakkar'

- ⊗ homo- and hemizygous phosphinothricin-tolerant plants from different transformants

<u>Aim</u>: to study inheritance and stability of the phenotypic expression of a transgene after selfing

- crosses between independent homozygous phosphinothricin-tolerant transformants

<u>Aim</u>: to study the effect of the introduction of a T-DNA locus in a recipient genome containing the same T-DNA locus in the same genetic background at a different position

* between-variety with *B. napus*

    B. napus 'Drakkar' x *B. napus* 'Westar'

<u>Aim</u>: to study transgene expression in the progeny of a cross between two homozygous phosphinothricin-tolerant oilseed rape varieties containing the same T-DNA locus in a different genetic background at a different position

Techniques

- Phosphinothricin tolerance tests

Tests to screen for PPT tolerance were performed as described earlier (Metz *et al.* 1995). In the present study, (transgenic) plants were sprayed as uniformly as possible with several Radicale® (150 gl^{-1} PPT) concentrations to determine an optimized Radicale® concentration for the selection of tolerant plants.

- Pollen stainability

Freshly harvested pollen was stained with Alexander's stain (Alexander 1969). Pollen stainability was expressed as the percentage of red pollen grains.

- DNA analysis

The DNA extraction method, the labelling system and the Southern blot analysis were described earlier (Metz *et al* 1995). As probes radioactively labelled *AphA2* (Koncz & Schell 1986) or *bar* (Wilmink 1996) gene fragments were used.

- Statistical analysis

Phenotypic classes were tested for goodness-of-fit. P-values for significant differences were calculated using a χ^2 test with $P < 0.05$.

- Herbicide spray and DNA analysis of PPT-sensitive plants

Pilot experiments have been made with different concentrations of the herbicide Radicale®. Transgenic, PPT-tolerant plants survived a treatment with 1, 0.5 and 0.05% (Fig. 3.1A), although especially at 1 and 0.5%, they showed a retarded growth compared to un-treated plants. Non-transgenic plants died within 12 days after the Radicale® treatment (Fig. 3.1B), while spraying with a solution of 5% Radicale® was also for the transgenic plants too heavy for survival. Spraying with different herbicide concentrations did not influence the quality and the quantity of the pollen produced in surviving plants (data not shown). For the experiments, it was decided to use 0.5% or 1% Radicale® for selection of PPT-tolerant progeny plants.

In case a progeny was completely sensitive, new seeds were sown. To be able to perform a DNA analysis of PPT-sensitive plants from all resown plants one or two leaves were harvested and kept at -80 °C before spraying with PPT. After spraying the PPT-sensitive plants could be identified and from their stored leaf material DNA was isolated. In this way, leaf material of these plants was saved for further analysis.
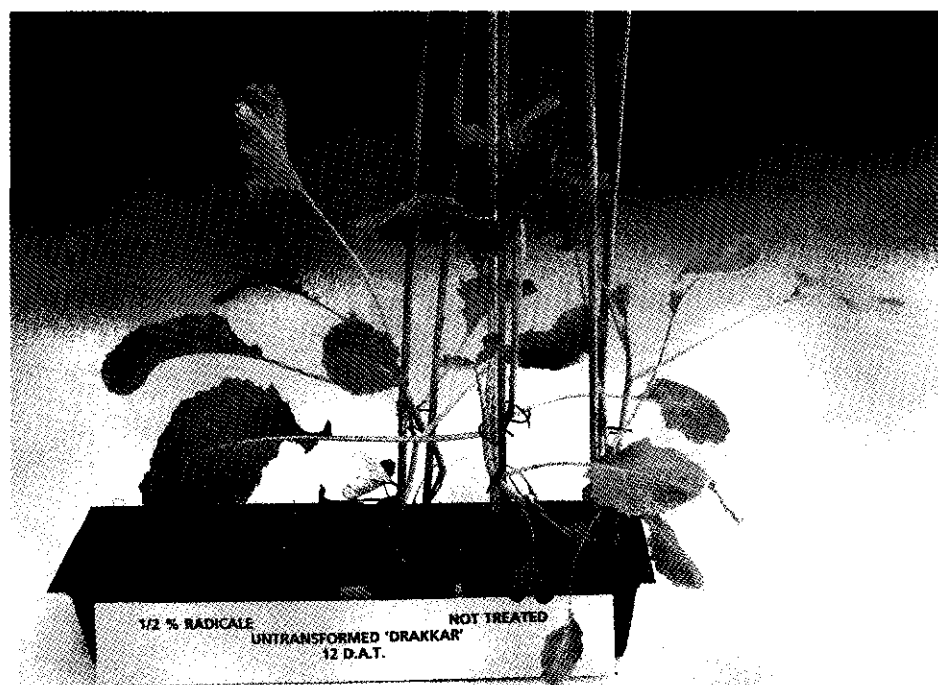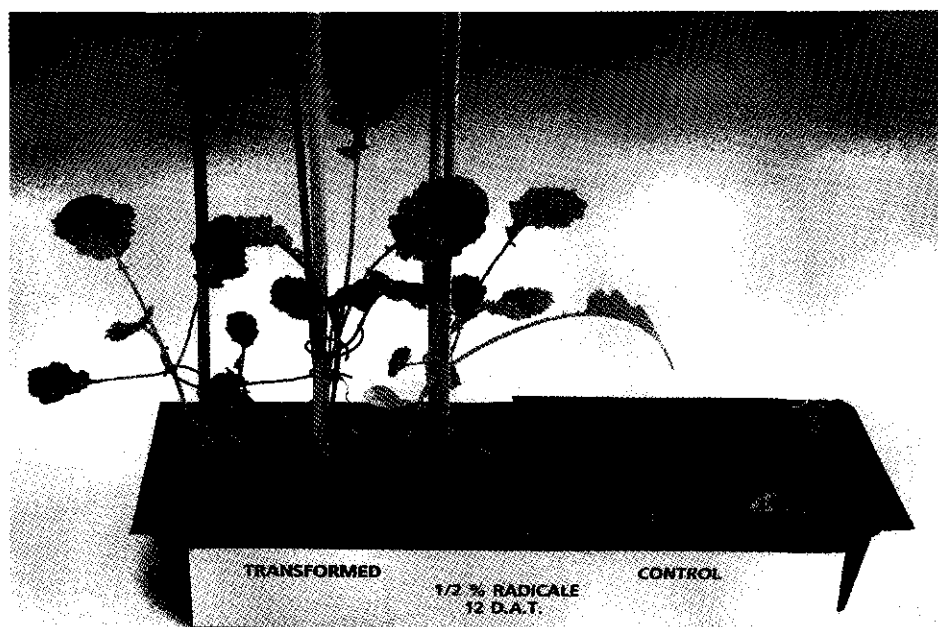
Figure 3.1 A-B Transgenic phosphinothricin-tolerant (A) and non-transgenic (B) oilseed rape cv. Drakkar after spraying with 0.5% Radicale, 12 days after treatment. Control means non-transgenic wildtype cv 'Drakkar' oilseed rape.

Results

- Monogenic inheritance of the PPT tolerance

Spraying of 30-45 plants of each of the four transgenic R_1 populations, showed that in all populations about 75% of the plants was PPT-tolerant as expected for a normal monogenic Mendelian segregation (Table 3.2). Backcrosses of a PPT-tolerant plant from two of these R_1 populations, TP2 and TP3, with non-transgenic oilseed rape gave an expected segregation ratio not deviating of 1:1 (Table 3.2).

Table 3.2 Number and percentage of phosphinothricin (PPT) tolerant plants in four transgenic R_1 populations (TP1 to TP4), in two backcross populations with non-transgenic 'Drakkar' (WT) and in the two non-transgenic varieties 'Drakkar' and 'Westar' of oilseed rape (*B. napus* L.) after spraying with 0.5% Radicale (150 gl^{-1} PPT).

| Population | Number | | Percentage | $\chi^2_{(3:1)}$ | $\chi^2_{(1:1)}$ |
	Tolerant	Sensitive			
'Drakkar'	0	36	0		
'Westar'	0	18	0		
TP1	35	12	75	0.95 > P > 0.90	
TP2	35	11	76	0.90 > P > 0.80	
TP3	22	7	76	0.95 > P > 0.90	
TP4	30	7	81	0.40 > P > 0.30	
WT x TP3	23	21	52		0.90 > P > 0.75
WT x TP2	15	18	46		0.75 > P > 0.60

- (In)stability of PPT tolerance

To study the stability of PPT-tolerance, the number of PPT-tolerant plants was determined in the sexual offspring of homozygous transgenic oilseed rape after selfings and in backcrosses with non-transgenic plants. The percentages of PPT-tolerant plants in the progeny during three generations of selfing are shown in Table 3.3. The progenies from plants TP1-101 and TP3-25 were already homozygous. After two more selfings their progenies remained, as expected, entirely PPT-tolerant using a PPT concentration of 0.5%. Although all TP1-101-2 plants did survive a treatment with 1% Radicale, using this concentration, two TP1-101-2-1 plants did not. This is in contrast to what was found for TP3 selfings.

Table 3.3 The number and percentage of phosphinothricin tolerant plants in three successive generations (S_1 to S_3) of selfed phosphinothricin tolerant oilseed rape originated from different transgenic populations (TP) and backcrosses to non-transgenic oilseed rape (NT) after spraying with 0.5% or 1% Radicale (150 gl^{-1} PPT). NT control plants did not survive either treatment. [1] Coding indicates that a PPT-tolerant progeny plant is again selfed, f.i. tolerant plant 2 from the progeny of TP1-101.

Generation	0.5%			1%		
Selfing	Tolerant	Sensitive	%	Tolerant	Sensitive	%
S_1, TP1-101	29	0	100			
S_2, TP1-101-2[1]	17	0	100	27	0	100
S_3, TP1-101-2-1	18	0	100	37	2	95
S_1, TP2-6	22	2	92			
S_2, TP2-6-2	22	0	100	8	0	100
S_3, TP2-6-2-2	22	0	100	40	3	93
S_1, TP2-9	13	10	57			
S_2, TP2-9-2	18	0	100	24	0	100
S_3, TP2-9-2-2	0	41	0	0	35	0
S_1, TP3-25	32	0	100			
S_2, TP3-25-3	27	0	100	6	0	100
S_3, TP3-25-3-16	7	0	100	9	0	100
S_1, TP4-14	23	6	79			
S_2, TP4-14-2	24	0	100			
Backcross						
NT x 2-6-2-2	25	0	100			
NT x 2-9-2-2	0	29	0			
NT x 3-25-3-16	23	0	100			

The progenies of the two plants of TP2 (2-6 and 2-9) showed neither a 1:0 nor a 3:1 ratio of PPT-tolerant and -sensitive plants, as expected for a homozygous or hemizygous plant, respectively. The occurrence of PPT-sensitive plants in the offspring of TP2-6 and TP2-9 might be due to a reduced transgene expression in individual plants. The TP4 progeny gave the expected 3:1 PPT tolerant:sensitive segregation ratio, indicating TP4-14 was hemizygous. After one extra selfing homozygous plants were identified in the progenies of TP2-6 (TP2-6-2), TP2-9 (TP2-9-2) and TP4-14 (TP4-14-2) on the basis of their

100% PPT-tolerant progenies irrespective of the Radicale dose used. However, after one more selfing of a tolerant progeny plant (2-6-2-2) using 1% Radicale, a few of its progeny plants was no longer PPT-tolerant in contrast to a treatment with 0.5%. Although TP2-9-2 was found to be homozygous, the selfed progeny of one of the surviving plants (TP2-9-2-2) was completely sensitive to both a 0.5 and 1% PPT treatment. These results were confirmed by the presence or absence of PPT-tolerant progeny plants in the backcrosses with non-transgenic oilseed rape. All plants of the backcross with TP2-9-2-2 were sensitive and those of both backcrosses with TP2-6-2-2 and TP3-25-3-16 were tolerant.

From the 41 sensitive TP2-9-2-2 selfing and the 29 sensitive TP2-9-2-2 backcross progeny plants, leaf material of respectively 7 and 5 randomly chosen plants was rescued as earlier described and tested on Southern blot using a *bar* probe. They all showed the 3 kb band, characteristic for TP2, indicating presence of the *bar* gene in these plants.

- Combination of different T-DNA inserts by intra-specific crosses
Within-variety crosses between independent transformants
From all four TP populations homozygous plants were selected on the basis of the absence of segregation for PPT tolerance after selfing (see Table 3.3). These plants were used
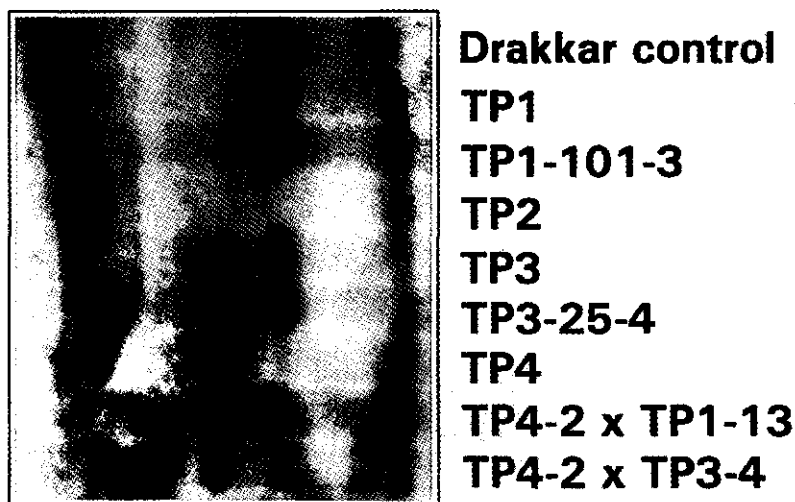

Drakkar control
TP1
TP1-101-3
TP2
TP3
TP3-25-4
TP4
TP4-2 x TP1-13
TP4-2 x TP3-4

Figure 3.2 DNA blot hybridization showing the specific banding pattern for plants from four independent phosphinothricin-tolerant oilseed rape (*B. napus*) transgenic R_1 populations of cv. Drakkar, designated TP1, TP2, TP3 and TP4 and from some crosses made with these plants using *bar* gene fragments as probe.

Table 3.4 The percentage phosphinothricin (PPT)-tolerant plants after crosses between homozygous and hemizygous PPT-tolerant oilseed rape plants originated from four independent transgenic populations (TP) after spraying with 0.5% Radicale (150 gl^{-1} PPT). N = number of plants tested. [1] Hemizygous TP4 plant.

♀ \ ♂	TP1	TP2	TP3	TP4
TP1	-		100 (N=48)	100 (N=4)
TP2	100 (N=47)	-	100 (N=51)	
TP3			-	
TP4	100 (N=21)		100 (N=49)	-
TP4[1]	100 (N=21)	100 (N=105)	100 (N=87)	

for the following crosses: TP1 with TP3 and TP4, TP2 with TP1 and TP3 and TP4 with TP1 and TP3. Furthermore, a hemizygous TP4 plant was crossed with a homozygous plant of each of the three other populations. After testing of the progeny plants, it was shown that in all cases these progenies were entirely PPT-tolerant (Table 3.4). This is in accordance with the expectation of no segregation for PPT tolerance. Also, when in the crosses one hemizygous crossing parent was involved, no PPT-sensitive plants were obtained in the progeny. On Southern blot using *bar* gene fragments as probe, all TP1, 2, 3 and 4 plants showed a banding pattern specific for each transformation event (Fig. 3.2). Combinations of plants from two TPs could be identified, showing the cumulative banding pattern of both TP parents.

Crosses between different varieties

In the progeny of crosses between homozygous PPT-tolerant variety 'Westar' plants and homozygous PPT-tolerant TP1 and TP3 plants of variety 'Drakkar' the number of PPT-tolerant offspring plants was determined. The number of plants tested for both crosses was 68 and 108, respectively and all progeny plants were PPT-tolerant as expected (Table 3.5). From both progenies, randomly, six plants were selected, which were analysed on a Southern blot. Hybridization with the *bar* probe showed for all progeny plants the two expected banding patterns of either parents. So, all bands were present and there was no loss of PPT tolerance in the progeny.

Table 3.5 Number and percentage of phosphinothricin (PPT)-tolerant plants in the progeny of crosses between plants of homozygous PPT-tolerant 'Drakkar' (TP1 and TP3) and 'Westar' (T5.5) oilseed rape varieties after spraying with 0.5% Radicale (150 g l^{-1} PPT). Plants of the three transgenic populations used in the crosses and non-transgenic (NT) 'Drakkar' and 'Westar' were sprayed for positive and negative control, respectively.

| Genotype/Cross | Number | | Percentage |
	Tolerant	Sensitive	
TP1-101-1	17	0	100
TP3-25-3	15	0	100
T5.5	15	0	100
TP1-101-1 x T5.5	68	0	100
TP3-25-3 x T5.5	108	0	100
NT 'Drakkar'	0	25	0
NT 'Westar'	0	16	0

Discussion

PPT tolerance of the transformants was shown to be dominant and monogenic. The segregation ratios indicated that the plants of the four transgenic PPT-tolerant oilseed rape R_1 populations contained a single T-DNA insertion locus. If two or more (unlinked) T-DNA loci would be involved, other segregation ratios of tolerant to sensitive plants should be expected (Heberle-Bors et al. 1988; Cherdshewasart et al. 1993).

In selfings and backcrosses involving transgenic PPT-tolerant TP1 and TP2 plants, deviating segregation ratios in PPT tolerant:sensitive were observed. In some selfings there was partial loss of phenotypic expression of PPT tolerance, which in the S_3 generation depended on the PPT concentration used. A more stringent selection resulted in a few sensitive plants possibly due to a weaker phenotypic expression of the transgene in some individual offspring plants. The sensitive plants could not be saved after the PPT treatment. The reduced phenotypic expression in both the S_1 and S_3 generation might be the result of somaclonal variation. In *Petunia* and tobacco either complete loss of the transgene, weak or variable phenotypic expression of the gene or non-Mendelian segregation ratios among seedlings have been observed resulting in irregular segregation patterns (Heberle-Bors et al. 1988; Deroles & Gardner 1988a).

In case of TP2-9-2-2 (Table 3.3), there was a complete loss of PPT tolerance both in the progeny after one generation of selfing and in the progeny of the backcross of a TP2-9-2-2 plant with the wild type *B. napus*. Southern analysis of selfing and backcross progeny plants of TP2-9-2-2 from newly sown seeds showed that there was loss of phenotypic expression of PPT tolerance despite the presence of the transgene. Possible explanations for the discrepancy between the presence of the transgene and absence of phenotypic expression of PPT tolerance might be methylation or co-suppression as reported in other studies (Jorgensen 1990; Matzke & Matzke 1991; Kilby *et al.* 1992; Matzke *et al.* 1993; Ingelbrecht *et al.* 1994). Other reports did not indicate a correlation between (reversible) gene inactivation and methylation, but the discrepancy of the presence of the transgene and phenotypic expression was the result of a reduced level of transcription of the transgene in the sensitive transformants (Mittelsten Scheid *et al.* 1991). Because only selfing of TP2-9-2-2 and not of TP1, TP3 or TP4 resulted in this gene inactivation, this could indicate that the integration position of the T-DNA locus is involved. Chromosomal position effects of the transferred gene have earlier been described for the maize A1 gene in *Petunia hybrida* (Linn *et al.* 1990). Because the transgene is present, instability and subsequent deletion of the inserted T-DNA locus can not explain the results obtained.

Another explanation for the complete gene inactivation leading to a loss of the phenotypic expression of PPT tolerance is a combination of a position effect of the T-DNA locus integration and the occurrence of somaclonal variation. When only somaclonal variation is involved, the result will be a reduced phenotypic expression. In our experiments in the case where complete gene inactivation was found, the loss of phenotypic expression of the transgene was independent from homozygosity or hemizygosity. Further studies on the plants that lost phenotypic expression of the PPT tolerance have to clarify the underlying mechanisms involved. Determination of the copy number, the presence and number of inverted repeats and the transcription level may give additional information. Treatment with a demetylating agent can indicate whether the suppression of gene expression was due to methylation or not. The possible influence of position effects of the T-DNA locus integration and/or somaclonal variation is difficult to study.

In crosses between independent homozygous PPT-tolerant 'Drakkar' plants and between homozygous PPT-tolerant 'Drakkar' and 'Westar' plants, which have different

genetic backgrounds, no gene inactivation was observed within the number of progeny plants tested. Southern blot analyses of progeny plants of some within- and between-variety crosses showed that the inserted T-DNA loci of both parents were present. The transgenes while both present, did not interact in such a way that altered phenotypic segregation ratios were found due to (partial) loss of expression of PPT tolerance, indicating that *trans*-inactivation does not occur often in our plant material. This is in contrast to the observation in *Petunia hybrida* where introduction of additional genes could lead to suppression of both the transformed and the corresponding endogenous genes (Napoli *et al.* 1990; van der Krol *et al.* 1990).

Meaningful predictions about long-term stability of transgenic DNA seem to be difficult and require better understanding of the mechanisms that regulate chromosome stability. Molecular cytogenetics might help to elucidate these mechanisms. In our study, within- and between-variety crosses did not show altered phenotypic segregation ratios, so this will not cause problems during breeding activities. However, in subsequent generations of selfing in some cases (partial) loss of phenotypic expression was observed, disturbing reliable transgene expression in plants necessary for a sound economic use, for instance during seed multiplication of varieties and in breeding programs. During seed multiplication spraying with the herbicide is no longer selective and thus might cause problems. The loss of phenotypic expression is not associated with the absence of the transgene and therefore, Southern blot analysis is no alternative for testing the occurrence of this loss. Thus, during seed multiplication and in breeding programs it should be tested whether selected lines stably express PPT tolerance during successive generations or not.

The observation that the level of transgene expression in transgenic plants varies between different transformants makes it necessary to generate a large number of independent transformants and to screen them for those plants that have the appropriate expression level. This labor intensive procedure can possibly be avoided by inclusion of nuclear scaffold or matrix-associated regions (MAR) flanking the transgene. In earlier studies, these MARs increased the expression level and reduced the variability in transgene expression in tobacco plants (Stief *et al.* 1989; Breyne *et al.* 1992; Allen *et al.* 1993; Mlynárová *et al.* 1994; 1995).

Molecular breeding does simplify the introduction of new traits, but to guarantee stable expression of such transgenes, breeders have to follow similar time-consuming selection

procedures as required for the generation of new lines and for seed multiplication in conventional breeding programs.

Acknowledgements

Chapter 4

The impact on biosafety of the phosphinothricin tolerance transgene in inter-specific *B. rapa* x *B. napus* hybrids and their successive backcrosses

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Summary

There is strong evidence indicating that gene flow from transgenic *B. napus* into weedy wild relatives is inevitable following commercial release. Research should now focus on the transmission, stability and impact of transgene expression after the initial hybridization event. The present study investigated the transfer of a phosphinothricin tolerance transgene by inter-specific hybridization between *B. rapa* and two transgenic *B. napus* lines. The expression of the transgene was monitored in the F_1 hybrids and in subsequent backcross generations. The transgene was transmitted relatively easily into the F_1 hybrids and retained activity. Large differences in the transmission frequency of the transgene were noted between offspring of the two transgenic lines during backcrossing. The most plausible explanation of these results is that the line showing least transmission during backcrossing contains a transgene integrated into a C-genome chromosome. Approximately 10% of offspring were retained the tolerant trait in the BC_3 and BC_4 generations. The implications of these findings for the stable introgression of transgenes carried on one of the chromosomes of the C-genome from *B. napus* and into *B. rapa* are briefly discussed.

Introduction

The genus *Brassica* includes several economically important species, such as *B. oleracea*, *B. rapa*, *B. napus*, *B. juncea* and *B. nigra*. The genomic relationships of these *Brassiceae* are described in the so-called triangle of U (1935). The A-genome occurs in *B. juncea*, *B. napus* and *B. rapa*, which are all grown for oil production. Oilseed rape (*B. napus*) is allotetraploid with the genome constitution AACC (2n=38). The cytogenetic relationships were confirmed by nuclear DNA content (Verma & Rees 1974), DNA analysis (Erickson *et al.* 1983) and genome-specific chromosome markers (Hosaka *et al.* 1990).

The production of transgenic *Brassicas* has raised the question of whether transgene dispersal into natural populations can be expected or not (Metz *et al.* 1997). In the Netherlands, (semi)spontaneous populations of *B. rapa* are found in the wild. These might be regarded as wild relatives of *B. napus*. In Denmark and Canada, *B. rapa* is a common weed in cultivated areas, mostly in oilseed rape fields.

Crosses between *B. rapa* and *B. napus* are frequently described as successful (U 1935; Palmer 1962; Nwankiti 1971; MacKay 1977; Beversdorf *et al.* 1980). Spontaneous hybridization with *B. napus* was observed in agricultural fields (Bing *et al.* 1991; Jørgensen & Andersen 1994; Jørgensen *et al.* 1996a). In the Netherlands spontaneous hybridization also occasionally occurs in nature (De Vries *et al.* 1992). In addition, under open pollination conditions small amounts of viable seeds from the hybrid plants were obtained, indicating that hybrids are able to survive to the next generations (Bing *et al.* 1991). Furthermore, it has been shown that inter-specific hybrids can backcross as female with *B. rapa* (Mikkelsen *et al.* 1996a; b), even under field conditions (Jørgensen *et al.* 1996a).

Most of the hybrids between *B. rapa* and *B. napus* possess 29 chromosomes. At metaphase I of meiosis most pollen mother cells have been shown to contain 10 bivalents, presumably between the A-genome chromosomes and 9 univalents, representing the C-genome chromosomes (U 1935; Beversdorf *et al.* 1980). During evolution of *Brassica* species, the chromosome structure seems sufficiently conserved for the potential occurrence of homoeologous pairing between chromosomes of the A and C genomes. Meiosis in hybrids of *B. napus* and *B. rapa* and in backcross generations, gives the opportunity for genomic recombination leading to introgression of (trans)genes (Quiros *et al.* 1994; Jørgensen *et al.* 1996b; Mikkelsen *et al.* 1996a; b). Introgression of traits by breeding has

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been reported from *B. rapa* into *B. napus* (MacKay 1977; Gowers 1982; Goring *et al.* 1992). Also reciprocally, there were reports of introgression of cold tolerance and black-rot resistance from *B. napus* into Pak choi and Chinese cabbage (Guo *et al.* 1990; Heath *et al.* 1994).

There is strong evidence that gene flow from *B. napus* and introgression into weedy relatives is inevitable (Timmons *et al.* 1995; 1996; Mikkelsen *et al.* 1996a; Kerlan *et al.* 1993), and so research on transgenic *Brassica* species should now focus on the impact and stability of transgene expression and its fate after inter-specific hybridization (Metz & Nap 1997). In our study, transgenic phosphinothricin-tolerant *B. napus* plants were crossed under controlled conditions with the *B. rapa* representatives Pak choi and Chinese cabbage to investigate whether the transgene could be transferred to the inter-specific hybrid and whether it remains active. In successive generations of backcrosses with *B. rapa*, the expression and fate of the transgene was monitored.

Materials and methods

Plant material

- Transgenic material

Two phosphinothricin (PPT)-tolerant oilseed rape (*B. napus*) transgenic R_1 populations obtained after selfing of two primary transformants of cv. Drakkar, were kindly provided by Dr. P. Rüdelsheim (Plant Genetic Systems, Ghent, Belgium). These populations were designated TP2 and TP3, respectively. Both lines were transgenic for a T-DNA insertion containing 3'ocs-*NPTII*-neo and pSsuAra-*bar*-3'g7 conferring kanamycin resistance and phosphinothricin - the active ingredient of Basta®/Radicale® - tolerance, respectively. For both populations it was not known whether the transgene locus was located on chromosomes of the A- or of the C-genome (Rüdelsheim pers. comm.). The two lines were the result of independent transformation events.

PPT tolerance is conferred by the *bar* gene (De Block *et al.* 1987; Thompson *et al.* 1987). The *bar* gene encodes an acetyltransferase that inactivates the PPT by acetylation of a free NH_2-group. PPT inhibits glutamine synthetase resulting in a rapid accumulation of ammonia leading to cell death (Tachibana *et al.* 1986).

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- Non-transgenic material

Non-transgenic oilseed rape cv. Drakkar, self-incompatible Pak choi (*B. rapa* Chinensis group [*B. chinensis*], origin China, accession number BC30.02) and self-compatible Chinese cabbage (*B. rapa* Pekinensis Group [*B. pekinensis*], origin China, accession number BC20.08) were used as crossing parents.

Crossing experiments

Twenty individual PPT-tolerant R_1 plants were selfed and two were crossed with non-transgenic *B. napus* to study the genetics of this trait. Crosses of Pak choi and Chinese cabbage were performed with two transgenic, PPT-tolerant *B. napus* R_1 plants, which were hemizygous for this trait. All plants that were used as female parent were emasculated and the pollinated flowers were bagged for three days prior to pollination to prevent uncontrolled cross-pollination.

The PPT-tolerant inter-specific hybrids were used as male parent and backcrossed with Pak choi or Chinese cabbage, producing BC_1 generations segregating for PPT tolerance. With Pak choi as female and PPT-tolerant backcross plants as male parent, another three rounds of backcrosses were made resulting in BC_2, BC_3 and BC_4 populations segregating for PPT tolerance. A schematic presentation of the crosses is given in Fig. 4.1. Crosses were conducted in a pollen cage, placed in the greenhouse, with underpressure to avoid transgenic pollen spread by air flow and a double-door entrance to prevent insects entering the cage, according to regulations ordered by the Dutch Committee of Genetic Modification (COGEM).

Techniques

- Phosphinothricin tolerance test

Plants, at the two- to four-leaf stage were sprayed from approximately 20 cm distance, using a normal household plant spray, with 0.5% Radicale® (150 gl^{-1} PPT) as uniformly as possible. Alternatively, leaf discs of hybrids were tested for the activity of the PPT tolerance gene non-destructively on MS-10 containing 7.5 mgl^{-1} Radicale® and 50 mgl^{-1} chlorophenol red (Metz *et al.* 1995). This test made it possible to screen the PPT-sensitive plants for presence or absence of the transgene.

B. rapa, A^RA^R, hh x B. napus, A^NA^NCC, H.
Pak choi
Chinese cabbage   ↓ PPT tolerance selection

B. rapa, A^RA^R, hh x F_1, A^RA^NC, Hh

↓ PPT tolerance selection

B. rapa, A^RA^R, hh x BC_1, A^RA^N or A^RR^A + (C), Hh

↓ PPT tolerance selection

B. rapa, A^RA^R, hh x BC_2, A^RA^N or A^RR^A + (C), Hh

↓ PPT tolerance selection

B. rapa, A^RA^R, hh x BC_3, A^RA^N or A^RR^A + (C), Hh

↓ PPT tolerance selection

BC_4, A^RA^N or A^RR^A + (C), Hh

Figure 4.1 Crossing scheme. Phosphinothricin (PPT)-tolerant inter-specific hybrid plants were backcrossed to *B. rapa* followed by three more backcrosses of PPT-tolerant plants on *B. rapa*. A^R and A^N represent the A-genome of *B. rapa* and *B. napus*, respectively. (C) indicates that only part of the C-genome may be present containing the PPT toleran- ce. H. indicating the presence of PPT tolerance in either homozygous (HH) or hemizy- gous (H0) configuration; hh indicates the absence of the *bar* gene implying herbicide sensitivity.

- Pollen stainability

Pollen stainability was assessed by staining freshly harvested pollen with Alexander stain (Alexander 1969). Ten samples were collected and per sample 100 pollen grains were counted. Stainability was expressed as the number of red pollen grains per number of pollen grains that was counted.

- Flow cytometry

Preparation of the nuclear samples and flow cytometry were performed as described by Bino *et al.* (1993). The fluorescence signals are presented as frequency distribution histograms, the DNA amount being expressed as relative C values. The 1C value repre-

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sents the DNA amount of the unreplicated haploid chromosome complement, which is for oilseed rape AC.

- Southern analysis

Southern analysis was conducted according to the method described by Metz *et al.* (1995). In brief, about 5 μg DNA, extracted from young leaf tissue (Dellaporta *et al.* 1983), was digested using *HindIII* and run on a 0.8% agarose gel overnight. DNA was transferred onto a Hybond N$^+$ filter (Amersham) by vacuum blotting (Pharmacia Bio-tech). Probes labelled with ^{32}P were hybridized onto the filter, washed with 2xSSC (with 1% SDS) and exposed to Kodak X OMAT-AR or Fuji RX films. The hybrid nature of the putative hybrid plants was analysed using *AphA2* (Koncz & Schell 1986) or *bar* (Wilmink 1996) gene fragments as probes.

- Statistical analysis

Phenotypic segregation ratios were tested for goodness-of-fit by χ^2 tests.

Results

The original transgenic R_1 PPT-tolerant *B. napus* TP2 and TP3 plants were sprayed with 0.5% Radicale. Selfings of individual tolerant R_1 plants resulted in R_2 populations and crosses between individual R_1 plants and non-transgenic *B. napus* were made in order to study the genetics of this transgenic trait. Inter-specific hybrid plants of either Pak choi or Chinese cabbage and PPT-tolerant *B. napus* and successive backcross populations were analysed for the presence and expression of the *bar* gene conferring PPT tolerance. Furthermore, plants from different generations were analysed using flow cytometry.

Fate of herbicide tolerance

- Intra-specific crosses

None of the 54 non-transgenic *B. napus* control plants survived the PPT spray. Forty-six and 29 randomly chosen plants of the primary R_1 transgenic *B. napus* TP2 (92.2) and TP3 (92.3), respectively, were tested for PPT tolerance by spraying with Radicale. These populations displayed a segregation ratio PPT- tolerant:sensitive plants not deviating significantly from a 3:1 ratio (Table 4.1). Selfed progenies of selected PPT-tolerant R_1 plants yielded, as expected, a 1:0 or 3:1 segregation (data not shown). This indicated that

Table 4.1 Number and percentage of the observed and expected phosphinothricin (PPT)-tolerant plants in transgenic R_1 populations (TP) and in intra-specific backcrosses after spraying with 0.5% Radicale (150 gl^1 PPT). T and S are PPT-tolerant and sensitive plants respectively. WT means wild type *B. napus*.

Population (number) /Cross	Observed			Expected probability	
	Number		Percentage	$\chi^2_{1:1}$	$\chi^2_{3:1}$
	T	S	T		
WT	0	54	0		
TP3 (92.3)	22	7	76		0.90-0.95
TP2 (92.2)	35	11	76		0.80-0.90
WT x 92.3.20	23	21	52	0.75-0.90	
WT x 92.2.12	15	18	46	0.60-0.75	

the PPT-tolerant R_1 plants were either homozygous or hemizygous for a single T-DNA insertion expressing PPT tolerance.

A cross between a non-transgenic 'Drakkar' *B. napus* plant with a hemizygous PPT-tolerant TP2 R_1 plant (92.2.12), yielded 33 plants of which 15 were PPT-tolerant. The same cross using a hemizygous PPT-tolerant TP3 R_1 plant (92.3.20) gave 23 tolerant plants of 44 plants tested. This is again in agreement with a monogenic (1:1) segregation ratio (Table 4.1).

- Inter-specific hybrids

Controlled inter-specific crosses and backcrosses were performed with the transgenic allo-tetraploid species *B. napus*. The PPT-tolerant inter-specific hybrid or backcross plants were male and Pak choi or Chinese cabbage plants were female parents. In gene dispersal from the (transgenic) crop to its wild relative, the initial hybridization event is most probably with *B. rapa* as the female parent. Crossing of Pak choi and Chinese cabbage with PPT-tolerant *B. napus* R_1 plants from TP3 (92.3.20) and Pak choi with TP2 (92.2.12) respectively, resulted in viable, fertile hybrids, denoted Pak choi*3, Chinese cabbage*3 and Pak choi*2. They were morphologically intermediate exhibiting traits from both parents. The hybrids had less trichomes on their leaves than *B. rapa*. The glaucous leaves resembled *B. napus* more than *B. rapa*. The inflorescence of the hybrid mirrored

the *B. napus* parent and had open flowers rising above the closed flower buds. The pollen stainability of the inter-specific hybrids was about 60%, while that of the parents was 100%. Backcrosses of the male-fertile hybrids with Pak choi or Chinese cabbage as female parent yielded over 600 seeds. However, selfing of Pak choi*2 and Chinese cabbage*3 hybrids did not yield any seed-bearing siliques. Selfings of Pak choi*3 hybrids were not performed.

Table 4.2 gives the number and percentage of PPT-tolerant hybrid plants in Pak choi*2 and Chinese cabbage*3. As expected from the selfings and test cross results, both inter-specific F_1 hybrid progenies segregated 1:1 for PPT tolerance:sensitivity. All PPT-tolerant F_1 plants which were tested by Southern blotting with an *AphA2* probe, displayed the presence of the transgene (Fig. 4.2). All PPT-tolerant Pak choi*2 hybrids showed the two bands characteristic for tolerant plants from TP2. Pak choi *3 and Chinese cabbage*3 hybrids showed the single band of tolerant plants from TP3. The results of the DNA analysis were in agreement with the chlorophenol red test. This indicated that the non-destructive *bar* enzyme activity determination was reliable. All 24 leaf discs of the three PPT-tolerant F_1 plants turned the colour of the medium into orange/yellow showing the presence of the active *bar* gene while the leaf discs of the PPT-sensitive F_1 plants and the non-transgenic *B. napus* coloured the medium purple.

- Backcrosses for indications of the presence of transgenes on chromosomes of the A- or C-genome
PPT tolerance could be transferred to the next generation by backcrossing PPT-tolerant Chinese cabbage*3 hybrids with Chinese cabbage or the PPT-tolerant Pak choi*3 hybrid with Pak choi (Table 4.2). In the first backcross the segregation ratio observed did not deviate significantly from a 1:1 ratio. This was expected when the PPT tolerance gene was present on one of the chromosomes of the A-genome of plants from TP3. Pollen stainability of Chinese cabbage*3 hybrid plants was about 20%.

By backcrossing PPT-tolerant Pak choi*2 hybrids with Pak choi, PPT tolerance could also be transferred to the next generation. However, only 26% of the progeny expressed PPT tolerance instead of 50%. The PPT tolerance:sensitivity segregation clearly deviated from 1:1, having a deficiency in the class of PPT-tolerant plants. This observation is different from the expected segregation if this trait was located on one of the chromo-

Table 4.2 Number and percentage of the observed and expected phosphinothricin (PPT) tolerant plants in inter-specific F_1's of transgenic *B. napus* with *B. rapa* (Pak choi, Pc or Chinese cabbage, Cc) and hemizygous PPT-tolerant *B. napus* plants from transgenic R_1 populations TP2 and TP3 and backcrosses (BC_n) onto *B. rapa*, after spraying with 0.5% Radicale (150 gl^{-1} PPT). T and S are PPT-tolerant and -sensitive plants, respectively.

Cross	Observed		Percentage	Expected probability $\chi^2_{1:1}$
	Number			
	T	S	T	
*Chinese cabbage*TP3*				
Cc x 92.3.20 (F_1)	6	6	50	1.00
Cc x F_1.1 (BC_1.1)	4	5		
Cc x F_1.2 (BC_1.2)	10	11		
Cc x F_1.3 (BC_1.3)	5	5		
Cc x F_1.4 (BC_1.4)	<u>14</u>	<u>17</u>		
BC_1 total	33	38	46	0.50-0.60
*Pak choi*TP3*				
Pc x 92.3.20 (F_1)	1	0	100	
Pc x F_1.1 (BC_1)	4	7	36	0.30-0.40
*Pak choi*TP2*				
Pc x 92.2.12 (F_1)	7	6	54	0.70-0.80
Pc x F_1.1 (BC_1.1)	9	30		
Pc x F_1.2 (BC_1.2)	7	23		
Pc x F_1.3 (BC_1.3)	15	40		
Pc x F_1.4 (BC_1.4)	<u>8</u>	<u>20</u>		
BC_1 total	39	113	26	<0.0005
Pc x BC_1.1.1 (BC_2.1)				
Pc x BC_1.3.1 (BC_2.2)				
Pc x BC_1.3.2 (BC_2.3)				
Pc x BC_1.4.2 (BC_2.4)				
BC_2 total	6	111	5	<0.0005
Pc x BC_2.1.2 (BC_3.1)				
Pc x BC_2.3.1 (BC_3.2)				
Pc x BC_2.4.2 (BC_3.3)				
BC_3 total	33	267	11	<0.0005
Pc x BC_3.2.1 (BC_4.1)	1	10	9	0.005-0.01

B. napus 'Drakkar'
Pak choi (Pc)
Chinese cabbage (Cc)
TP2
TP3
Cc x TP3 pl.1
Cc x TP3 pl.2
Cc x TP3 pl.4
Pc x TP3 pl.1
Pc x TP2 pl.1
Pc x TP2 pl.2
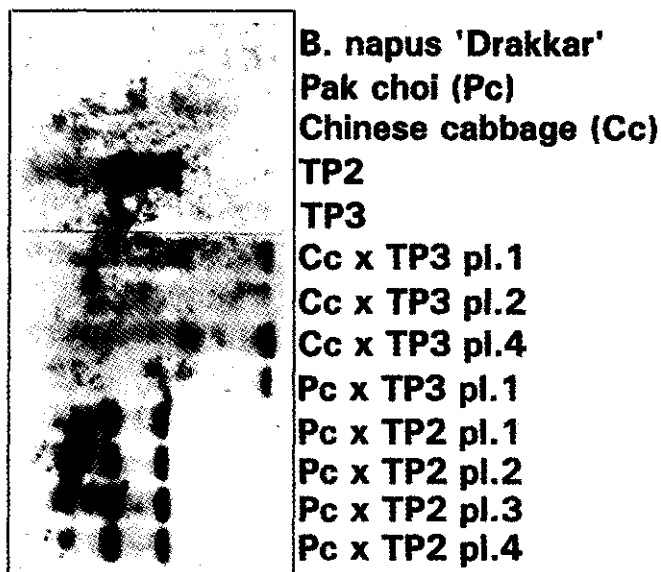**Pc x TP2 pl.3**
Pc x TP2 pl.4

Figure 4.2 Southern blot, using an *AphA2* probe, of non-transgenic *Brassica napus*, Pak choi, Chinese cabbage, plants from two independent transgenic *B. napus* populations (TP) and several F_1's of Pak choi or Chinese cabbage with transgenic *B. napus*.
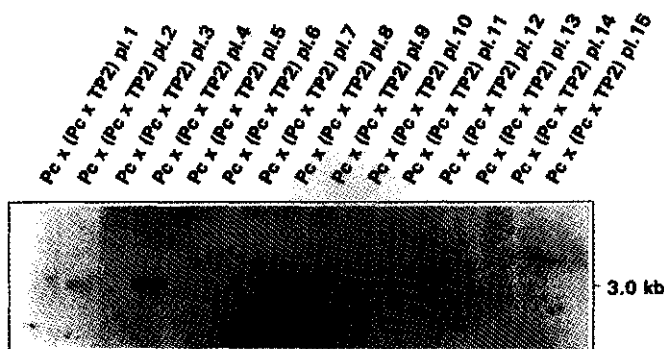

Figure 4.3 Southern blot, using a *bar* probe, of 15 randomly chosen Pak choi x (F_1, Pak choi x transgenic *Brassica napus*) BC_1 plants indicated as Pc x (Pc x TP2).

somes of the A-genome. On a Southern blot, using a *bar* probe, three PPT-tolerant Pak choi BC_1 plants out of 15 plants tested showed the band of TP2, which was not found for the 12 sensitive plants (Fig. 4.3). The pollen stainability of the BC_1 plants was about 40%.

In the successive BC_2 generation, using a PPT-tolerant BC_1 plant as male, only 5% of the plants was PPT-tolerant (Table 4.2). The male and female (self)fertility of the BC_2 plants was completely restored. The pollen stainability was found to be 100% and after selfing one of the tolerant BC_2 plants, viable seeds were obtained.

The following BC_3 generation, with a PPT-tolerant BC_2 plant used as male parent, gave only 11% PPT-tolerant plants, while for the BC_4, testing a limited number of plants, only one out of 11 plants was found to be PPT-tolerant (Table 4.2). A random sample of ten PPT-sensitive BC_3 plants was screened for the presence or absence of the PPT-tolerance gene on Southern blot. The PPT gene was absent in all ten PPT-sensitive plants. As expected, the BC_3 and BC_4 plants that survived a PPT spray resembled the Pak choi parent in morphology.

The number of PPT-tolerant plants transmitted to the BC_1 population produced with TP2 differed from those made with TP3. The most plausible explanation for these results is that in TP2 the PPT tolerance gene is present on one of the chromosomes of the C-genome. This explanation is supported by the low transmission percentages found in the BC_2, BC_3 and BC_4 generations.

Flow cytometric analyses
Flow cytometric analyses of a mixed sample with Pak choi, the Pak choi*2 hybrid and *B. napus* nuclei, showed that the peak of the hybrid was clearly between the peaks of both parents (Fig. 4.4A). This confirmed that the DNA content of the Pak choi*2 hybrid was intermediate between the DNA contents of Pak choi and *B. napus*, which are 1.05 pg/2C peak and 2.45 pg/2C, respectively (Arumuganathan & Earle 1991). This indicated that the inter-specific hybrid had the expected triploid genomic constitution (see also Fig. 4.1). Compared to this F_1 hybrid, the DNA content of the BC_1 plant showed a shift towards the Pak choi peak (Fig. 4.4B). The peaks of the tested BC_2 and BC_3 plants coincided with that of the recurrent Pak choi parent (Fig. 4.4C and D). It was not easy to define the presence of only 1 or 2 additional (parts of) C-chromosomes using flow cytometry.
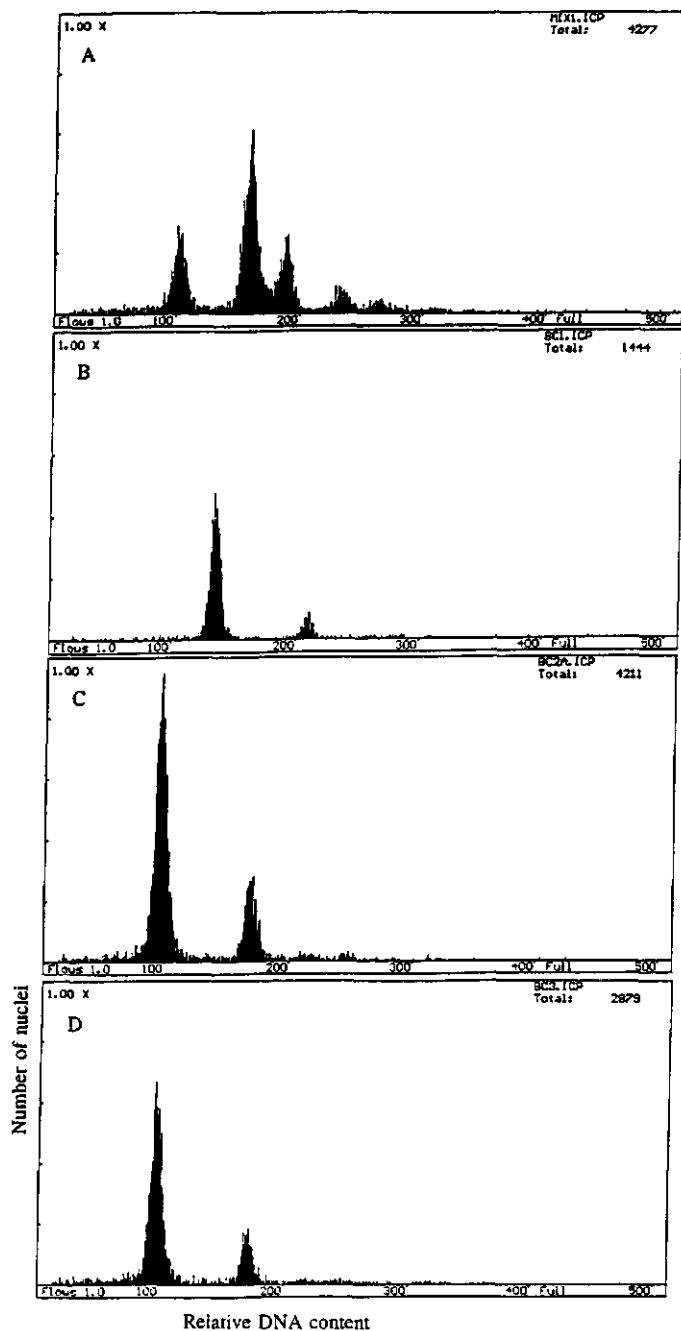
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Figure 4.4 A-D Relative DNA content. Histograms of flow cytometric analyses of nuclei from leaves of a mixed sample of Pak choi, the Pak choi-*Brassica napus* hybrid and transgenic *B. napus* (A) and the subsequent BC$_1$ (B), BC$_2$ (C) and BC$_3$ (D) on Pak choi. Nuclei from leaves show peaks at the 2C and 4C level. Totals refer to the number of nuclei measured.

Discussion

Crosses between transgenic, PPT-tolerant and non-transgenic *B. napus* and selfed pro-
genies of PPT-tolerant *B. napus* exhibited the normal Mendelian segregation. In both
populations (TP2 and TP3) PPT tolerance was inherited as a stable, single dominant trait.
From transmission genetics and male and female fertility of the transgenic *B. napus,*
introduction of the PPT tolerance gene did not indicate a fitness disadvantage due to the
transformation event. Controlled crosses between Pak choi and Chinese cabbage with
PPT-tolerant *B. napus* showed that the transgene could relatively easily be transmitted to
the inter-specific hybrids and that it was still active in the hybrid. This inter-specific
transfer occurred in an expected ratio and was confirmed by several analyses. The flow
cytometric analysis indicated that the genomic constitution of the hybrid was probably
triploid.

The formation of the *B. rapa* x *B. napus* hybrid in controlled crossing experiments has
been reported previously by several researchers (Palmer 1962; Nwankiti 1971; MacKay
1977; Mikkelsen *et al.* 1996a). In our study only controlled inter-specific crosses were
performed with the allotetraploid species as male parent. In subsequent backcrosses PPT-
tolerant (backcrossed) hybrid plants were used as male parent. The initial hybridization
event is most probably with *B. rapa* as female parent. In subsequent generations,
however, the hybrid will be more fertile as female rather than as male. It has been found
that *B. rapa* x *B. napus* hybrids could be good female parents (Mikkelsen *et al.* 1996a).
As expected, in agricultural fields and field trials spontaneous hybridization between both
species have also been observed (Bing *et al.* 1991; Jørgensen & Andersen 1994; Jørgen-
sen *et al.* 1996a; Mikkelsen *et al.* 1996a).

In the literature, hybrids regularly were reported to have good pollen production, but
they showed reduced fertility (Beversdorf *et al.* 1980). Values similar to the pollen staina-
bility of about 60% found here, were observed in other studies (MacKay 1977; McNaug-
hton 1973). Jørgensen & Andersen (1994) reported hybrid pollen stainability that ranged
from 16-86%, while in a later study (Jørgensen *et al.* 1996a) stainability was reduced to
35%. In our hands, pollen production and fertility of the hybrids were sufficient to obtain
viable BC_1 seeds, but not to obtain selfed progenies.

The 'Pak choi and Chinese cabbage BC_1' progeny using TP3 segregated 1:1 for PPT-

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tolerance, as expected. In this case the PPT tolerance gene was probably inserted in the A-genome. Previous studies have reported backcross plants with 2n=20 in controlled crosses between (*B. napus* x *B. rapa*) and *B. rapa* (Quiros *et al.* 1987; McGrath & Quiros 1990), and also (spontaneous) backcrossing under field conditions, in the first backcross generation producing *B. rapa*-like plants with 20 chromosomes and a high pollen fertility was found (Jørgensen *et al.* 1996a; Mikkelsen *et al.* 1996b). These data support the hypothesis that a transgene located on the A-genome of *B. napus* can be transferred to *B. rapa* within two backcross generations.

The 'Pak choi BC_1' progeny using TP2 yielded only 26% PPT-tolerant plants, while 50% was expected as a result of the earlier observed monogenic inheritance. The difference in transmission of PPT-tolerant plants in the BC_1 generations made with TP3 and TP2 must be due to the specific integration position of the transgene. The most plausible explanation for this deficiency in PPT-tolerant plants is the presence of the transgene on one of the chromosomes of the C-genome in TP2. In the backcrosses of the inter-specific hybrid to Pak choi the C-chromosomes have no homologous partners during meiosis. Due to irregular transmission of the single C-chromosomes to the gametes, a (trans)gene located on the C-genome of *B. napus* is expected to be transmitted at a low frequency in the gametes and, to be lost after one or a few generations. In studies with RFLP and isozyme markers, transmission of the C- chromosomes from inter-specific hybrids to BC_1 and F_2 populations was often found to be lower than 50% and varied between individual C-chromosomes (McGrath & Quiros 1990; Chen *et al.* 1990). Deviations from expected co-segregation of markers belonging to the same linkage group indicated the occurrence of the possibility of inter-genomic recombination or breakage of chromosomes (Mikkelsen *et al.* 1996b). Transmission of three out of 33 *B. napus*-specific RAPD markers from the inter-specific hybrid to the backcross progeny was significantly different from 50%.

Also in successive backcross generations with Pak choi, under selective conditions, much lower percentages of PPT-tolerant plants were found than normally expected for monogenic transmission. These results confirm that the transgene must be present on one of the chromosomes of the C-genome. Our results indicate that the transmission of a C-genome linked transgene is stabilized at about 10%. The underlying mechanisms explaining these results might be inter-genomic recombination between the A- and C genome, chromosome substitutions or disomic chromosome additions (Jacobsen *et al.*

1994; Jørgensen *et al.* 1996a). In *Brassica* species, both intra- and inter-genomic recombination have been described (Armstrong & Keller 1982; Attia *et al.* 1987) and inter-genomic recombination between A- and C-chromosomes have been reported (Quiros *et al.* 1987; Chen *et al.* 1990). Partial homology between the A, B and C-genomes has been revealed by studies of inter-specific hybrids and marker analysis (U 1935; Hosaka *et al.* 1990; Kerlan *et al.* 1993; Frello *et al.* 1995) which incidently may trigger cross-overs between the chromosomes of these genomes. Additional studies using chromosome-specific (RAPD) markers, which are available for both the A- and C-genome (Quiros *et al.* 1991; 1994; Jørgensen *et al.* 1996b; Mikkelsen *et al.* 1996b) and molecular cyto-genetics using genomic *in situ* hybridization (GISH) might help to monitor the presence of the C-chromosome carrying the PPT transgene and possibly demonstrate interchanges between the A- and C-genome.

By studying the possible gene flow from transgenic *B. napus* to its weedy relative *B. rapa*, it was shown that inter-specific hybridization and backcrossing of these hybrids with *B. rapa* occurred spontaneously under field conditions (Jørgensen & Andersen 1994; Jørgensen *et al.* 1996a; Mikkelsen *et al.* 1996a). Also the results we obtained suggest that gene flow from (transgenic) *B. napus* to *B. rapa* is inevitable. However, the data of our study support the hypothesis of Mikkelsen *et al.* (1996b) of possible 'safe' integration sites, chromosome regions with a low probability of transfer to backcross generations with *B. rapa* via homo(eo)logous recombination. Specifically, the presence on either chromosomes of the A-genome or C-genome determined the transmission frequency of the PPT tolerance gene in subsequent backcross generations. Concerning the aspect of gene dispersal in the specific case of transgenic *B. napus* to *B. rapa* we suggest that the transgene is selected for its presence on chromosomes of the C-genome. This study showed that this might limit the transfer to *B. rapa*. Integration of the transgene on chromosomes of the C-genome would also reduce the probability of gene transfer to *B. juncea*. It might, however, increase the chances of exchange to some other related species containing (parts of) the C-genome, such as *B. oleracea* and *B. carinata*.

Acknowledgements

Chapter 5

Hybridization of radish (*Raphanus sativus* L.) and (phosphinothricin-tolerant) oilseed rape (*Brassica napus* L.) through a flower-culture method

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Summary

Hybridization between radish and oilseed rape has been cumbersome, requiring elaborate embryo rescue techniques. With a modified flower culture method, we have achieved successful hybridization of radish with non-transgenic and transgenic phosphinothricin-tolerant oilseed rape without the laborious and technically demanding *in vitro* ovule or embryo rescue techniques.

The hybrid nature of the inter-generic hybrids was demonstrated using morphological traits, and DNA analyses. Transgenic inter-generic hybrids contain the *bar* gene and, therefore, survived a treatment with phosphinothricin. The described method will facilitate the generation of *Raphanobrassica* hybrids useful for biosafety studies of the potential for transgenes to spread in weedy *Cruciferae* as well as for breeding programs aimed at introducing useful radish genes, e.g. nematode resistance genes, into oilseed rape.

Introduction

Within the tribe of *Brassiceae* natural hybridization has resulted in several amphidiploid or allotetraploid species. For example, *B. campestris* (syn. *B. rapa*), turnip hybridized with *B. oleracea*, cabbage resulting in oilseed rape (*Brassica napus* L.). The interrelationships between different *Brassicaceae* was shown by U (1935) and Prakash & Hinata (1980). More recently, Demeke *et al.* (1992) concluded from comparisons of *Brassica*, *Raphanus* and *Sinapis* species that Random Amplified Polymorphic DNA (RAPD) markers were useful for taxonomic studies. RAPD bands revealed the classical 'U triangle' relationship between diploid and amphidiploid *Brassica* species. The results of Thormann *et al.* (1994), comparing Restriction Fragment Length Polymorphism (RFLP) and RAPD markers to estimate genetic relationships, indicate that RAPD markers were similar to RFLP markers for estimating intraspecific genetic relationships, while estimating interspecific genetic relationships RAPD markers may be less reliable than RFLP markers.

Useful genes from other members of the *Cruciferae* family are introduced into *Brassica* to transfer certain characteristics such as disease resistances. Chèvre *et al.* (1991) attempted to introduce *Alternaria* resistance from white mustard (*Sinapis alba* L.) into oilseed rape, and Hagimori *et al.* (1992) transferred resistance to clubroot disease from Japanese radish into cauliflower (*B. oleracea*) through somatic hybridization. To obtain resistance against the white beet cyst nematode (BCN, *Heterodera schachtii* Schmidt 1871) in oilseed rape, both radish (*Raphanus sativus* L.) and white mustard were used. For both, sexual (Dolstra 1982; Thierfelder *et al.* 1992; Lelivelt *et al.* 1993a) and somatic (Lelivelt & Krens 1992; Lelivelt *et al.* 1993b; Rosén & Olin-Fatih 1993) hybridization was performed with oilseed rape. Lelivelt (1993) showed that BCN resistance is expressed at a high level in the few hybrid plants she obtained.

Turesson & Nordenskiöld (1943) were successful in crossing tetraploid radish with diploid oilseed rape. With oilseed rape as female parent, six hybrids were obtained, while the reciprocal cross yielded three hybrids. Also, McNaughton & Ross (1978) obtained a few hybrids from a large number of pollinations. In contrast, Chopinet (1944) only obtained hybrids with oilseed rape when a colchicine-doubled 4x radish was used as female parent. Successful hybridization between radish and oilseed rape has also been reported after a bridge cross of *B. rapa* with *Raphanobrassica* (Clauss 1978) or with *in ovulo* embryo rescue tech-

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niques (Paulmann & Röbbelen 1988; Thierfelder *et al.* 1992).

Oilseed rape has whole genomes in common with four of the *Brassica* species belonging to the *Brassica* triangle. Therefore, oilseed rape might hybridize with most of them. This may imply that when transgenic varieties of oilseed rape are released in the field (trans)genes may move to related species and become established in wild populations. The opportunities of such a gene transfer from transgenic oilseed rape to related species were recently reviewed (Scheffler & Dale 1994). A relative ranking of species by their ability to form F_2 and backcross progeny when crossed to oilseed rape indicated that both progenies were reported for turnip and neither of them for radish.

In order to study the gene transfer between members of *Brassica*, we attempted crosses between radish and (transgenic phosphinothricin-tolerant) oilseed rape through a flower-culture method which simulates natural systems. Lardon *et al.* (1993) developed a flower-culture method to study pollination, fertilization and early seed development in oilseed rape. Our study shows that, providing normal crosses do not yield viable seeds, such flower-culture methods can be used as a simple way to obtain hybrids and circumvent the need for *in vitro* embryo rescue techniques.

Materials and Methods

- Plant material

The plants used in this study were (1) oilseed rape (*B. napus* L.) cv. Drakkar, (2) transgenic oilseed rape cv. Drakkar tolerant for the herbicide phosphinothricin (PPT, sold as Basta® or Radicale®) and (3) radish (*R. sativus* L.) cv. French Breakfast. PPT tolerance is due to the presence of the *bar* gene (De Block *et al.* 1987; Thompson *et al.* 1987). The *bar* gene, which originates from *Streptomyces hygroscopicus*, was isolated and characterized by Murakami *et al.* (1986). The *bar* gene encodes an PPT-acetyltransferase (PAT) that inactivates PPT by acetylation of a free NH_2-group. PPT inhibits glutamine synthetase, resulting in a rapid accumulation of ammonia and leading to cell death (Tachibana *et al.* 1986).

- Hand pollination and flower culture

Closed buds of radish were emasculated and hand-pollinated with pollen of oilseed rape. In the flower culture method (Lardon *et al.* 1993) these pollinated flowers were cut off, and their flower petioles were surface-sterilized by immersion in a 2% sodium hypochlorite
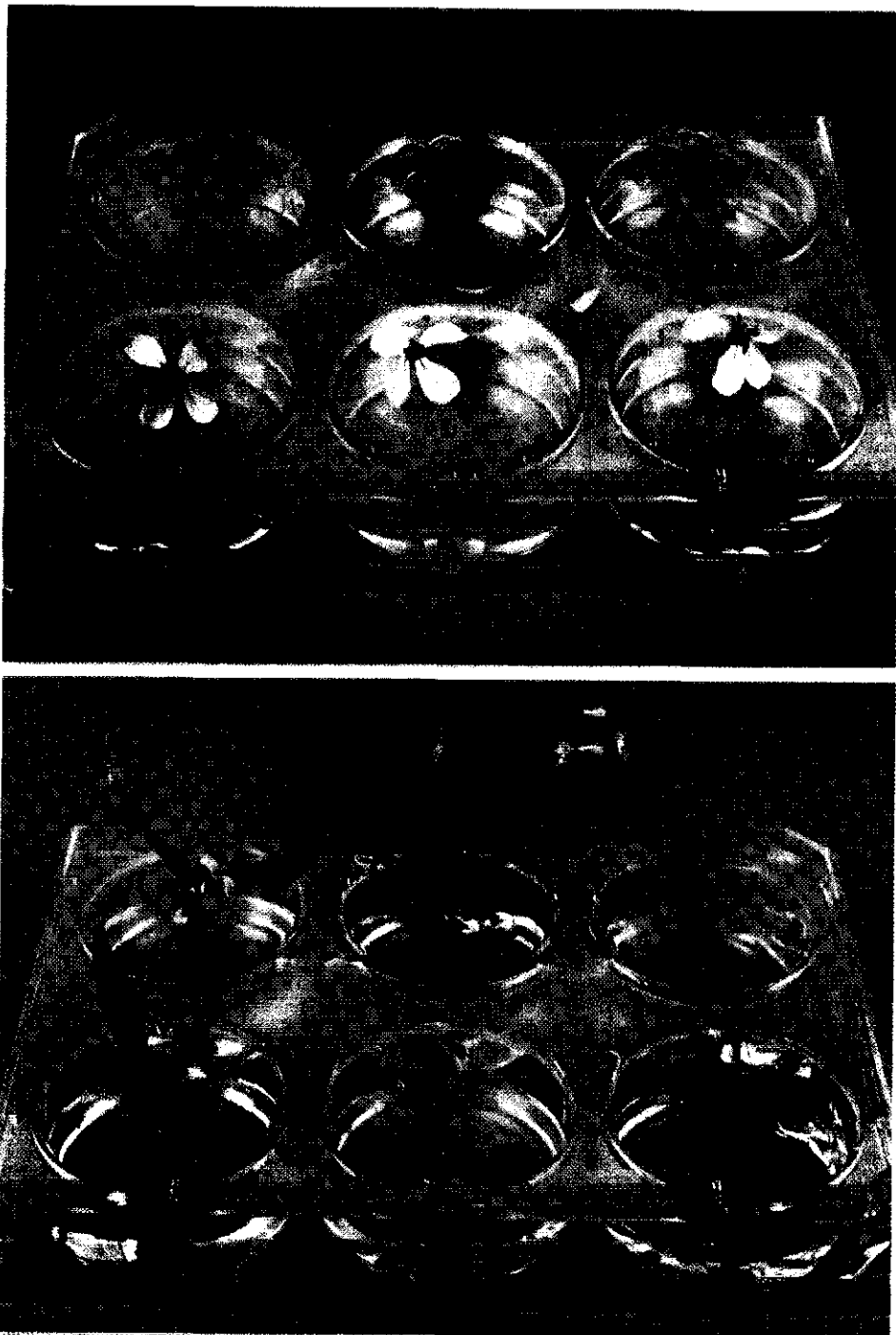
65

Figure 5.1 A-B Flower culture method of oilseed rape and radish flowers (A) and the siliques of the radish x oilseed rape hybrids, formed 21 days after pollination (B).

solution for two minutes followed by rinsing with sterile water twice for five minutes. The flower petioles were put in the holes of the cover of 6-well plates (Fig. 5.1 A-B). The wells contained 9 ml of liquid Murashige & Skoog medium (Murashige & Skoog 1962) with 10 or 30 g/l sucrose (MS-10 and MS-30 resp.). Seeds formed were put on MS-10 medium to speed up growth of the hybrids.

- Flow cytometry

Preparation of the nuclear samples and flow cytometry were performed as described by Bino *et al.* (1993). The fluorescence signals are presented as frequency distribution histograms, the DNA amount being expressed as relative C values. The 1C value represents the DNA amount of the unreplicated haploid chromosome complement.

- DNA analysis

From approx. 1 g of young leaf tissue DNA was extracted as described by Dellaporta *et al.* (1983). Aliquots of 5 μg DNA were digested overnight at 37°C with 50 u/μl HindIII, EcoRI, BamHI and BglII with addition of 1mM spermidine. After a sodium-acetate/isopropanol DNA precipitation, the samples were run overnight on a 0.7% agarose gel at 4°C (28V, 30mA). After electrophoresis, the gel was coloured in an ethidiumbromide (EtBr) containing buffer, put in 0.25 M HCl for 10 minutes, soaked twice in 0.4 M NaOH and vacuum-blotted with a 2016 VacuGene (Pharmacia) on Hybond-N+ (Amersham). The DNA was probed with the 5' end of the Male Sterility 2 gene (*MS2*) of *Arabidopsis thaliana* (Aarts *et al.* 1993), a transposon-tagged cDNA of *A. thaliana* (Aarts, in preparation) and a pea ribosomal DNA (r-DNA) probe (Nap, unpublished), which were radioactively labelled according to the USB random primed labelling kit or the Life Technologies RadPrime labelling system. Hybridization took place at 65°C overnight. The excess of radioactive label was removed by washing 5 minutes with 2x SSC and 30 minutes with 2xSSC 1%SDS at 65°C. If the number of counts was still too high a similar 30 minutes wash step was performed. Hybridizing bands were visualized by autoradiography using Kodak X OMAT-AR or Fuji RX films. The hybridity of the putative hybrid plant was analysed by Southern analysis using the radio-actively labelled *bar*-gene (Murakami *et al.* 1986).

- Pollen viability

Pollen viability was assessed by staining freshly harvested pollen with 0.5 mg/ml fluoresceïne diacetate (FDA) in a 9% sucrose solution. Pollen viability was determined as the number of yellow-green fluorescent pollen per about 100 pollen grains.

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- PPT tolerance tests

Rooted cuttings of putative PPT-tolerant hybrids with three or four leaves were sprayed with 0.5% Radicale® (150 gl⁻¹) to test PPT tolerance. Furthermore, in order to test non-destructively, leaf discs of putative hybrids, expected to contain the PPT tolerance gene, were screened on MS-10 containing 7.5 mg/l Radicale® and 50 mg/l chlorophenol red (CPR) according to a method developed by Kramer et $al.$ (1993). CPR is a pH indicator which is red at pH 6, purple at higher pH and yellow/orange at lower pH. PPT-tolerant leaf discs turn the red medium into yellow/orange due to the acetylation of the free NH_2-group of PPT, while control leaf discs colour the medium purple as a result of the accumulation of ammonia.

Results

In total, 447 hand pollinations between radish and oilseed rape resulted in 50 siliques, but no viable seeds were obtained due to seed abortion (Table 5.1). With the flower culture method, the percentage of seeds formed per pollinated flower was about 7% for both wildtype and transgenic PPT-tolerant oilseed rape. Both types did not differ in siliques production per pollinated flower and the seed yield per siliques obtained in the flower culture method (Table 5.1). Hybrid plants were obtained for both wildtype and transgenic oilseed rape, although the yield of hybrid plants per pollinated flower was low.

Table 5.1 Number of pollinated flowers, siliques, seeds, and hybrid plants from the cross of radish with wildtype or transgenic, phosphinothricin-tolerant oilseed rape (osr) either by hand pollination or flower culture.

Method	# pollinated flowers	# siliques	# seeds	# hybrid plants
Radish x wildtype osr				
Hand pollination	111	31	-	-
Flower culture	15	7	1	1
Radish x transgenic osr				
Hand pollination	336	19	-	-
Flower culture	215	114	13	2

Figure 5.2 Vigour of the hybrid radish-oilseed rape plant (middle) and its parents radish (left) and oilseed rape (right)

Non-transgenic inter-generic hybrid

The radish-oilseed rape hybrid showed very strong vigour (Fig. 5.2) and exhibited traits from both parents (Table 5.2). It had the blue-green colour, that is characteristic for the wax layer of oilseed rape. The leaves of the hybrid lacked the broadened, undeeply cordate base half-clasping the stem, characteristic for radish. On the other hand, they have, like radish, lyrate-pinnatifid leaves with some pairs of smaller distant laterals and a large rounded terminal lobe. The hybrid contained bristles, as does radish, but less many and not on the stem, but on the leaves only. In contrast to radish which showed anthocyanin coloration in the tuber, stem and petioles, the hybrid was purple only at the base of the petioles.

The flower colour of the hybrid was the same as that of radish, although the purple veins typical for the radish flowers were absent from the hybrid flowers. The inflorescences of the hybrid and both its parents were similar. The flowers of the hybrid developed normally with four sepals, four petals and six stamens. No flower abortion was observed. The amount of pollen formed per flower was about half that produced by the flowers of the parent plants, and FDA staining showed about 1% to be viable. Selfing of the hybrid and reciprocal back-crosses were not successful.

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Table 5.2 Morphological characteristics of radish (♀), oilseed rape (♂) and the radish-oilseed rape hybrid.

Trait	Radish	Hybrid	Oilseed rape
Flower colour	white	white	yellow
Tuber formation	yes	no	no
Wax layer	no	yes	yes
Anthocyanins	yes	yes	no
Bristly	yes	yes	no

Flow cytometric analyses for radish, the hybrid and oilseed rape, respectively are shown in Fig. 5.3. A mixed sample showed that the peak of the hybrid was clearly between the peaks of both parents (Fig. 5.3D). This confirmed that the DNA content of the hybrid is intermediate between the DNA contents of radish and oilseed rape, which are 1.09 pg/2C peak and 2.34 to 2.56 pg/2C, respectively (Arumuganathan & Earle 1991).

Using the pea r-DNA as probe, it was found that the putative hybrid contained bands of both radish and oilseed rape (Fig. 5.4A). Also by Southern analyses with the *MS2* and a transposon-tagged CDNA of *Arabidopsis thaliana* as a probe, the hybrid nature was demonstrated on the basis of similarities in band pattern with both parents (Fig. 5.4 B-D).

Transgenic inter-generic hybrids

In crosses with PPT-tolerant oilseed rape as male parent, two putative hybrids could easily be distinguished by spraying the progeny plants with 0.5% Radicale® and by the non-destructive pH indicator test. Both progeny plants survived a treatment with PPT. Fig. 5.5 shows the result of the pH indicator test for one of the PPT-tolerant hybrids. Their hybrid nature was also confirmed by hybridization of a Southern blot with a *bar*-probe. The specific band, indicating the presence of this gene from the male parent, was detected in these plants (Fig. 5.6).

The morphology of the PPT-tolerant hybrids was similar to that of the non-transgenic hybrids, exhibiting traits from both parents. After FDA staining, about 3% of the pollen of tolerant hybrids was shown to be vital which is not significantly different from the pollen viability of the non-transgenic hybrid. Also, these PPT-tolerant hybrids could not be selfed, and backcrosses on radish yielded only two very small seeds which did not germinate.
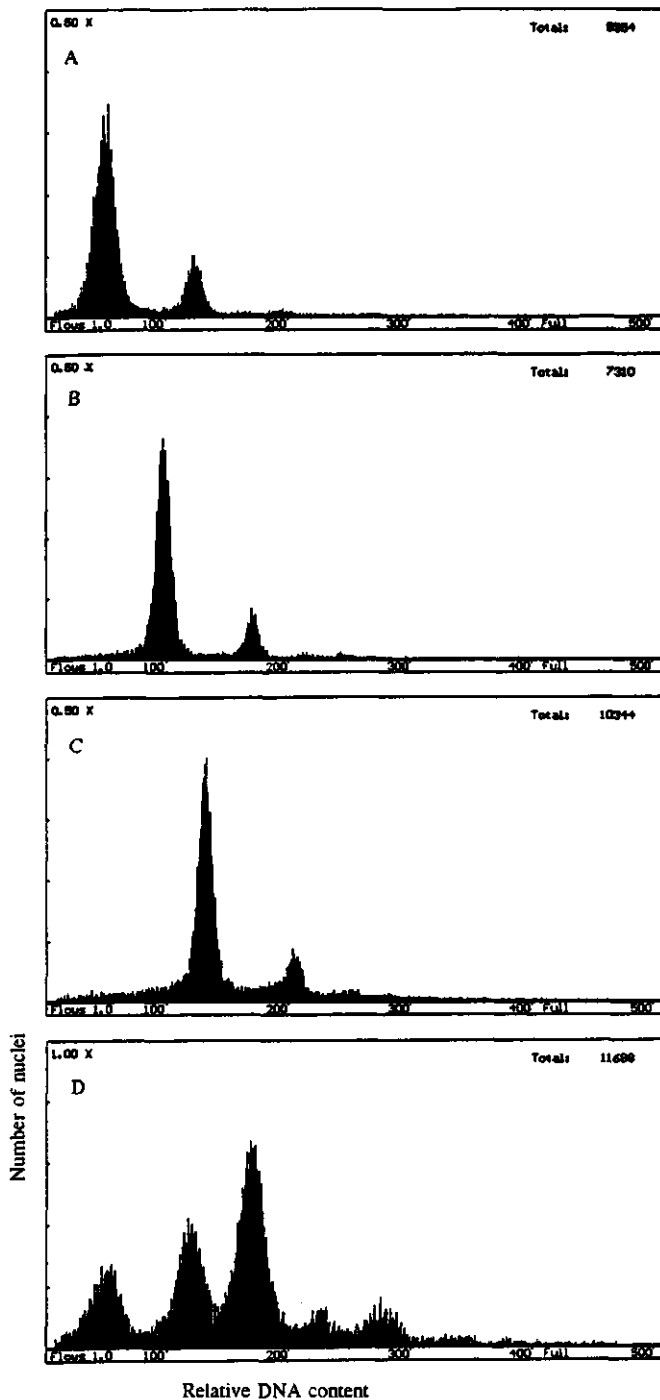
Figure 5.3 A-D Histograms of flow cytometric analysis of nuclei from leaves of radish (A), the radish-oilseed rape hybrid (B) and oilseed rape (C). A mixed sample of leaf material gives the peaks of A, B and C in one histogram (D). Nuclei from leaves show peaks at the 2C and 4C DNA level. Totals refer to the number of nuclei measured
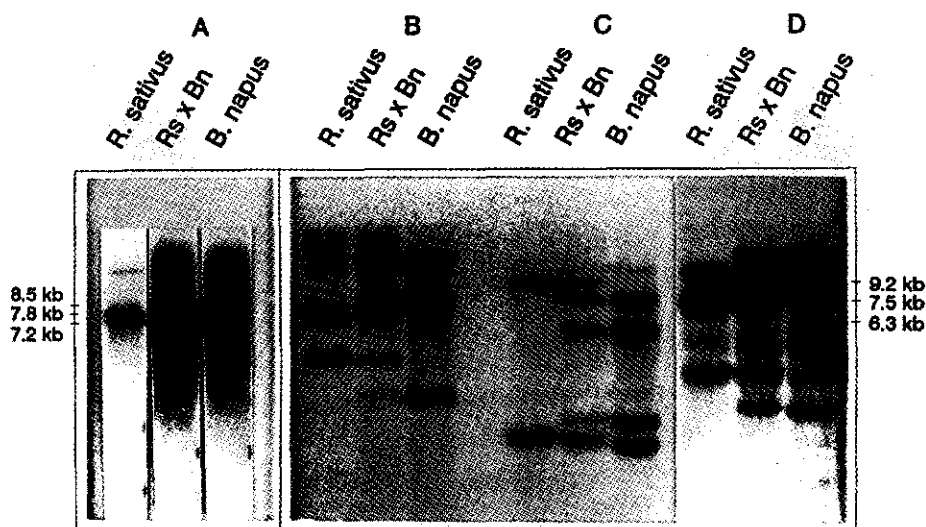
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Figure 5.4 A-D Southern blot hybridizations of DNA of radish, the radish-oilseed rape hybrid and oilseed rape, digested with HindIII (A, D), BamHI (B) and BglII (C) and hybridized with pea r-DNA (A), *Arabidopsis thaliana* MS2 (B, C) and *Arabidopsis thaliana* transposon-tagged CDNA (D) probes.
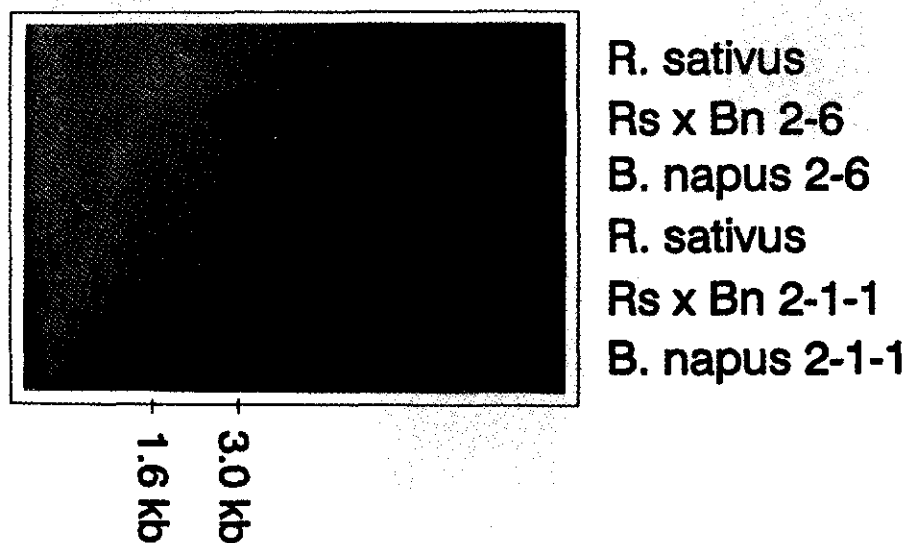

Figure 5.6 Southern blot hybridizations of DNA of two hybrids of radish and PPT-tolerant oilseed rape (2-6 and 2-1-1) and their parents digested with HindIII and hybridized with the bar probe.
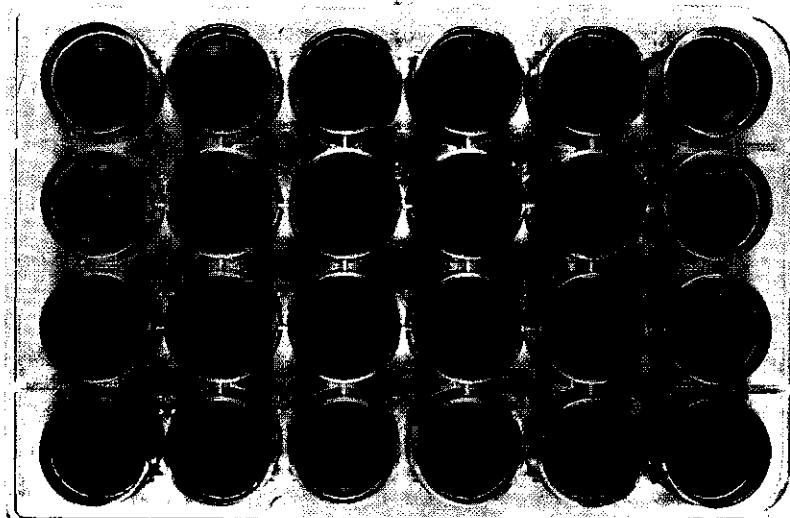
Figure 5.5 Chlorophenol red pH-indicator test of the hybrid of radish and PPT-tolerant oilseed rape (Rs x 2-6) and the wild type oilseed rape (control).

Discussion

The flower culture method in which the flowers were cut from the plant after pollination, resulted in a total of three hybrids of radish and oilseed rape out of 230 pollinated flowers (1.5%). When the pollinated flowers were left on the plant, seed abortion occurred probably due to the malfunctioning of the endosperm (Kato & Tokumasu 1976). Becker (1951), Tokumasu (1965), Takeshita *et al.* (1980) and Lelivelt *et al.* (1993a) also failed to obtain hybrid plants by crossing oilseed rape with radish. Crosses with diploid oilseed rape were only successful with natural tetraploid radish (Turesson & Nordenskiöld 1943) or with a colchicine doubled 4x radish as female parent (Chopinet 1944). McNaughton & Ross (1978) reported radish- oilseed rape hybrids, but they did not mention the plant material used. Paulmann & Röbbelen (1988) obtained 34 hybrids by *in vitro* embryo culture after pollinating 765 buds (4.4%), and Thierfelder *et al.* (1992) obtained 1.6% hybrids by dissecting ovules. These percentages are similar to those obtained in this study. By radish/oilseed rape protoplast fusion, Lelivelt & Krens (1992) obtained one hybrid out of 286 regenerants.

The method described here is less artificial and less laborious than the above-mentioned procedures. The only difference to reproduction in nature is the removal of the pollinated flowers from the plant. Apparently, when the flowers are still attached to the plant mechanisms are effective that prohibit the formation of viable seeds after inter-generic crosses. These can be circumvented by removal of the pollinated flowers, and their transfer to a flower culture medium. These mechanisms are not yet understood. Lardon *et al.* (1993) found the culture of isolated flowers a suitable tool for studying pollination and early seed development. This method may therefore also be useful in unravelling the mechanisms preventing successful inter-generic hybridization.

All three hybrids had normally developed anthers but produced only few viable pollen grains (1 to 3%) which reduces the chance for successful selfing or backcrossing. Paulmann & Röbbelen (1988) and Thierfelder *et al.* (1992) obtained (partially) fertile AACCRR ($2n=56$) individuals after colchicine treatment. Backcrosses with the monogenomic ancestral species *B. rapa* and *B. oleracea* yielded only offspring after the *in vitro* culture of ovules. Studies on chromosome pairing at meiotic metaphase I indicated partial homology of the A and C genomes of oilseed rape with the R genome of radish (Dolstra 1982; McNaughton 1973a; Mizushima 1980; Namai 1976; 1978). This means that introgression of *Raphanus* genes into a *Brassica* species is possible.

The vigour of the hybrid plant is probably due to a heterosis effect and not to the tetra- or hexaploidy of the plant, as shown by the flowcytometric measurements which suggest that the genomic constitution of the hybrid is ACR. The crosses with the transgenic, PPT-tolerant oilseed rape showed that with the flower culture method a dominant, monogenic trait can be transferred to the hybrid. If fertility can be partially restored, for example by doubling the chromosome number with colchicine, such hybrids can be used in a backcross programme for the introduction of genes-of-interest in the recurrent parent. For biosafety studies, these hybrids are interesting because they afford an opportunity to study the expression of a transgene in different genetic backgrounds. The possibility that these hybrids will have an ecological impact, by transferring the PPT tolerance gene from oilseed rape to radish, is negligible, as hybridization is only successful in the laboratory, and the hybrids produce only a small amount of viable pollen. Scheffler & Dale (1994), who reviewed the opportunities of gene transfer from transgenic oilseed rape to related species, reported that in nature, hybrids between radish and oilseed rape were not able to form F_2 and backcross progeny.

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In conclusion, the described flower culture method is likely to be a good alternative for embryo rescue techniques and will facilitate the generation of hybrids. It is less laborious and does not require any specific skills in preparing embryos or ovules.

Acknowledgements

Chapter 6

General discussion

Introduction

Using standard transformation techniques, isolated genes of plants and other organisms can be introduced into plants. Because genetic modification hypothetically may result in unforeseen consequences, prior to the release of transgenic crops in the environment their biosafety has to be assessed following a 'case by case' and 'step by step' policy in order to build up more familiarity (OECD, 1993a). This means that, subsequently, greenhouse experiments, small-scale field experiments and large-scale field trials have to precede the release of transgenic plants in the environment before market introduction and normal agricultural practice will be allowed. Such transgenic crops will significantly contribute to agriculture in the near future. However, genetic modification will not be a substitute for traditional plant breeding, but will be an additional tool to improve the applicability and quality of crop plants.

Legislation

In the specific case of the release of transgenic herbicide-tolerant crops three legal frameworks are relevant: that for genetically modified organisms (GGO), that for herbicides and that for new agricultural and horticultural varieties (Bijman & Lotz 1996). The EU plays an important role in the legislation of biotechnology and in the near future there will be a further shift from national legislation by national competent authorities to EU legislation. Table 6.1 shows the Dutch frameworks and the Ministries concerned in addition to the EU Directives regulating the same aspects at EU level. The Dutch framework for the admission of a GGO comprises two aspects: environmental safety and food safety. For the environmental safety the Minister of Housing, Spatial Development and the Environment is the competent authority, which can ask the Dutch Committee of Genetic Modification (COGEM) for advice. The competent authority for food safety is the Minister of Health, Welfare and Sports, which can obtain additional advice from the Provisional Committee on the Safety of Novel Food (safety and food aspects) and the Subcommittee on Novel Foods of the Food and Commodity Act (for social aspects, mainly labelling). With respect to herbicide-tolerant crops it is important whether or not the current regulations for the application of the herbicide involved can be applied to its novel use in combination with plants tolerant to the herbicide, for instance post-emergence instead of pre-emergence use of the herbicide. Based on governmental guidelines, the

Table 6.1 Survey of the Dutch legal frameworks with respect to herbicide-tolerant transgenic plants with the Ministries concerned and the EU Directives regulating the same aspects on EU level. Abbreviations: GGO genetically modified organisms; VROM Housing, Regional Development and the Environment; VWS Health, Welfare and Sports; LNV Agriculture, Nature Management and Fisheries; SZW Social Affairs and Employment.

Framework	Ministry	EU Directive	Regulated in
GGO			
- Environment	VROM	90/220/EEG	Decree GGO
- Food	VWS	Draft Novel Food and Food Ingredients	Novel Food Order
Herbicides	VROM, VWS LNV, SZW	91/414/EEG	Pesticides Act 1962
Plant Varieties	LNV	70/457/EEG (Agriculture) 70/458/EEG (Vegetables)	Seed and Plant material Act 1966

Board for the Authorization of Pesticides (College voor de Toelating van Bestrijdingsmiddelen) will decide whether or not the use of a herbicide is allowed on transgenic tolerant crop plants and under which conditions. Furthermore, genetically modified crop plants should, as conventionally bred varieties, comply with the Distinct, Uniform, Stable (DUS) criteria and prove their cultural and economic value to be legally protected and to get permission to be traded.

Scope of this thesis

In the field release of transgenic crops and their commercialization transgenic phosphinothricin (PPT)-tolerant oilseed rape (*Brassica napus* L.) is at the forefront (OECD, 1993c). At the beginning of the project this fact played a decisive role in the choice of this particular trait-crop combination for a biosafety assessment. The aim of the study described in this thesis was to obtain more insight in and familiarity with the biosafety of transgenic PPT-tolerant oilseed rape. Knowledge about the biosafety of PPT-tolerant oilseed rape was gained in two ways. In Chapters 1 and 2 existing biological, biochemical, ecological and toxicological data on oilseed rape, the herbicide PPT, the *bar* and *pat*

transgenes, the transgene product phosphinothricin-N-acetyltransferase (PAT) and putative metabolites were reviewed. In Chapters 3, 4 and 5 experiments were performed to obtain data about the transfer of PPT tolerance to different genetic backgrounds and the fate of the expression of the transgene in successive sexual offspring. This research was complementary to simultaneously running EU-funded biosafety projects (Biotechnology Action Program, 1990; Biotechnology Research for Innovation, Development and Growth in Europe, 1992), where field trials were performed to assess pollen dispersal of transgenic oilseed rape and hybridization of transgenic PPT-tolerant oilseed rape and several related species, others than used here (see Chapter 1).

The results of biosafety studies, as presented here, are of help for competent authorities responsible for the release approval of herbicide-tolerant oilseed rape. In Denmark, for instance, the application for marketing PPT-tolerant oilseed rape in the EU was negatively assessed, because there was no analysis of the effect of PPT use and of long-term environmental effects of such crops (Bijman & Lotz 1996). However, the EU approved this release, based on the opinion that the questions about agricultural effects were no part of EU directive 90/220/EEG, which regulate the release of transgenic plants in the environment, but of directive 91/414 (pesticide regulations).

Oilseed rape

Chapter 1, reviewing the taxonomy, cytogenetics and reproduction system of oilseed rape and its possible hybridization with (wild) relatives showed that complete containment of transgenic oilseed rape in the field is not possible. For the related oilseed Brassicas, *B. rapa* and *B. juncea*, hybridization has been found in artificial crossings and spontaneously - which means without the elaboration of *in vitro* techniques - under field conditions, resulting in fertile inter-specific offspring (Bing *et al.* 1991; Jørgensen *et al.* 1996a; Mikkelsen *et al.* 1996a). Although reported outcrossing frequencies were shown to be low, the spread of the transgene from transgenic oilseed rape to (wild) relatives could not be ruled out. Therefore, attention was focused on the ecological and toxicological impact of the introduced transgenes.

Transgene-centered evaluation of the PPT tolerance transgene

As a consequence, Chapter 2 describes the transgene-centered approach to evaluate transgenic plants. In this approach all characteristics of a particular transgene and its product(s) are assessed. The added value of this approach is two-fold. It helps to generalize outcomes irrespective of the plant species into which the transgenic trait is introduced. Furthermore, concentration on the characteristics of the transgene and the gene product allows the formulation of definite questions. The evaluation of biochemical, ecological and toxicological data may help to identify what kind of further data should be required before a safe release can be approved.

To illustrate the transgene-centered approach, the *bar* and *pat* transgenes, as well as the gene products conferring PPT tolerance were reviewed. Transgenic PPT-tolerance is currently applied in the development and use of plant material as selection marker during transformation, as agronomic character and in female line multiplication for hybrid seed production. The specific and relatively little change brought about by the introduction of the *bar* or *pat* transgene will theoretically result in a transgenic plant identical to the untransformed parent plant, with the exception of the added PPT tolerance.

Biosafety concerns with respect to PPT and transgenic PPT tolerance enclose both ecological and toxicological issues. Following the transgene-centered approach, it can be concluded that the use of PPT and PPT-tolerant crops in the production of hybrid seeds and the use of PPT tolerance as selection marker are ecologically fully biosafe. The agronomic application of PPT and PPT-tolerant plants could imply a considerable environmental gain compared to the application of current-day herbicide cocktails and can be considered to be ecologically biosafe too. However, by spread of the *bar* or *pat* gene at some places some wild relatives of a crop plant will acquire PPT tolerance. It is not expected that this will give uncontrollable situations in agronomy.

Toxicologically, the consequences of consumption of PPT-tolerant plants are less clear. Because consumption of plants containing *bar* or *pat* transgenes or the gene product, the PAT enzyme, will have no adverse effects, without spraying, transgenic PPT-tolerant plants are toxicologically fully biosafe. Upon spraying with PPT, the amount of PAT activity determines whether all L-PPT is acetylated or not. In the most likely case this activity is sufficiently high and only D-PPT and acetyl-PPT, which themselves will not pose concern for consumption, may be present in the plant material. However, at present

it is unknown how these metabolites will behave upon food processing. In plants having a low PAT activity, in addition to acetyl-PPT and D-PPT, PPT-derived metabolites may be formed. To what extent such metabolites might accumulate is unclear and no toxicological data on these metabolites have been found in the available literature.

A protocol to assess the levels of various PPT metabolites in transgenic PPT-tolerant plants or food will indicate if and if so which further toxicological data might be necessary before these crops can be considered safe for consumption. Such assessment might also answer the question whether or not regulations, now valid for the use of PPT as total herbicide, can also be applied for the use of the herbicide in combination with PPT-tolerant crops. PPT is used for post-emergence, pre-harvest desiccation in potato, legumes and oilseed rape (Trinks *et al.* pers. comm.). In the Netherlands this use of PPT is allowed by the Board for the Authorization of Pesticides (see Table 6.1) only for desiccation in potatoes and the Board has still to decide upon the new post-emergence application of PPT in combination with PPT-tolerant crops. Another example of the transgene-centered evaluation in which familiarity with a transgene was obtained is the study on the biosafety of the kanamycin resistance gene (Nap *et al.* 1992). This example and our findings (Chapter 2) showed that the transgene-centered approach, by gathering various data of the transgene, its product, substrates and putative degradation products, is powerful in identifying if and if so, which further data should be required for a safe release of transgenic crops. The availability of such data most likely will make biosafety discussions about a particular gene more transparent and possibly more constructive.

Transmission and expression of the PPT tolerance transgene
The PPT-tolerance *bar* gene was successfully transmitted in intra-specific, inter-specific and inter-generic crosses (Chapters 3, 4 and 5). Successive generations of selfings and mutual within-variety crosses showed that PPT tolerance is dominantly and monogenically inherited. A predictable, consistent and stable transmission and expression of transgenes are prerequisites for commercial success of genetically-modified crops. Studying the segregation ratios of PPT tolerance stable transmission and expression was found in within- and between-variety crosses. However, due to gene inactivation occasional loss of the phenotypic expression of the PPT tolerance gene was observed after selfing of indi-vidual PPT-tolerant plants and after backcrosses with non-transgenic plants (Chapter 3).

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For seed multiplication of varieties and in breeding programs this (partial) loss of pheno-typic expression implies that spraying with PPT is sometimes no longer selective for all oilseed rape plants of the same transgenic variety. Possible explanations for the discrepan-cy between the presence of the transgene and absence of phenotypic expression of PPT tolerance might be methylation or co-suppression as reported in other studies (Jorgensen 1990; Matzke & Matzke 1991; Kilby *et al.* 1992; Matzke *et al.* 1993; Ingelbrecht *et al.* 1994) or a position effect of the T-DNA locus integration as described earlier for *Petunia* (Linn *et al.* 1990). We would like to hypothesize a combination of such a position effect and the occurrence of somaclonal variation. Further molecular analyses of the PPT-susceptible transgenic plants and their offspring have to clarify the underlying mechanisms involved. These observations support the view that breeders with transgenic plants have to follow similar time-consuming selection procedures to ensure stable expression of transge-nes as required for the generation and multiplication of new lines or varieties in conventi-onal breeding.

In inter-specific hybrids of *B. rapa* and *B. napus* and their successive backcrosses, the frequency of transmission of the PPT tolerance transgene was suggested to be dependent on its presence on chromosomes of either the A- or C-genome of *B. napus* (Chapter 4). Comparing the percentages of PPT-tolerant plants in BC_1 generations which were made with plants from populations of two independent transgenic parents, the specific integrati-on position of the transgene gave different transmission frequencies. In case expected frequencies of PPT-tolerant plants were observed in the BC_1 and BC_2's the location of the transgene on one of the chromosomes of the A-genome of *B. napus* was suggested. When located on one of the chromosomes of the C-genome, a high deficit in PPT-tolerant offspring plants was observed. In three generations of backcrosses involving plants accommodating the transgene on a chromosome of the C-genome, the percentage PPT-tolerant plants was low (Table 4.2). Only approximately 10% of offspring had retained the tolerant trait in the BC_3 and BC_4 generations.

From a biosafety point of view the inter-generic crosses between *B. napus* and radish (*Raphanus sativus* L.) will have no impact (Chapter 5). Only by the use of a modified flower culture method the PPT tolerance transgene could be transferred to hybrids, which produced a small amount of stainable pollen. The hybrid plants could not be selfed and backcrosses on radish did not yield any seeds which germinated. This makes potential

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spread of the PPT tolerance transgene from oilseed rape to radish negligible.

Biological containment

The outcome of the inter-specific hybridization studies can be predicted theoretically. In backcrosses of the inter-specific hybrid to Pak choi having only the A-genome, the C-chromosomes have no homologous partners during meiosis. Due to irregular transmission of the single C-chromosomes to the gametes, a transgene located on one of the chromosomes of the C-genome of *B. napus* is expected to be transmitted in a much lower frequency to the gametes than with transgene location on one of the chromosomes of the A-genome. This difference is clearly supported by our experimental data (Table 4.2) which suggested that, although gene flow from (transgenic) *B. napus* to *B. rapa* can not be ruled out, this transfer can be limited considerably through selection for the presence of the PPT tolerance transgene on one of the chromosomes of the C-genome of *B. napus*. The probability of gene transfer from *B. napus* to *B. juncea*, with the genomic constitution AABB, might also be reduced by integration of transgenes on chromosomes of the C-genome. However, the probability for the exchange to *B. oleracea* (CC) and *B. carinata* (BBCC), related species containing the C-genome, might be increased. Both these species do not occur in nature in the Netherlands.

To ensure containment of a transgene in a certain crop plant a new approach has become available. Only recently, a technique for plastid transformation has been developed. Stable chloroplast transformants of tobacco were obtained by Maliga and co-workers following particle bombardment (Svab *et al.* 1990; Carrer *et al.* 1993; Svab & Maliga 1993) and by PEG-mediated DNA uptake by protoplasts (O'Neill *et al.* 1993; Golds *et al.* 1993). Since the plastid in most plant species are maternally inherited (Gillham *et al.* 1991), plastid transformation will prevent the spread of transgenes to (wild) relatives through transfer of pollen (Dix & Kavanagh 1995; McBride *et al.* 1995). As additional advantage, this method simplifies the introduction of transgenes in commercial hybrids (Maliga 1993; McBride *et al.* 1995).

Herbicide tolerance in a broader perspective

Part of this thesis was focussed on transgenic oilseed rape in which the *bar* gene had been introduced conferring tolerance to PPT and the impact of this trait has been discussed in Chapter 2. Here, herbicide tolerance in general will be discussed in a broader perspective, because in the whole process of modification, testing, introduction into the environment and commercialization of transgenic crops, the herbicide-tolerant crops are at the forefront and the first products now on the market.

At the end of 1996 transgenic soybeans grown in the US, which were tolerant to another herbicide, glyphosate (Roundup®), after introduction of a bacterial mutant *epsp* gene, were shipped to Europe for further processing to end products. Transgenic soybean is difficult to ban based on the agreements within the World Trade Organisation, unless human health is at risk. Import of these transgenic soybeans into Europe led to strong protests from (Dutch) environmental organisations such as "Greenpeace" and "Natuur en Milieu". According to their argumentations the use of glyphosate during growth of tolerant soybeans would have a negative effect on the environment and drinking water supplies. The use would imply a negative effect on human fertility and the danger of exposure to putative carcinogenic substances. The first two arguments were refuted by findings in numerous studies, such as by the American Food and Drug Administration, the British Advisory Committee on Novel Foods and Processes, the Dutch Centrum voor Landbouw en Milieu and the World Health Organization, which all indicate that glyphosate, which is already used for over 20 years without problems, has a low environmental burden and does not impose risks for animal and human health and is not carcinogenic. Glyphosate and its metabolites do not leach into the ground water, but can be found in the surface water after incompetent use or heavy rainfall. Due to their low toxicity this has little effect for the environment. Weed control by glyphosate in soybean fields led to a 30% reduction in the use of other herbicides, which often have a higher environmental burden. Furthermore, glyphosate application reduces the necessity for mechanic weed control which is in some parts of the US the main cause of erosion.

In soil, high concentrations of glyphosate - much higher than the recommended application ratios- in combination with high concentrations of sodium nitrite may generate N-nitrosoglyphosate (Khan & Young 1977). This compound belongs to the family of the N-nitrosoamines which can be toxic, mutagenic and/or carcinogenic (Reddy & Hayes

1989). In plants, both nitrite and glyphosate occur in the chloroplast. In a kinetic study, the pH optimum for nitrosation was determined to be 2.5, suggesting that in plants the reaction would not occur. In soils, however, nitrosation was pH independent, what theoretically also might be the case in the chloroplast. Possibly, formation of N-nitrosoglyphosate occurs when sufficient amounts of glyphosate and nitrite are combined in the acid environment of the human stomach. No study concerning the toxicology or carcinogenic properties of N-nitrosoglyphosate was found, although the compound was reported to be weakly mutagenic (Seiler 1977). More data for the putative formation of this compound and its toxicological characteristics seem required, which was also one of the outcomes of the transgene-centered evaluation of glyphosate tolerance transgenes (Nap *et al.* 1996).

Although there is no convincing evidence, environmental organisations are critical concerning possible negative effects of glyphosate on human fertility. This is based on two publications from one research group (Yousef *et al.* 1995, 1996). One is a model study to develop *in vitro* toxicity tests for sperm cells, which did not allow to draw conclusions about the glyphosate toxicity for human sperm cells. Because the applied dose and the mode of application were not well described no conclusions could be drawn from the second study in which glyphosate was given to two groups of four rabbits. In other toxicity studies with rodents no effect of glyphosate on reproductive organs was observed (World Health Organization 1994).

It is noteworthy that the arguments put forward by environmentalists against glyphosate-tolerant soybeans concern primarily the herbicide itself and not the transgenic trait or the transgene (product). However, glyphosate may currently be applied pre-harvest in cereals not later than 4 days before harvest to better get rid of weeds compared to in stubble. Spot application is allowed in other consumption crops, like potato, beet and legumes in the Netherlands as determined by the Board for the Authorization of Pesticides. In the USA, glyphosate is currently also allowed to be applied pre-harvest in soybean (unpublished). The use of glyphosate-tolerant crops allows, if necessary, a much earlier application of herbicide during the plant development. This can imply that, for instance, the current soybean contains more residues of glyphosate than the glyphosate-tolerant soybean. Admission of the glyphosate-tolerant soybean was tested against the EU directive 90/220/EEG, to determine if such a crop did not impose a burden on the environment. Furthermore, this transgenic crop was approved to be safe for food application by the

governments of the US, Canada, Japan, the UK and The Netherlands. Based on findings of experts, the European Committee decided to give permission for the import, storage and processing of these soybeans.

'Narrow sense' and 'Broad sense' biosafety

In the assessment of the biosafety of genetically modified crops a distinction can be made between 'biosafety in narrow sense' and 'biosafety in the broad sense' (Metz & Nap 1997). Concerns with respect to the biosafety in the narrow sense involve the ecology and toxicology of both release and use of transgenic herbicide tolerant crop plants. The ecological concerns focus on weediness and vertical and horizontal spread of the gene. The toxicological concerns focus on food safety and consumption. The concerns and issues with respect to the biosafety in the broad sense in plants reflect, in addition, social, ethical and/or economic views with respect to current agriculture. A few examples, which not only apply for herbicide tolerant transgenic crops, but for transgenic crops in general will be mentioned in random order of arguments that have been put forward and should ideally be included in assessments of biosafety in the 'broad sense'. Genetic modification that overcomes species barriers is seen as tampering with the natural order of life. Evolutionary 'boundaries' should be considered as provisional warning signs of danger (Suzuki & Knudtson 1989). Transgenic crops could threaten the centers of crop diversity (Rissler & Mellon 1993). Resources used for genetic modification are thought to be better spent on more important issues. Research into transgenic herbicide-tolerant crops, for example, could distract from research into alternatives such as mechanical weed control (Reijnders 1993). The combination herbicide tolerance transgene/transgene-containing crop/transgene substrate, being the herbicide, is generally owned by the 'agro-industrial complex'. This may limit the options of farmers, may impair development of agriculture in third-world countries and generally will result in too high profits for only a few (Lucassen *et al.* 1990; Rissler & Mellon 1993).

Companies, on the other hand, point out that the investments made into agricultural biotechnology are high. Preferably early in its development a product should promise to be cost-effective. Cost-effectiveness will depend on patent and license fees, alternatives, environmental impact, environmental policies and taxes (Bijman 1994). Such concerns play also a role in the social and public acceptance of the transgenic crops. Related topics

are the information of consumers about risks and benefits; the developments with respect to property rights; as well as the necessity for and methods of labelling foods derived from modern biotechnology. Each of these topics is currently generating a respectable bibliography (*e.g.* Scholten *et al.* 1991; Durant 1992; Bryant & Leather 1992; Dunwoody 1992; van Wijk *et al.* 1993; Barefoot *et al.* 1994). Full clearance and/or clarity for particular transgenes in an early stage of development would be advantageous especially for small and medium-sized enterprises.

Unfortunately, between (and within) EU member states there are clear differences in the conceptualization of 'risk' and disagreements with respect to the environmental impacts that should be taken into consideration. For example, application of a herbicide-tolerant transgenic crop might cause, upon outcrossing, that the herbicide can no longer be used, whereas the herbicide itself is considered to be more environmentally friendly than alternatives. The latter effects, the potential loss of the applicability of the herbicide and the environmental impact of the herbicide, are clearly secondary or indirect effects, or, in the terminology we propose, issues of biosafety in the broad sense. Regulatory authorities in EU member states such as the UK and The Netherlands tend to consider mainly the narrow sense effects of the transgenic plants to be a biosafety issue (Bijman & Lotz 1996). Broad sense effects are not seen as an issue of biosafety. Such effects are considered to be the competence of other committees and/or are covered by different laws and jurisdiction (see Table 6.1). Other member states, such as Austria, Denmark and Sweden, however, indicate that broad sense effects should be more included in assessments of transgenic plants. Their national legislation links biotechnology with broader criteria, such as sustainability, socioeconomics and ethics. Commandeur *et al.* (1996) recently described the situation in various EU countries.

In such a complex and politically sensitive context, full assessments of all aspects of the 'biosafety in the broad sense' are highly demanding and interdisciplinary tasks. Consensus on the 'narrow sense' issues of individual transgenes may contribute to 'broad sense' assessments. It is possible that a herbicide tolerance transgene is evaluated to be biosafe in the narrow sense, but that it poses undesirable characteristics with respect to its biosafety in the broad sense. An example would be a particular transgene conferring herbicide tolerance to a herbicide with an adverse environmental or toxicological impact. The presence of the transgene and the transgene product in plants could be fully biosafe,

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but the associated increased use of the environmentally or toxicologically adverse herbicide would imply a negative effect in the broad sense. Examples of this are bromoxynil-tolerance and tolerance to some acetolactate synthase (ALS) herbicides. The agronomic application of bromoxynil-tolerant plants is considered to be biosafe with respect to its ecological and toxicological consequences. However, the herbicide has been qualified as harmful to mammals and various fish species. Bromoxynil butyrate and its commercial formulations might form an unacceptable risk of toxicity in persons handling these compounds (Campt 1989).

Tolerance to persistent and non-persistent herbicides

In the discussion about the biosafety of herbicide tolerant crops a distinction should be made between tolerance to non-persistent herbicides such as PPT and glyphosate and persistent herbicides such as some ALS herbicides, like chlorsulfuron. Although crops tolerant to either of these three herbicides were considered to be ecologically and toxicologically biosafe, the use of ALS herbicide tolerant crops may stimulate the use of a persistent herbicide. Depending on the soil type and environmental conditions, chlorsulfuron for instance applied to monocots like wheat, can preclude farmers from growing dicot crops for at least 4 years or longer after application (McHughen & Holm 1991). On the other hand it has been argued that transgenic ALS-tolerant crops such as flax, can be grown on soils 'polluted' with ALS herbicides used to control weeds in cereal crops and therewith provide farmers with an option to flexible crop rotation by taking advantage of the residual activity of these herbicides (McHughen 1989; McHughen & Holm 1991). However, it is expected that the use of these ALS herbicide tolerant crops in combination with the herbicide will not be restricted to those treated areas. Therefore, a critical consideration of the type of crop into which this trait should be introduced and under which conditions they should be used seems justified.

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Summary

Genetic modification is an additional tool for conventional plant breeding to improve the application and quality of crop plants. No longer hampered by natural crossing barriers, application of genetic modification results in a nearly infinite pool from which genes, after isolation, can be introduced in crop plants. At this moment more and more genetically modified crops are coming on the market and these crops will probably significantly contribute to near-future agriculture. However, since the introduction of transgenic plants in the environment the biosafety regulation of these crops has been discussed and developed. Preceding the release of transgenic plants within existing legal frameworks several stages of containment were passed through and a new regulation concerning biotechnological products was set up. In the process leading to commercialization of transgenic plants, herbicide-tolerant crops, such as phosphinothricin (PPT)-tolerant oilseed rape are at the forefront and the first varieties are at present on the market. This fact played five years ago a decisive role to use this particular trait-crop combination for biosafety studies.

The aim of the study described in this thesis was to gain knowledge about and familiarity with transgenic PPT-tolerant oilseed rape in relation to its biosafety. This was made in two ways: 1) scientific data concerning oilseed rape were reviewed and the ecological and toxicological impact of the PPT tolerance transgene and the herbicide PPT was evaluated (Chapters 1 and 2); 2) experiments were performed to investigate whether or not PPT tolerance could be transmitted to intra-specific, inter-specific and inter-generic hybrids and if so, what the fate of the transgene and its expression was in these different genetic backgrounds and in successive selfings and backcrosses (Chapters 3, 4 and 5).

Reviewing the taxonomy and cytogenetics of the family of *Cruciferae* revealed that there were ample possibilities for inter-specific and inter-generic hybridization, either with or without embryo-rescue techniques (Chapter 1). Pollen dispersal by both insects and wind is the main factor through which transgenes in oilseed rape may spread. Gene dispersal from transgenic oilseed rape to its (wild) relatives can not be ruled out. Hybridizations, such as oilseed rape (genome constitution AACC) with *B. rapa* (AA) and *B. juncea* (AABB) have been described to occur spontaneously under field conditions.

Assuming outcrossing occur, attention should be given to the ecological and toxicological impact of the introduced PPT tolerance transgenes in combination with the use of PPT

(Chapter 2). To illustrate the so-called 'transgene-centered approach' in which all characteristics of a particular transgene and its product are assayed the *pat* and *bar* transgenes, whose gene products, confer PPT tolerance were reviewed. The use of PPT-tolerant crops in combination with PPT could imply a considerable environmental gain compared to currently used herbicide cocktails. Assuming responsible use of PPT-tolerance in agronomy, the consequences with respect to weediness or spread of this trait are minor. Consumption of unspread transgenic PPT-tolerant plant material containing the *bar* or *pat* transgenes and/or the gene product PAT will have no adverse effects. *Bar* and *pat* DNA will not differ from any other DNA that passes the digestive tract daily and all data found, indicate that no toxicity or allergenicity of PAT are to be expected. Upon spraying PPT-tolerant plants, PPT or derivatives might be present in food and feed. To date, it is insufficiently clear to what extent consumers are exposed to PPT (metabolites) and what the toxicological impact of such exposure might be. As long as there is not much familiarity with the trait, pre-market evaluation of the levels of PPT metabolites in PPT-tolerant plant food will indicate, which further toxicological data are necessary for safe consumption.

Because biological containment cannot be obtained for oilseed rape, studying the transgene transmission and its fate in different genetic backgrounds and over generations can indicate the transgene impact in time in sexual offspring. Only when the transgenes are transmitted and expressed in a predictable, consistent and stable manner in subsequent generations during seed multiplication or subsequent steps in a breeding program, transgenic crops have commercial value. The *bar* gene was successfully transmitted to intraspecific, inter-specific and inter-generic hybrids. In crosses among independent transgenics of one variety and between transgenics of different varieties, no transgene inactivation was observed (Chapter 3). This is what has been expected, because the phenotypic expression of the transgene in homozygous and hemizygous nature in these transgenics was stable. However, independent from its homozygous or hemizygous nature, infrequent loss of expression of the PPT tolerance transgene was found after selfings and backcrosses of some individual transgenic plants with non-transgenic oilseed rape. Molecular analyses of susceptible plants showed that the transgene was still present. Gene inactivation might be caused by methylation or co-suppression while also somaclonal variation might be one of the mechanisms responsible for a reduced of even a loss of phenotypic expression in later generations. These observations indicate that breeders should test whether selected lines

stably express PPT tolerance during subsequent generations as is also required in conventional breeding programs.

By inter-specific hybridization between *B. rapa* (AA) and two transgenic oilseed rape lines, the PPT tolerance transgene was relatively easily transmitted into the F_1 hybrids and retained active (Chapter 4). During backcrossing, between offspring of the two investigated transgenic lines large differences in transmission frequency of the transgene were noted. The line showing low transmission contained the transgene most probably integrated into a C-genome chromosome and in the line showing high transmission it was probably integrated into a chromosome of the A-genome. Therefore, gene transfer from oilseed rape (AACC) to *B. rapa* (AA) and *B. juncea* (AABB) can be limited considerably by integration of the transgene on chromosomes of the C-genome. An alternative approach to prevent gene dispersal through pollen transfer is integration of the transgene into the DNA of plastids, since these organelles are maternally inherited in most plants.

The inter-generic crosses between transgenic PPT-tolerant oilseed rape and radish (*Raphanus sativus*) have no biosafety impact (Chapter 5). Potential spread of transgenes from oilseed rape to radish is negligible, because hybridization can only be accomplished using a modified flower culture method. Hybrids produced small amounts of stainable pollen, but they could not be selfed and backcrosses on radish did not yield any viable seed.

In the biosafety assessment of genetically modified plants a distinction between 'biosafety in the narrow sense' and 'biosafety in the broad sense' was proposed (Chapter 6). With respect to 'biosafety in narrow sense', ecological concerns focus on weediness and vertical and horizontal transgene spread and toxicological concerns focus on food safety and consumption. With respect to 'biosafety in the broad sense', concerns also reflect social, ethical and/or economic views related to current agriculture. Regulatory authorities in the UK and the Netherlands tend to consider mainly the 'narrow sense' biosafety questions of transgenic plants. Austria and the Scandinavian EU members take the position that 'broad sense' effects should also include linkage of safety aspects of transgenic plants with criteria such as sustainability, socio-economics and ethics.

At the end of 1996 transgenic glyphosate-tolerant soybeans were shipped to Europe, which led to protests. Arguments put forward by environmentalists against these soybeans concern primarily the herbicide, which was already allowed to be applied pre-harvest for

wild type soybean (USA) and other crops (The Netherlands). Both application of the herbicide and tolerant plants are approved following Dutch and EU regulations. Permission was given to import, store and process in food these glyphosate-tolerant soybeans. However, this does not mean that all herbicide-tolerant crops are biosafe. When a herbicide tolerance transgene is evaluated to be 'biosafe in the narrow sense', it might still possess undesirable characteristics with respect to its 'biosafety in the broad sense'. For example, a particular transgene confers tolerance to a herbicide with an adverse environmental or toxicological impact, such as bromoxynil or the persistent herbicide chlorsulfuron. Introduction of crops tolerant for such herbicides might stimulate the use of these herbicides. In the cases of bromoxynil and chlorsulfuron it is questionable whether or not this is a benign development due to respectively their toxicity and persistence in the soil for years.

The major issues described in this thesis are summarized as follows:
- the spread of the PPT tolerance transgene from oilseed rape to (wild) relatives occurs, especially when the transgene is integrated into a chromosome of the A-genome
- gene flow from PPT-tolerant oilseed rape to *B. rapa* (AA) and *B. juncea* (AABB) can be limited considerably through selection for the presence of the PPT tolerance transgene on one of the chromosomes of the C-genome of oilseed rape or through integration of the transgene into the chloroplast genome
- the transgene-centered approach shows that without spraying with PPT, PPT-tolerant crops are ecologically and toxicologically biosafe. Upon spraying, however, it is currently insufficiently clear whether consumers are exposed to what levels of PPT and/or its metabolites. No toxicological data are available of PPT-derived metabolites or how they behave upon food processing
- in intra-specific crosses involving PPT-tolerant oilseed rape occasional loss of phenotypic expression of the PPT tolerance was observed. This implies that breeders have to follow time-consuming selection procedures to ensure stable expression of transgenic traits, which are similar to those followed in conventional breeding
- because inter-generic hybrids between transgenic oilseed rape and radish are difficult to make and almost sterile, transgenes cannot spread in the environment through radish and these hybrids, therefore, have no impact from a biosafety point of view

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- in the assessment of the biosafety of genetically modified crops a distinction can be made between 'biosafety in the narrow sense' and 'biosafety in the broad sense'. 'Biosafety in the narrow sense' involves the ecology and toxicology of both release and use of transgenic plants. 'Biosafety in the broad sense' also implies social, ethical and/ or economic aspects of transgenic crops with respect to current agriculture
- permission for commercialization of a particular herbicide tolerance-herbicide-crop combination does not create a precedent for other herbicide tolerance-herbicide-crop combinations, but their 'biosafety in the broad sense' should be evaluated as a new case.

Samenvatting

Al dan niet biologisch veilig

- een evaluatie van transgeen fosfinothricine-tolerant koolzaad (*Brassica napus* L.) -

Bij de verbetering van kwaliteit en toepassingsmogelijkheden van cultuurgewassen vormt genetische modificatie een uitbreiding van de technieken die in de plantenveredeling gebruikt kunnen worden. Bij de toepassing van genetische modificatie zijn natuurlijke kruisingsbarrières niet langer beperkend. Alle organismen vormen hierdoor een bijna onuitputtelijke bron van eigenschappen waarvan de verantwoordelijke genen geïsoleerd en vervolgens in cultuurplanten ingebracht kunnen worden. Op dit moment worden meer en meer genetisch gemodificeerde gewassen geïntroduceerd in het milieu en op de markt gebracht en verwacht mag worden dat deze gewassen in de nabije toekomst een significante bijdrage zullen leveren aan de landbouw. Sinds de introductie van transgene planten in het milieu worden er discussies gevoerd over hun biologische veiligheid en over de regulering voor de markttoelating van deze gewassen. Voorafgaand aan het in het milieu en op de markt brengen van transgene planten moeten binnen vastgestelde wettelijke kaders verschillende stadia van inperking doorlopen worden en zijn wettelijke regels met betrekking tot het op de markt brengen van biotechnologische voedingsproducten opgesteld. Herbicide tolerante gewassen, waaronder fosfinothricine (FFT)-tolerant koolzaad, vormen de voorhoede bij het op de markt brengen van transgene gewassen. Daarom werd deze specifieke gewas-eigenschap combinatie als model gekozen voor deze studie naar de biologische veiligheid van transgene gewassen.

Het doel van het in dit proefschrift beschreven onderzoek was het vergaren van kennis over en het vervolgens evalueren van de biologische veiligheid van FFT-tolerant koolzaad. Ten behoeve van een tweeledige aanpak is een literatuurstudie naar de eigenschappen van koolzaad en naar de ecologische en toxicologische impact van het FFT tolerantie gen tezamen met het FFT herbicide uitgevoerd (Hoofdstukken 1 en 2). Tevens zijn experimenten uitgevoerd om te bepalen of FFT tolerantie via kruising kan worden overgebracht naar intra-specifieke, soort- en geslachtshybriden en wat het lot van de activiteit van het transgen in de verschillende genetische achtergronden en in opéénvolgende zelfbevruchtingen en terugkruisingen is (Hoofdstukken 3, 4 en 5).

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Gegevens over de taxonomie en cytogenetica binnen de familie der cruciferen geven aan dat soort- en geslachtskruisingen mogelijk zijn, al dan niet met behulp van *in vitro* technieken (Hoofdstuk 1). Pollen overdracht door insecten en de wind is de belangrijkste manier waarop transgenen in koolzaad zich kunnen verspreiden. Onder veldomstandigheden zijn spontane kruisingen tussen koolzaad (genoomsamenstelling AACC) en *B. rapa* (AA) en *B. juncea* (AABB) mogelijk en genoverdracht van transgeen koolzaad naar de (wilde) verwanten is dan ook niet uit te sluiten.

Omdat uitkruising kan optreden dient er aandacht besteed te worden aan de ecologische en toxicologische gevolgen van het geïntroduceerde herbicide tolerantie transgen in combinatie met het gebruik van het herbicide FFT (Hoofdstuk 2). Ter illustratie van de aanpak waarbij het transgen centraal staat, de zogenaamde 'transgene-centered approach' werden de *bar* en *pat* transgenen, waarvan de producten tolerantie voor FFT geven, bestudeerd. Uit deze evaluatie waarbij alle eigenschappen van het transgen en het genproduct werden onderzocht, bleek dat het gebruik van FFT-tolerante gewassen in combinatie met FFT in vergelijking met de huidige in gebruik zijnde herbicide cocktails een aanzienlijke milieuwinst kan opleveren. Indien FFT landbouwkundig verantwoord wordt gebruikt, dan zijn de consequenties met betrekking tot veronkruiding en verspreiding van FFT tolerantie gering. Consumptie van onbespoten FFT-tolerant plant materiaal heeft geen nadelige effecten omdat het *bar* en *pat* DNA niet verschillen van ander DNA dat dagelijks het spijsverteringskanaal passeert. Bovendien wijzen alle gevonden gegevens erop dat het genproduct, het fosfinothricine acetyltransferase eiwit, niet toxisch of allergeen is. Na bespuiting van FFT-tolerante planten met FFT, kunnen FFT of omzettingsproducten van FFT in voedsel en veevoer voorkomen. Op dit moment is het niet duidelijk of en zo ja in welke mate mens en dier aan FFT of FFT-metabolieten worden blootgesteld bij consumptie van FFT-tolerante gewassen en wat daar de toxicologische gevolgen van zijn.

Omdat biologische inperking van koolzaad niet mogelijk is, is het lot van het transgen in sexuele nakomelingen in de tijd gevolgd. Hiervoor is de transgenoverdracht bestudeerd en tevens de expressie van het transgen in verschillende genetische achtergronden en (terugkruisings)generaties. Bij commercialisatie van transgene gewassen is het noodzakelijk dat een transgen voorspelbaar en stabiel overerft en een stabiele expressie in opéénvolgende generaties tijdens zaadvermeerdering of in een veredelingsprogramma laat zien.

Het *bar* gen kon worden overgebracht naar intra-specifieke, soort- en geslachtshybriden. Er werd geen transgeninactivatie waargenomen in kruisingen tussen onafhankelijke transgene lijnen van één ras of van verschillende rassen (Hoofdstuk 3). Dit was conform de verwachting, omdat de fenotypische expressie van het transgen in homozygote en hemizygote toestand daadwerkelijk stabiel bleek te zijn. Soms werd na zelfbevruchting van individuele FFT-tolerante koolzaad planten of na terugkruising van deze planten met niet-transgeen koolzaad echter verlies van expressie van het FFT tolerantie gen gevonden. Moleculaire analyse van deze FFT-gevoelige planten toonde aan dat het transgen wel aanwezig was. De gevonden geninactivatie kan veroorzaakt worden door methylering of co-suppressie. Ook somaclonale variatie kan één van de verantwoordelijke mechanismen zijn die tot verlies of een verminderde fenotypische expressie van het FFT-tolerantie gen in latere generaties leidt. Deze waarnemingen geven aan dat, net zoals in conventionele veredelingsprogramma's, ook geselecteerde lijnen van transgene gewassen getest moeten worden op het stabiel tot expressie komen van het ingebrachte transgen tijdens opééénvolgende generaties.

Door soortkruising tussen *B. rapa* (AA) en twee transgene koolzaadlijnen (AACC) kon de FFT-tolerantie relatief gemakkelijk naar F_1 hybriden worden overgebracht waarin het stabiel tot expressie kwam (Hoofdstuk 4). Gedurende vier terugkruisingen met *B. rapa*, werd er echter tussen de twee transgene ouderlijnen een verschil waargenomen in de frequentie waarmee het transgen naar de volgende generatie werd overgebracht. In de ouderlijn met de laagste FFT-tolerantie overdracht, was het transgen hoogstwaarschijnlijk in één van de chromosomen van het C-genoom en in de ouderlijn met de hoogste overdracht in één van de chromosomen van het A-genoom geïntegreerd. Dit gegeven laat zien dat genoverdracht vanuit koolzaad (AACC) naar *B. rapa* (AA) en *B. juncea* (AABB) aanzienlijk beperkt kan worden door integratie van het transgen in één van de C-genoom chromosomen. Een alternatieve manier om genoverdracht via pollenverspreiding tegen te gaan is de integratie van het transgen in het DNA van plastiden, omdat deze organellen in de regel maternaal overerven.

De geslachtskruising tussen koolzaad en radijs heeft geen consequenties voor de biologische inperking van transgeen koolzaad. Mogelijke verspreiding van transgenen vanuit koolzaad naar radijs is verwaarloosbaar, omdat hybridisatie slechts met behulp van een *in vitro* bloemcultuurmethode lukte. Aldus verkregen hybriden produceerden een kleine hoe-

veelheid vitaal pollen, maar de hybriden konden niet worden zelfbevrucht en terugkruisingen op radijs leverden geen kiembare zaden op.

Bij de analyse van genetisch gemodificeerde planten kan een onderscheid gemaakt worden tussen 'biologische veiligheid in engere zin' en 'biologische veiligheid in ruimere zin' (Hoofdstuk 6). Bij 'biologische veiligheid in engere zin' concentreren de ecologische aspecten zich op veronkruiding, verticale en horizontale genoverdracht, terwijl de toxicologische aspecten zich concentreren op voedselveiligheid en -consumptie. Bij 'biologische veiligheid in ruimere zin' wordt ook rekening gehouden met maatschappelijke, ethische en economische standpunten betreffende de huidige landbouw. Regelgevende autoriteiten in het Verenigd Koninkrijk en Nederland neigen bij transgene planten vooral biologische veiligheidsaspecten in 'engere zin' in beschouwing te nemen. Oostenrijk en de Scandinavische EU lidstaten staan echter meer op het standpunt dat biologische veiligheidsaspecten in 'ruimere zin' ook beschouwd moeten worden en dus gekoppeld moeten worden aan criteria, als duurzaamheid, sociaal-economische aspecten en ethiek.

Eind 1996 is het eerste glyfosaat-tolerante soja naar Europa verscheept, wat tot protesten van diverse actiegroepen heeft geleid. Argumenten van de milieubeweging tegen deze soja hebben voornamelijk betrekking op het gebruik van het herbicide glyfosaat, dat echter in Nederland voor andere gewassen al toegelaten is voor het doodspuiten van het gewas vlak voor de oogst. Zowel toepassing van dit herbicide als van de glyfosaat-tolerante planten zijn goedgekeurd op basis van Nederlandse en Europese regelgeving. Deze glyfosaat-tolerante soja mag dus worden ingevoerd, opgeslagen en verwerkt in voedingsmiddelen. Dit betekent echter niet dat alle herbicide-tolerante planten biologisch veilig zijn. Als een herbicide tolerantiegen na evaluatie 'in de engere zin' biologisch veilig is bevonden, kan het nog steeds ongewenste eigenschappen bezitten in relatie tot biologische veiligheid 'in ruimere zin'. Voorbeelden hiervan zijn transgenen die gewassen tolerant maken voor herbiciden die een ongewenst toxicologisch of milieu effect hebben, zoals het herbicide bromoxynil of persistente herbiciden, zoals chlorsulfuron. Introductie van gewassen tolerant voor dergelijke herbiciden zal het gebruik van deze herbiciden stimuleren, hetgeen een ongewenste ontwikkeling is gezien hun respectievelijke toxiciteit en jarenlange persistentie in de bodem.

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De belangrijkste in dit proefschrift beschreven aspecten kunnen als volgt worden samengevat:

- verspreiding van het FFT tolerantie gen vanuit koolzaad naar (wilde) verwanten treedt op. Dit is vooral het geval als het transgen op een chromosoom van het A-genoom van koolzaad is gelocaliseerd, omdat zowel *B. rapa* als *B. juncea* het A-genoom bezitten

- genoverdracht van FFT-tolerant koolzaad naar *B. rapa* kan biologisch aanzienlijk beperkt worden door integratie van het transgen op één van de chromosomen van het C-genoom van koolzaad of door integratie in het chloroplastgenoom dat normaliter maternaal overerft

- de analyse die het transgen centraal stelt, laat zien dat zonder FFT bespuiting, het FFT-tolerante gewas, ecologisch en toxicologisch biologisch veilig is. Na FFT bespuiting van een dergelijk gewas is het echter onduidelijk of en in welke mate consumenten worden blootgesteld aan FFT en/of omzettingsproducten van FFT en of dit een effect heeft op hun gezondheid

- in intra-specifieke kruisingen wordt soms onverwacht het verlies van de expressie van het FFT transgen waargenomen. Dit impliceert dat, net als in de conventionele veredeling, tijdrovende selectie procedures gevolgd moeten worden om zeker te zijn van stabiele transgenexpressie gedurende een reeks van generaties

- omdat geslachtshybriden tussen koolzaad en radijs moeilijk zijn te maken en bovendien bijna steriel zijn, kunnen transgenen niet via radijs verspreid worden in het milieu en hebben deze hybriden geen consequentie voor de biologische veiligheid van transgeen koolzaad

- er kan een onderscheid gemaakt worden tussen 'biologische veiligheid in engere zin' en 'biologische veiligheid in ruimere zin'. 'Engere zin' heeft betrekking op de ecologische en toxicologische gevolgen van de introductie en het gebruik van transgene planten. 'Ruimere zin' heeft ook betrekking op sociale, ethische en/of economische aspecten van transgene planten in relatie tot de huidige landbouw

- goedkeuring voor het vermarkten van een bepaalde herbicide tolerantie-gewas combinatie schept geen precedent voor andere combinaties. Hun biologische veiligheid 'in ruimere zin' moet iedere keer opnieuw beschouwd worden, zeker als het tolerantie voor een herbicide betreft dat persistent is of een nadelig toxicologisch effect heeft.

Account

Parts of the work described in this thesis have been or will be published elsewhere:

Metz, P.L.J., Jacobsen, E., Nap, J.P., Pereira, A. & Stiekema, W.J. The impact on bio-safety of the phosphinothricin tolerance transgene in inter-specific *B. rapa* x *B. napus* hybrids and their successive backcrosses. Theor. Appl. Genet. in press. (Chapter 4).

Metz, P.L.J., Jacobsen, E. & Stiekema, W.J. (1997). Aspects of the biosafety of trans-genic oilseed rape (*Brassica napus* L.). Acta Bot. Neerl. **46**: in press. (Chapter 1).

Metz, P.L.J., Jacobsen, E. & Stiekema, W.J. Occasional loss of expression of phosphi-nothricin tolerance in sexual offspring of transgenic oilseed rape (*Brassica napus* L.). Submitted to Euphytica. (Chapter 3).

Metz, P.L.J., Nap, J.P. & Stiekema, W.J. (1995). Hybridization of radish (*Raphanus sativus* L.) and oilseed rape (*Brassica napus* L.) through a flower-culture method. Euphytica **83**:159-168. (Chapter 5).

Metz, P.L.J., Stiekema, W.J. & Nap, J.P. A transgene-centered approach to the biosafety of transgenic phosphinothricin-tolerant plants. Submitted to Nature Biotechnology. (Chapter 2).

Other publications related to the topic of this thesis:

Metz, P.L.J. & Nap, J.P. (1997). A transgene-centered approach to the biosafety of transgenic crops: overview of selection and reporter genes. Acta Bot. Neerl. **46**: in press

Nawoord

Dit proefschrift beschrijft de resultaten van een 'niet-promotie' onderzoek. Lange tijd zag het er namelijk naar uit dat het moeilijk zou zijn om op dit project te promoveren. Immers kon een onderwerp als biologische veiligheid wel wetenschappelijk benaderd worden en konden er in een drie-jarig onderzoek voldoende resultaten verkregen worden? Gaandeweg kwam het besef dat het er misschien inzat en groeide het vertrouwen op een goede afloop. Dat er uiteindelijk toch een proefschrift voor u ligt is mede te danken aan de bijdrage van vele mensen die ik hierbij wil bedanken.

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Het was wel even wennen in het begin als veredelaar tussen moleculaire biologen, maar Moleculaire Biologie bleek een leuke afdeling en het werken binnen deze afdeling gaf mij de gelegenheid mijn kennis en ervaring uit te breiden. De goede sfeer en de belangstelling en betrokkenheid van mijn afdelingsgenoten heb ik altijd als heel prettig ervaren. Zonder anderen tekort te doen wil ik hier mijn kamergenoten Jos en Fred bedanken voor de gezellige tijd en Bas en Andy voor het begeleiden van mijn eerste schreden op het 'moleculaire pad'.

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Curriculum vitae

Petrus Lambertus Johannes Metz werd op 10 februari 1962 te Amsterdam geboren. Na het doorlopen van VWO-B van 1974 tot 1980 aan het Pius X Lyceum in Amsterdam, werd in 1980 begonnen met de studie Plantenveredeling aan de Landbouwuniversiteit (destijds Landbouw Hogeschool) te Wageningen. In september 1986 werd de kandidaats-fase afgerond. Het doctoraalexamen werd in november 1988 behaald met als hoofdvakken plantenveredeling (Prof. dr. ir. J.E. Parlevliet en dr. ir. M.S. Ramanna, uitgevoerd bij het toenmalige Instituut voor de Veredeling van Tuinbouwgewassen onder begeleiding van dr. ir. W.H. Lindhout) en plantenfysiologie (Prof. dr. C.M. Karssen) en als bijvak erfe-lijkheidsleer (dr. ir. P. Stam en ir. J.W. van Ooijen). Zijn praktijktijd werd doorgebracht bij twee veredelingsbedrijven, Bruinsma te Naaldwijk en Rijk Zwaan in de Lier, en bij het Instituut voor Plantenziektenkundig Onderzoek in Wageningen. Na enige tijd werkloos te zijn geweest, werd januari 1990 begonnen met een Na-Doctoraal Onderzoeksproject bij de vakgroep Plantenfysiologie van de LUW hetgeen in juli van dat jaar resulteerde in een 'echte' baan als onderzoeker bij de afdeling Dicotyle Akkerbouwgewassen van het CPO-DLO, de voorloper van het CPRO-DLO, voor anderhalf jaar. Aansluitend hierop werd in januari 1992 bij de afdeling Moleculaire Biologie van het CPRO-DLO begonnen met het onderzoek waarvan dit proefschrift het resultaat is.

Sinds maart 1996 is hij bij dezelfde afdeling als onderzoeker werkzaam binnen een EU-project dat moet leiden tot de ontwikkeling van plantmateriaal voor transposon tagging in arabidopsis.