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Environmental risks related to the release of genetically modified plants with the focus on oilseed rape (*Brassica napus*)

Kirsti Kvaløy

NINA Project Report 15

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Abstract

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The risk of gene flow associated with growing genetically modified plants is important to evaluate when the GM-plants are released into the environment. Gene flow is expected to have its greatest effect on weed species that are closely related to crop plants or as contamination of neighbouring crops. Because of its many weedy relatives, dispersal of transgenes from GM oilseed rape and cultivated *B. rapa* via pollen could potentially hybridise with related *Brassica* species or neighbouring crops. Dependent on the transgene being transferred, the transgenes may cause difficulties with weed and volunteer management in agricultural fields or influence ecological balances and interactions. There is some urgency for knowing more about weed-complexes of *B. napus* / *B. rapa* and survival of such hybrids since *B. rapa* populations already is a serious weed of more than 20 crops in more than 50 countries and many transgenic types of oilseed rape is now being commercialised.

This project is part of an initiative to increase the knowledge of potential environmental risks related to the release of genetically modified plants with the main focus on possible gene dispersal and cross hybridisation between genetically modified oilseed rape and weedy *Brassica rapa*. The project comprises a theoretical description of general risks related to the release of genetically modified oilseed rape and cultivated *B. rapa*. In addition, the project includes an experimental part which involves the development of expertise on using genetic marker methodology to detect *B. napus* / *B. rapa* hybrids and the use of such techniques to evaluate the genetic diversity in *B. napus* and *B. rapa* cultivars.

The presence of *B. napus* / *B. rapa* hybrids derived from plots where both ratios between *B. napus* and weedy *B. rapa* and density (high, intermediate and high) between each plant varied, was investigated in this project. Six hybrids out of 281 samples tested were detected. Three genetic marker-systems were used: inter-SSR, one microsatellite and AFLP. The investigation suggested a tendency of a higher number of hybrids when plants were organised in a 1:1 ratio at a high density. The AFLP-technique demonstrated genetic diversity within both cultivated *B. napus* and *B. rapa* specific agricultural lines. The results indicated higher genetic diversity within cultivated *B. rapa* than *B. napus*, which is in agreement with the expected since *B. rapa* is known to cross-pollinate. The inter-individual genetic differences that were demonstrated proclaim the importance of including several samples of the same cultivar when executing this kind of surveys.

Important issues to consider and to increase our knowledge on in general are base-line information on previous occurrence of hybridisation events in regions with extensive growth of cultivated *Brassica* species. Furthermore, an accurate measure of the separate agricultural areas of oilseed rape and cultivated *B. rapa* and present-day agricultural practise in Norway would help us to estimate potential impact of future releases of genetically modified types. At present no genetically modified crops are grown in Norway. This situation could change for example as a result of a change in consumers' demand for potentially quality improved GMPs or by modified crops possessing great agricultural advantages for Norwegian farmers. To foresee which types of plants this could entail would help us to get an idea of which risks that are most urgent to increase our knowledge on.

Key words: GMO, environmental monitoring, oilseed rape, *Brassica napus*, *Brassica rapa*, hybrids, introgression, gene flow, molecular fingerprint techniques.

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Preface

Against a background of a global increase in production and release of genetically modified plants, it is important to expand our knowledge of the potential environmental effects such activity might have. Good ecological monitoring designs are essential to enable predictions of potential environmental effects to be made. Such designs are best with as much knowledge as possible on the environment to which the GM plant is being released, the GM plant itself and its potential interactions with the environment.

The Norwegian Directorate for Nature Management provided funding for this project. A travel grant was also received from the European Science Foundation (ESF). The main focus of this project involves a theoretical assessment of the environmental risks related to the release of genetically modified oilseed rape *(B. napus*). In addition experimental work is described which was performed during a one-month stay in Dr. Rikke Bagger Jørgensens group at the Plant Genetics Section, Environmental Science and Technology Department, Risø National Laboratory, Roskilde, Denmark. This work focus on the use of molecular fingerprint markers to investigate *B. napus / B. rapa* hybridisation events. Molecular markers are also used to obtain distinct fingerprint patterns in *B. napus*, and cultivated and weedy *B. rapa*. Material used was mostly developed during Marina Johannesens PhD work. I am very grateful to Rikke Bagger Jørgensen, Marina Johannesen and Bente Andersen for their support and help during my stay at Risø. I also aknowledge the economic support from the ESF AIGM (Assessment of the Impact of Genetically Modified Plants) Programme. I would also like to thank Esten Ødegaard (Directorate for Nature Management) for help and comments on the report manuscript.

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Kirsti Kvaløy

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1 Introduction

The release, import and use of GMOs both related to research and commercial trade might have negative effects on health and environment. It is necessary to monitor these potential effects. The responsible authorities must ensure that control and monitoring is implemented in the best possible way based on good theoretical and practical knowledge.

This project is part of an initiative to increase the knowledge of potential environmental risks related to the release of genetically modified plants with special focus on possible gene dispersal and cross hybridisation between genetically modified oilseed rape and weedy *Brassica rapa*. The project will involve the development of general expertise on aspects related to experiments for targeted analysis of GMPs.

1.1 Genetically modified plants

Modern biotechnology offers benefits to medical and biological research, and in the future possibly to nutrition and agriculture in large parts of the world. There are however concerns that the release, import and use of genetically modified organisms (GMOs) both related to research- and commercial trade might have unforeseen negative effects on health and environment.

New plant biotechnology offers the possibility to transfer new traits of agronomic importance into crop species. The first series of transgenic plants released in the late 1980s and early 1990s mostly contained traits such as improved herbicide tolerance and insect or microbial resistance. From the early 1990s, there have been an increasing number of releases of transgenic plants that involve traits for quality improvement.

The Regulatory authority's responsibility is to see that control and monitoring is undertaken in the best possible way by ensuring that knowledge is present both at the theoretical and practical level. The development and release of GMOs is subject to regulation in the EU under the directive 90 / 220 / EEC on deliberate release of GMOs into the environment.

1.2 General risks related to the release of genetically modified plants - GMPs

There is a long list of concerns regarding genetically modified crops. Transgenes that escape and persist can be detrimental if they confer higher fitness to the plants carrying them or if the phenotype of the transgenic plant alters species interaction in the environment that surrounds them (Tiedje et al., 1989; Crawley et al., 1993). Regarding the transfer of inserted genetic material or the dispersal of transgenes to the environment, most studies and concerns have emphasised dispersal in space, especially the movement of pollen. However, transgenes may also disperse in time through persistence of dormant seeds in the soil. The ability of this to happen depends on seed bank characteristics, which may vary depending on for instance maternal effects specific for certain crop-wild hybrids (Linder & Schmitt 1994).

Here, I will focus on the dispersal of inserted traits through pollination of other crops or wild plants by wind or insects giving rise to hybrid native plants (Bergelson, 1998). Several crops are known to interbreed with weedy relatives under field conditions, i.e. oilseed rape, rice, sunflower, sugar beet, carrot, strawberry, wheat, oats, rice, etc. (Raybold & Gray 1993; Snow & Palma 1997; Zemetra et al., 1997). These could become more competitive than the original plants and potentially more invasive depending on the trait inserted and the environment to which the plant is released. Important factors affecting the chance of these events to occur are the ability for two plants to cross hybridise with each other, form hybrids, and whether these are able to produce seeds to further propagate the novel trait. The transgenic plant itself might also have an increased ability under certain circumstances to disperse, persist and compete compared to the original non-transgenic plant. All these factors could change the population dynamics at the re-

lease site and the surrounding environment. Gene flow between crops and weeds, or "introgressive hybridisation", is sometimes difficult to demonstrate as morphology varies as a continuum within a gene pool, and intermediate forms may originate both from interspecific crosses and species variability. Molecular markers, makes such an investigation easier to perform and is often a prerequisite to be able to understand the situation.

1.3 Brassica

Brassica as crop encompasses very diverse types of plants. They are grown as vegetables, fodder and the source of oils or condiments. Rape and mustard are a source of vegetables and oil, which constitute an important part of the human diet. The oil is also utilised for various industrial purposes. Oleiferous Brassica ranks fifth in importance among oilseed crops. Here, important species are *B. rapa* ssp. oleifera (before *B. campestris*) or turnip rape and *B. napus* (rape) in temperate Europe and Canada, *and B. rapa* and *B. juncea* (mustard) in Asia.

The nomenclature of the different species of *Brassica* has presented a great deal of confusion. After much confusion, it was agreed that crop *Brassicas* consist of six species, three, *B. nigra*, *B. oleracea* and *B. campestris* of which *campestris* are regarded as elementary or basic species. The other three, *B. carinata*, *B. juncea* and *B. napus*, as amphidiploids derived from any two of the basic species (Prakash & Hinata 1980). The *Brassica* oilseed and condiment crops have evolved within closely related species, which form part of a larger interspecific, intergenic genepool. Cytological evidence suggests that all the *Brassica* derived crops originated from an extinct, common ancestor with a basic chromosome number of x = 5 or 6 (Heyn, 1977). The species with the lower chromosome numbers (n = 8-12) apparently developed into separate monogenomic species through a process of autoploidy followed by chromosomal loss and structural differentiation (Downey et al., 1980). It has been found that the genus includes a number of species with diploid chromosome numbers ranging from 14 to 38, forming a partially continuous series. The three species with higher chromosome complements, *B. juncea*, *B. napus* and *B. carinata* (tetraploid with chromosome numbers: 38, 36 and 34 respectively), are thought to have resulted from natural crosses between the monogenomic species *B. campestris* (syn. *B. rapa*), *B. oleracea* L. and *B. nigra* (diploids with chromosome numbers: 20, 18 and 16 respectively)(Heyn, 1977). This cytogenetic relationship of different species was established by U and is known as U's triangle after him (see Fig. 1; U, 1935). The A genome was assigned to *campestris*, B to *nigra* and C to *oleracea*. Similarly the genomes of higher chromosome species or amphidiploids were designated AB for *juncea*, BC for *carinata* and AC for *napus*. The probability of obtaining crosses within and between *Brassica* species and forms will vary with species and cultivar, in addition to condition of the plants and the environment.

Interspecific hybridisation in *Brassica* was first reported having been attempted by Herbert in 1834 when he successfully crossed *napus* and *rapa* and obtained F_1-hybrids (Sikka 1940). At the same time, Sageret (in 1826) also succeeded in hybridising *oleracea* with *rapa* and *napus*.

B. napus and *B. juncea* are self-fertile, with approximately 80% of the seeds arising from self-pollination. Most *B. rapa* and *B. hirta* strains, are highly self-incompatible, and self-pollinated seeds are difficult to produce. The ability to either be self-fertile or self-incompatible depends on whether or not the species contain a self-incompatibility (SI) system which forces natural outcrossing. Both wind and insects are effective pollen vectors of the *Brassica* oilseed and condiment species. However, under large-scale commercial production, wind is the main pollinating agent in these species, with insect pollinators of less importance (Downey et al., 1980).

There are basically two different ways of reproduction, vegetative propagation and sexual reproduction where the latter include self-pollination and cross-pollination. The different means of reproduction will strongly affect the genetic variability. Continual vegetative propagation will almost exclude genetic variability within a clone. Self-pollinating plants tend to be homozygous because the fraction of heterozygous decreases at each generation of self-pollination (review by Suzuki et al., 1989). In a cross-pollinating population each plant is genetically distinct and will produce distinct gametes. Depending on the number of potential hybridisation partners being

high enough, this kind of reproduction has the highest chance of maintaining high genetic diversity.

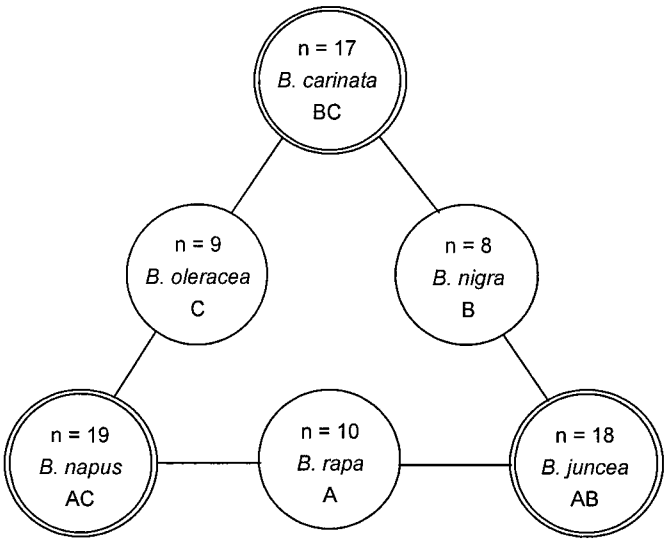

Figure 1
U's triangle that show the evolutionary relationship between three diploid and three allotetraploid Brassica-species. A, B and C represent the three original genomes.

1.3.1 Oilseed rape (*Brassica napus*)

Oilseed rape belongs to the family *Brassicaceae*, genus *Brassica*, and the species name is *Brassica napus*. *Brassica napus* is thought to originate from an interspecific hybridisation between garden cabbage (*Brassica oleracea*) and *Brassica rapa*. Thus it could only have originated in nature in those regions where these two species were growing together. *B. napus* does not occur in wild populations.

B. napus has 38 chromosomes in the vegetative cells (2n=38. genome composition AACC). Genetic constitution of AACC originate from a chromosome doubling because of the previously mentioned hybridisation between *B. oleracea* (2n=18. genome composition CC) and *B. rapa* (2n=20. genome composition AA). Approximately 1 mg pollen is produced per flower. These are quite dry and are therefore easily transported by wind. However, primary pollinators for oilseed rape are insects, especially honey bees. *B. napus* is to a high degree self-fertile. It is both annual and biennial.

1.3.2 *Brassica rapa* (syn. *B. campestris*)

The present day *campestris* comprises a vast number of morphologically divergent forms. The specific name "campestris" was given to annual weed plants by Linnaeus (in 1753). The various taxa in the *campestris* complex can be divided into three well-defined groups purely on morphological basis but within the limits of homology: oleiferous, leafy and rapiferous (for more information, see Prakash & Hinata 1980). Wild *campestris* is regarded as the species from which different forms originated. It is native throughout Europe, Western USSR, Central Asia and the Near East. Storage organs, leaves and seeds are all utilised in the varying forms of *B. campestris*. Turnip forms are important as forages for sheep and cattle, especially in northern Europe and

New Zealand, but are also eaten as a vegetable in many parts of the world. In China and Japan there is a range of leafy forms developed for salad and pickling purposes. Oilseed forms, both annual and biennial (summer and winter varieties) are of considerable economic significance. The annual form predominates in Canada where it provides a substantial proportion of the worlds' rapeseed crop. Annual forms are also widely cultivated in India and Pakistan. The biennial forms are important in Sweden and Norway where it is found to be hardier than the winter form of *B. napus*. Winter turnip-rape tolerates late sowing better than the *napus* analogue. It is hardier and earlier to ripen but is inferior in seed yield and oil content (McNaughton 1973).

Brassica campestris (2n = 2x = 20) has shown a very high level of polymorphism. The group has been classified into a number of subspecies on the basis of complete inter-fertility (Olsson, 1954). The wild type, ssp. *campestris*, is a slender rooted, branching, annual plant. The wild *campestris* is a common weed both in Europe (wild rape) and in North America (field mustard). The ssp. *oleifera*, turnip rape, appears closest morphologically, and probably phylogenetically, to wild type. *B. campestris.* It crosses readily with *B. napus*, particularly when the latter is used as a female parent. Both spontaneous and artificial hybrids have been reported (Frandsen & Winge 1932; McNaughton 1973; Olsson 1963). Hence, there are possibilities of introgression of characters from species into another.

1.4 Risks related to genetically modified oilseed rape (*Brassica napus*) and cultivated *Brassica rapa* (ssp. *oleifera*)

Oilseed rape was among the first of the major agricultural crops to be genetically modified. It is a crop grown on large areas, producing millions of flowers, each having approximately 60,000 pollen grains. Oilseed rape is a likely candidate for crop species that could exchange genes with its many weedy relatives. Among these, *B. rapa* (syn. *B. campestris*, "åkerkål" in Norwegian) is generally considered the most compatible (Scheffler & Dale, 1994) and naturally hybrids have been reported in the British Isles (Stace, 1975).

The knowledge of the extent of pollen dispersal from an agricultural field is an important prerequisite for estimating risk and setting up monitoring strategies. There have been several investigations of pollen dispersal from fields of oilseed rape. The data from these investigations, however, are not in total agreement with each other. This might be due to many things such as local conditions or the experimental designs that have been used. For instance demonstrated McCarteny and Lacey (1991) that pollen 100 metres from the source was reduced to 2-11%, which is much less than 27-69% documented by Timmons and colleagues (1994) at the same distance. Recent investigations in England have indicated higher frequency then expected of dispersed rapeseed pollen at long distances from the field (Newsweek, BBC, summer 1999). Wind borne rapeseed pollen was demonstrated to have dispersed to at least 475 m from the source and with bees even up to 4.5 km. It is important to consider whether the observations and results of experiments done in other countries are transferable to Norwegian conditions.

In Norway, we have several wild relatives present with the possibility of hybridisation with oilseed rape. The frequencies of such events are unknown since very little investigations have been performed. Potential hybridisation partners that are present in Norway are: *Brassica rapa* ssp. *campestris* which is common, *Raphanus raphanistrum* which is quite common, *Sinapsis arvensis*, *B. nigra*, *B. juncea* and *Hirschfeldia incana* which all exist but are quite rare.

1.5 Introgression between oilseed rape (*Brassica napus*) and weedy *Brassica rapa (ssp. campestris)*

When hybridisation occurs between weedy *B. rapa* (AA, 2n=20) and *B. napus* (AACC, 2n=38). F_1-hybrids are formed with an unbalanced chromosome number: 2n=29 (AACC). When these then back-cross to *B. rapa* the progenies will have between 20 and 29 chromosomes because they receive 10 A chromosomes from both parents and from the F_1-plant from 0-9 chromosomes. In these unbalanced genomes there might occur intergenomic recombinations. These

are thought to occur primarily in the hybrid and the first backcrossed generations (Lydiate et al., 1993; Song et al., 1995).

Hybridisation between *B. napus* and weedy *B. rapa* occurs spontaneously both ways (Jørgensen & Andersen 1994; Mikkelsen et al. 1996), but seems to happen more frequently with *B. rapa* as maternal plant. This can partly be explained by the difference in the growth rate of the pollen tube in the two species (Hauser *et al.*, 1998a). In seed lots harvested on *B. rapa* from Denmark, 0-69% hybrids with oilseed rape was detected depending on environmental conditions. (Landbo *et al.*, 1996). Field trials have shown that F_1-hybrids in the field backcrosses spontaneously with *B. rapa*, and that some of the backcross plants were fertile (Jørgensen *et al.*, 1996; Mikkelsen *et al.*, 1996). Although the *B. napus* x *B. rapa* F_1-hybrids tend to be more similar to *B. rapa*, they are larger than both parents (heterosis) with morphological characteristics that are intermediate. F_1-hybrids also possess fitness intermediate to their parents (Hauser *et al.*, 1998a). Investigations have shown that even if fitness of offspring from F_2 and back-crosses between weedy *B. rapa* and oilseed rape was reduced relative to their parents, some hybrids were as fit as the parents and significantly more fit than *B. rapa* (Hauser *et al.*, 1998b).

Most documentation of hybridisation between oilseed rape and *B. rapa*, has been based on trials carried out under controlled conditions either in the field or in greenhouses. Recently, introgression in naturally mixed populations of weedy *B. napus* and *B. rapa* have been documented in a field where conventional organic cultivation of oil seed rape had been going on 11 years ago (Hansen et al., 1999).

1.6 Agronomic practise related to oilseed rape and cultivated *B. rapa* in Norway

The cultivated acreage of the two crops, oilseed rape and *Brassica rapa* ssp. *oleifera* ("ryps" in Norwegian) together was in 1998 on 63 917 decare in Norway. It is hard to get exact data on the portion of each of these crops because the agricultural statistics mix them together. However, the growth of oilseed rape is limited to the regions, which are climatically most optimal parts of Norway; i.e. within the counties Østfold and Vestfold in the Oslofjord area while cultivated *B. rapa* ssp. *oleifera* is the dominant in the more northern parts. Therefore, probably less than half of the total oilseed production area is covered by rape production.

Agricultural practises in Norway related to oilseed rape, is that of growth rotation with grain. This has been shown to have a favourable effect on grain production. Limitations to increased oilseed cultivation are mainly climatic; there is no ongoing work in Norway for producing varieties that grow better under the harsh Norwegian conditions. Instead, seeds are imported (mainly from Sweden), and tested, especially for being able to produce under short growing seasons. The acreage is also limited by the «growth rotation» in that the agricultural policy is in favour of barley and wheat within the actual areas. Pests also play an important role here, to avoid «lump root» (a disease caused by fungi infection which generally is a problem on *Brassica*-species) oilseed rape is normally produced in the same field only each sixth year. There is also problems with insects belonging to the family *Nitidulidae* (pollen beetles, sap beetles, dried fruit beetles) which sometimes require the use of pesticide.

With the use of genetic manipulation one can foresee a situation where several of the aspects limiting cultivation of oilseed rape in Norway today will be abolished such as drought resistance, faster growth, faster germination, pest resistance, etc. In addition, the possibility of changing the composition of fatty acids and proteins would make the crop more suitable for various uses in agriculture, fisheries and industry. Increased cultivation acreage will lead to an enlarged chance of potential effects from large-scale releases of genetically modified oilseed rape; hence the need for increased focus on risk assessments. Table 1 is included to give an idea of the traits inserted and extent of genetic manipulation of *B. napus* and *B. rapa* at present. The data is extracted from the OECD-database (internet-address: http//:www.oecd.org/biotrack.nsf).

Table 1. Field trials in OECD of B. napus and B. rapa, 1988-1998

Inserted property	*Brassica napus* (% of 1070 properties in 886 plants in total)	*Brassica rapa* (% of 30 properties in 29 plants in total)
Fungi resistance	3	3
Insect tolerance	2	3
Herbicide resistance	45	66
Stress tolerance	2	-
Selectable markers	8	3
Male sterility / fertility restorer	15	24
Modified oil content	15	-
Increased nutrition	1	-
Protein / enzyme production	3	-
Others	6	-

2 Introduction to experimental part

Different cultivars of oilseed rape and hybrids between oilseed rape and weedy *B. rapa* are difficult to distinguish on the basis of their morphology. Alternative means of identifying oilseed rape cultivars have been based on seed oil fatty acid profiles (White & Law 1991), HPLC analysis of leaf glucosinolates (Adams et al., 1989), and starch-gel isozyme electrophoresis (Mundges et al., 1990). These techniques enable only a limited degree of polymorphisms to be detected and are often sensitive to environmental and developmental parameters. In contrast, some nuclear DNA markers are believed to be unaffected by external influence and has been successfully used to distinguish species within the *Brassica* genus. Restriction fragment length polymorphism (RFLP) analysis was the first molecular technique to be used for this purpose. Although this method generally produces low number of discriminating loci (Waugh & Powell 1992), and requires high yields of DNA of high purity. PCR-based techniques have advantages to the RFLP-technique such as for example the need for only minute quantities of DNA, which in many cases does not need a high degree of purity. In addition, PCR-based techniques based on the amplification of random nuclear DNA-fragments has been proven to be a useful tool in population genetic analysis of species devoid of genetic information. Random amplified polymorphic DNA (RAPD) is such a technique which is quick and simple. This technique was used to discriminate between 23 cultivars of *B. napus* and *B. rapa* (Mailer et al., 1994). The greatest disadvantage of this method is that it is based on unspecific and low stringent amplification of multiple DNA-regions and therefore has been shown to often be dependent on highly reproducible conditions both regarding reagent composition and machinery. The development of other PCR-based methods, which still amplifies non-coding, multiple regions of the genome, but which require more specific conditions, have been found to give more reproducible results. Such techniques are for example the so-called 5'-anchored simple sequence repeat (SSR)-technique and the amplified fragment length polymorphism (AFLP)-technique. Both methods have been used in this study to enable the discrimination between *B. napus* / *B. rapa* hybrids and the original *B. napus* line that was used in the study. The techniques are further discussed below in addition to microsatellite-analysis, since one such marker was used in the study.

2.1 Background on project

Dr. Rikke Bagger Jørgensen is the head of a research group at RISØ National Laboratory located in Roskilde, Denmark. She and her colleagues have been working on issues related to *B. napus* and possible hybridisation / introgression with wild relatives to this crop plant for several years. This work has been very important in assessment of the possibility of such events to happen. In addition to this research issue, research scientists at the RISØ National Laboratory are working on topics related to evaluation of various types of genetically modified plants and their performance in the field regarding issues like transgene dispersal, plant fitness, gene expression and gene silencing.

In this project, I have spent approximately one month at RISØ National Laboratory. I took part in a project with the title "Gene dispersal from winter varieties of oilseed rape with genetically modified organelles to the weedy *Brassica rapa*". The main purpose of this ongoing project, which mainly is part of Marina M. Johannessens PhD-work, is to assess the amount of introduction, persistence and dissemination of cytoplasmic transgenes into wild relatives by doing studies to:

- Estimate the relative frequency of interspecific hybrids produced between *B. rapa* and *B. napus* with *B. napus* as the female, (chloroplast DNA originating from *B. napus*).
- Estimate the relative frequency of BC_1 (first generation of backcrosses) plants produced between F_1 hybrids with oilseed rape cytoplasm and *B. rapa* with F_1 as the female.
- Investigate effects of plant density and abundance on both hybridisation partners, *B. napus* and *B. rapa*, to reveal potential effects of plant composition / competition on hybridisation frequencies.

This project is a two-year project where winter varieties of oilseed rape lines are used. These winter varieties are potentially more prone to give fit hybrids and to introgress with a higher frequency than spring varieties. The oilseed rape will be crossed with different *B. rapa* lines, which are clearly differentiated genetically to be able to discriminate between hybrids formed during the different crosses. The investigation will include analyses of seed and plant material appearing from various crosses and backcrosses with the basis on morphological or molecular characterisation (ISSR or AFLP).

2.1.1 Materials and methods

2.1.2 Plant material

Plant material that was used in this investigation originated from cultivated lines of oilseed rape (*Brassica napus* ssp. *napus*) (used and referred to in Johannesen 1998), and *Brassica rapa* ssp. *oleifera* from different manufacturers. Weedy *Brassica rapa* ssp. *campestris* material (used and referred to in Hansen 1999) that was used in the investigation was all derived from wild individuals found in Danish fields.

Oilseed rape varieties:
Matador – Matador is a Swedish variety of winter oilseed rape. It is from "Allmänna Sv. Utsädes Ab" and was released on the market in 1949. It originated by selection from Lembkes oilseed rape. The plant material that was used in the analyses was derived from seeds achieved from the Nordic Gene Bank, number ngb# 594.

Vestal – Vestal is a Swedish variety of winter oilseed rape. It is from "Olson & sons" and was released on the market in 1956. It was obtained by selection from Hammenhög 0117. The plant material originated from seeds from Nordic Gene Bank, number ngb# 591.

Viktor – Viktor is a Swedish variety of winter oilseed rape. It is from "Svalöf" and was released on the market in 1963. This variety was obtained by selection from a heavily storm damaged cultivated Matador and therefore Viktor and Matador should be expected to show some relatedness. Viktor is distinguished from matador by its unique combination of good rigidity of the stem, not-shattering pods and the higher oil content of the two (Jönsson et al., 1986). The plant material used originates from seeds from the Nordic Gene Bank, number ngb# 590.

Express – Express is a German variety of winter oilseed rape. It has been bred by "Norddeutsche Pflanzensucht (NPZ)" by traditional breeding techniques and was released in 1993. The variety has the following features: It is a traditional self-pollinator and was obtained by the following four-way cross/double cross: (NPZ2/84 x Darmor) x (Bienvenu x 1775/82). It has a short stem, a high standability, has a medium ripening time, a very high seed yield, an extremely high oil content and it is healthy (low susceptibility to fungi). The plant material was derived from seeds from Pajbjergfondet, which has stated that there presumably not should be expected much genetic variation within this variety.

Cultivated *B. rapa* (ssp. *oleifera*) varieties:
Kova – Kova is a Swedish variety of cultivated spring *B. rapa*.
Per – Per is a Swedish variety of cultivated autumn *B. rapa*.
01582 – 01582 is a Swedish variety of cultivated autumn *B. rapa*.
Agena – Agena is a Swedish variety of cultivated spring *B. rapa*.
Ac Parkland – Ac Parkland is a Canadian variety of cultivated spring *B. rapa*.

Weedy *B. rapa* (ssp. *campestris*):
The weedy *B. rapa* individuals that were used have all been collected from various populations in fields in Denmark.

Control sample of *B. napus* and *B. rapa* (ssp. *campestris*):
The samples that were used as controls in all the experiments, were the same lines as had been used both during the controlled hybridisations in the green house and the spontaneous crosses

that were performed outside in the field. The *B. napus* control was a variety of winter rape called Capitol.

2.1.3 Seed germination

The cultivated *B. rapa* seeds were germinated in petri dishes on moist filter paper. The seeds were kept in the dark during germination, and placed on the bench during further growth. DNA was extracted when the shoots were approximately 1 cm tall.

2.1.4 DNA extraction

The DNA extraction was performed from fresh or frozen leaves or small, germinated plants using the procedure by Edwards et al. (1991), with the modifications described by Johannesen et al. (1999).

2.1.5 Experimental set-up

Experimental set-up to increase knowledge on the topics which are described under "background on the project" is carried out both investigating 1) spontaneous hybridisation events in specific field experiments (see figures 2a, 3, and 4 of field set-up) and 2) controlled hybridisation experiments at RISØ's indoor facility. The latter comprise an advanced system for conducting genetic, physiological, biogeochemical and ecological experiments (RERAF = Risø Environmental Risk Assessment Facility) (figure 2b shows controlled crossing between *B. napus* and weedy *B. rapa*).

The outdoor plots were placed in a field of rye. The organisation of the plots is described in fig. 3 and 4. Within each square the composition of plants varied both related to density of plants and ration between *B. napus* and *B. rapa*. The density of plants was ranged as high – 10 cm between each plant, intermediate – 15 cm between each plant, and low – 25 cm between each plant.

Figure 2
Photos of experimental set-ups.
a) 10 metres between each plot. b) Controlled hybridisation.

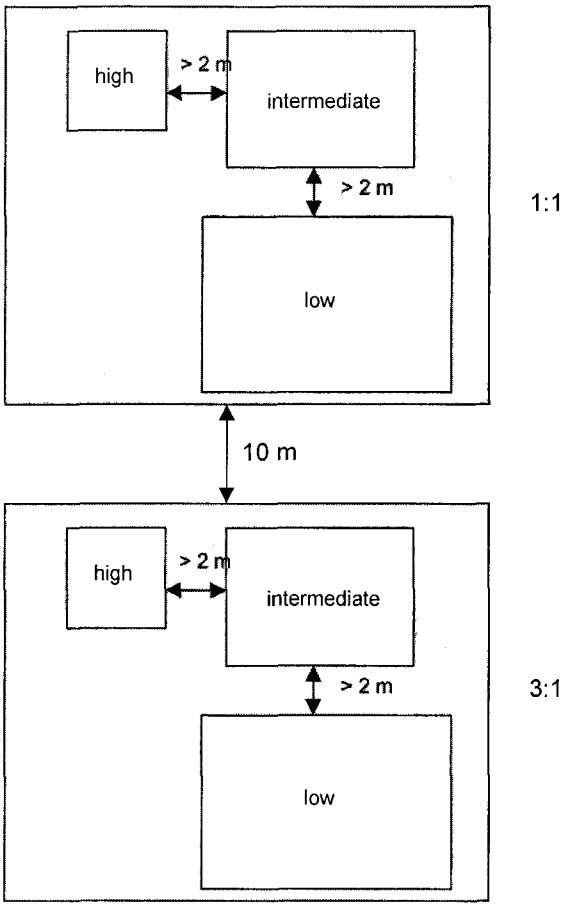

Figure 3
Plants were organised at different densities: high (10 cm between each plant), intermediate (15 cm between each plant) and low (25 cm between each plant). Plants were planted in the ratios 3:1 or 1:1 (see figure 4 for further details).

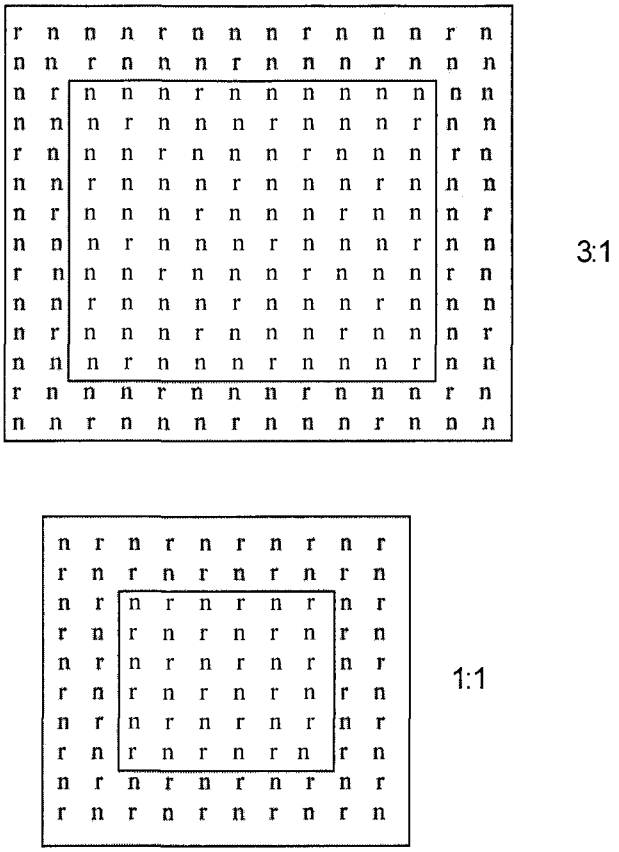

Figure 4
Field experimental set-ups related to ratios.
Ratios of hybrid partners were 3:1 or 1:1 (B. napus; n and weedy B. rapa; r, respectively).

2.2 Molecular methodology

2.2.1 ISSR-technique

PCR analyses using anchored SSR (simple sequence repeats) – primers is a method used for analysing complex genomes. Anchored SSR primers are complementary to microsatellites and contain short oligonucleotide "anchor" sequences that ensure the primers to anneal to either end of a microsatellite repeat. Since the microsatellites are numerous within eukaryotic genome and the length of these are highly variable between individuals, the length between each annealed primer will vary and PCR result in polymorphic banding patterns.

The inter-SSR marker that was used here is called 888 and is described by Charters et al. (1996). In most cases it discriminates between *B. napus* and *B. rapa*.

2.2.2 Microsatellites

Microsatellites consist of tandem repeated sequence units where the polymorphic unit is between one and five bp long (Litt & Luty, 1989; Weber & May, 1989). Microsatellites are widely distributed in the eukaryotic genome (and chloroplast genome in plants) and may because of their polymorphic nature be used related to for instance population genetic studies (Tautz, 1989)

and related to individual recognition. Microsatellites are small enough to be investigated by use of PCR where the allelic variability corresponds to the various lengths of the PCR-products. These are identified by gelelectrophoresis or by other means of DNA-fragment separation. A disadvantage with such molecular markers, is the required knowledge of DNA-sequence organisation around the microsatellites to enable primer construction prior to amplification. Gradually, the availability of knowledge on genome organisation for various organism will improve this situation. Primers constructed for one organism may also be used in a related taxa. (Bruford & Wayne, 1993).

The microsatellite marker that was used here is called 12A and is described by Szewc-McFadden et al. (1996).

2.2.3 AFLP

AFLP (Amplified fragment length polymorphism) is a relatively new PCR-based technique that give co-dominant markers. This means that both alleles within a locus are present. This makes it possible to investigate the origin of each progeny. In the AFLP-technique, the genome is digested with two restriction enzymes: one "frequent-cutter", where the restriction site is four base pairs long, and one "rare-cutter" with a recognition sequence of six or eight base pairs (Blears *et al.*, 1998; Parker *et al.*, 1998). This results in three groups of DNA-fragments; fragments that are cut with either enzymes at both ends, or a mixture of the two enzyme restriction sites at each separate end. Fragments that are digested with the frequent-cutter at both ends of the DNA-fragment are in majority. The digested fragments are ligated to adapters, which consist of synthetically constructed short DNA-fragments composed of the restriction site and another 10-30 bp where primers can anneal. The ligation do not reform the restriction site because a baseshift is incorporated, therefore restriction reaction and ligation can be performed simultaneously, without the formation of ligation of multiple fragments (Blears *et al.*, 1998) (see figure 5).

The AFLP procedure performed here was conducted according to Vos et al. (1995), with modifications described by Johannesen et al. (1999). The pre-amplification was carried out with one selective nucleotide. Further in the specific amplification step, two or three nucleotides were added to the primer. Adapters are described by Maughan et al. (1996). Primer combinations are shown in the appendix.

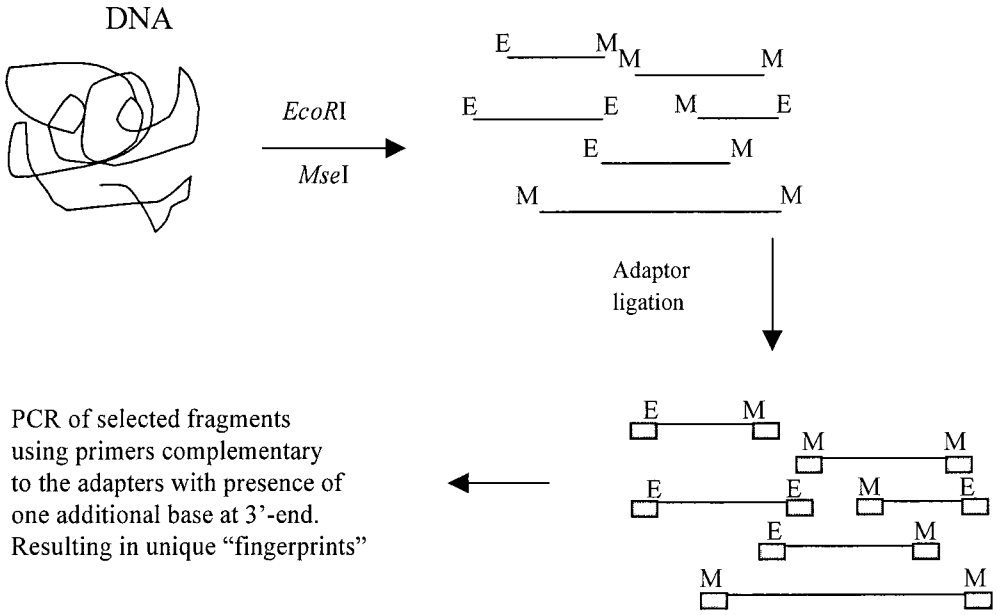

Figure 5
AFLP (Amplified Fragment Length Polymorphism)

2.3 Results

2.3.1 Analyses of *B. napus* / *B. rapa* hybrids using ISSR, AFLP and one microsatellite marker

DNA was isolated from plants derived from seeds originating from *B. napus* plants. The *B. napus* plants had been spontaneously fertilised by pollen from either *B. napus* or *B. rapa*. The field plots where the plant seeds had been collected were from plots with ratios 1:1 between the two species. The samples were called D3-x, E3-x and F3-x, which represented three different plots: D, E and F. The different plots reflected various plant density differences. The densities within the plots were defined as low (25 cm between each plant) called D, intermediate (15 cm between each plant) called E and high (10 cm between each plant) called F. Number (here number 3) refer to position within the plots. x refers to the plant derived from one seed taken from plant number 3. In addition, plant-DNA previously extracted from seeds from a similar set of plants within other plots was included in the analyses. The organisation of *B. napus* and *B. rapa* in these latter plots was on a 3:1 ratio respectively, but with the same density measures as for the previous. These were called A (low), B (intermediate) and C (high). Plants where the seeds had been collected were called A7, B7 and C7 (plant number 7 within the plots).

To check for possible *B. napus* / *B. rapa* hybrids in some of the material, both ISSR and amplification of the microsatellite that distinguish the two species in most cases were performed. The two PCR-reactions were run together in one common lane for each sample on polyacrylamid gels. The gels were further silver-stained to elucidate banding DNA-fragment patterns. The results of such runs are illustrated in figure 6. The following six plants seemed to be hybrids: A7-12, C7-9, E3-29, F3-17, F3-45 and F3-50. The frequencies of hybrids are given in table 3.

Table 3. Frequency of hybrids within separate plots

Samples (number of plants analysed)	**Frequency of hybrids (hybrid name)**
Ratio 3:1:	
A7 (50)	0.02 (A7-12)
B7 (49)	none
C7 (49)	0.02 (C7-9)
Ratio 1:1:	
D3 (49)	none
E3 (34)	0.03 (E3-29)
F3 (49)	0.06 (F3-17, F3-45, F3-50)

To investigate the hybrids found in plots E and F further (because of the DNA extraction method used only these samples contained DNA of good enough quality for AFLP-analyses); AFLP-analysis was performed according to what is described in materials and methods. The results of this analysis verified the presence of hybrids found in the ISSR-microsatellite analyses. Examples of such results are shown in figure 7.

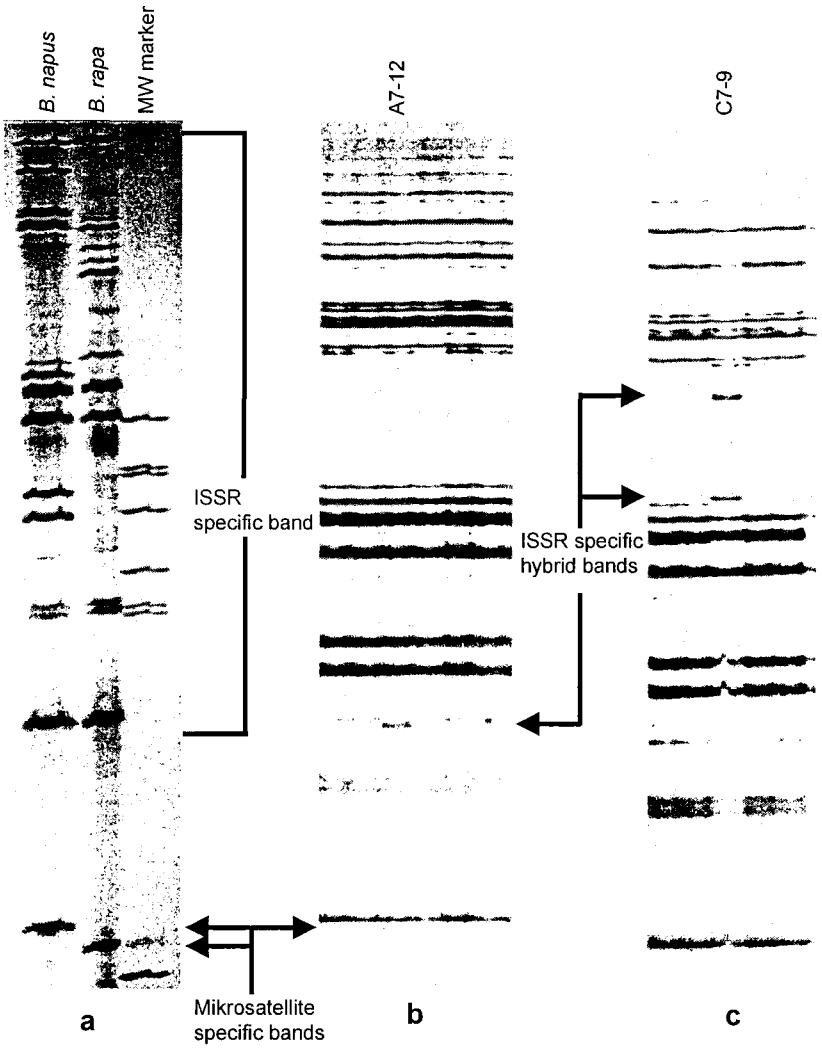

Figure 6
ISSR and microsatellite specific banding patterns.
a) Shows banding-profile of control, B. napus (B.n), and the B. rapa (B.r) that were used. MW is a molecular weight standard. b) shows banding profile of A7-12 hybrid compared to B. napus which serves as the female donor. c) shows the same for hybrid C7-9. Primers 888 was used in the ISSR-banding profile. Primers for microsatellite was 12A (both sets listed in appendix).* shown in surrounding lanes.*

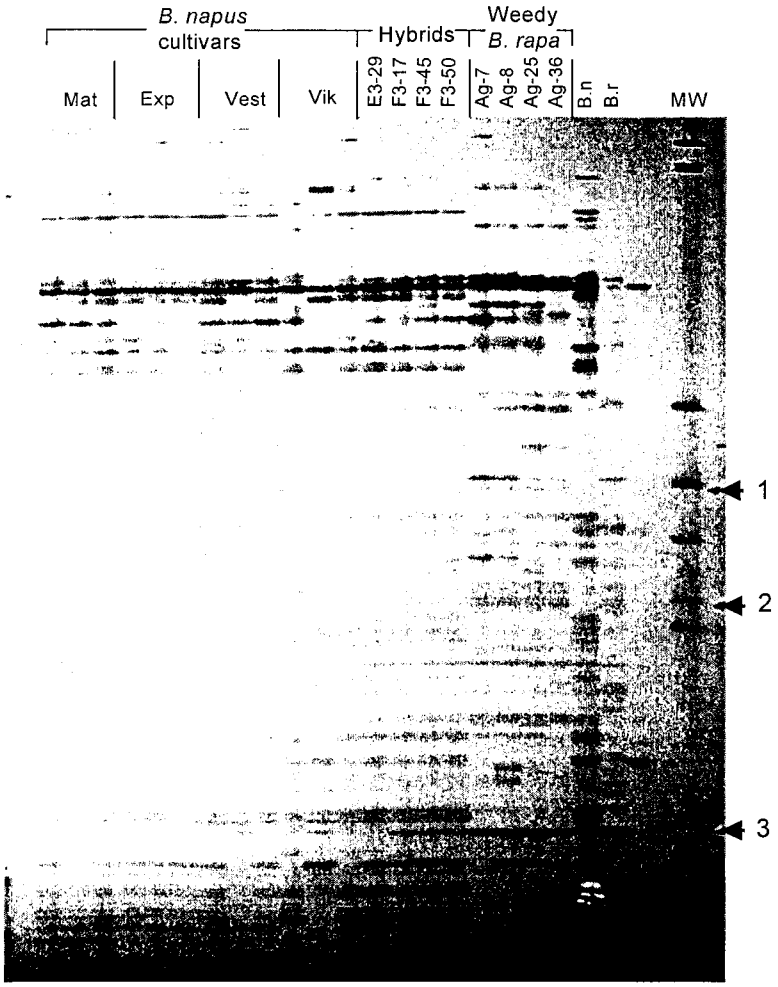

Figure 7
AFLP-analysis confirming hybrids and demonstrating genetic diversity.
Banding-profiles of four B. napus / B. rapa hybrids, four B. napus cultivars and four weedy B. rapa individuals. The specific AFLP-amplification was performed with primers E-AC and M-ACG (listed in appendix). Number 1,2 and 3 indicate B. rapa specific bands.

2.3.2 AFLP-analyses of various *B. napus* and *B. rapa* cultivars

The AFLP-method was performed to give an idea of the degree of genetic diversity within each cultivar of both *B. napus* and cultivated *B. rapa*, and to investigate the ability of this method to distinguish between different genotype. AFLP was also used to investigate the presence of *B. rapa* specific DNA-markers that could be used in further analyses and for verification of some of the hybrids found in the previous study.
Cultivated *B. rapa* is known to be genetically diverse, partly because of its ability to cross-pollinate, hence it was particularly interesting to investigate this diversity within some of the commercially available *B. rapa* cultivars. The *B. napus* plants that were investigated belonged to four different cultivars (Matador, Express, Vestal and Victor). The *B. rapa* cultivars belonged to five different cultivars (Agena, Ac Parkland, Kova, Per and 01582). DNA from the *B. napus* cultivars were already available as DNA, the *B. rapa* cultivars were grown to shoots from seeds placed in petri dishes before DNA was isolated. AFLP was performed in two PCR-steps, first round with the nucleotide A linked to the adapter (E-A and M-A), second round with two or three nucleotides linked to the adapter, either E-AC and M-ACG or E-AG and M-AGG (for further specification of the PCR-reaction see appendix for primers). Three plants of each *B. napus* culti-

var and four for each of the *B. rapa* cultivars were included in the analysis. In addition, four weedy *B. rapa* plants (called Ag-7, Ag-8, Ag-25 and Ag-36) and some of the potential hybrids found with the two other markers were included (E3-29, F3-17, F3-45 and F3-50).

Some results from this investigation are shown in figure 8. Demonstrated by the various individual fingerprints within the same cultivars, I could conclude from this experiment that individuals within the same cultivars are genetically quite different. This was especially striking in *B. rapa*. Furthermore, these cultivars seem to contain genetic alleles both present in weedy *B. rapa* and *B. napus*, which is in agreement with what our expectations on an evolutionary basis. The weedy *B. rapa* individuals that were included in the investigation definitely showed a "fingerprint" different from their *B. napus* origin. On the basis of comparisons of AFLP-fingerprints between *B. napus*, *B. rapa* and hybrids, I could verify the presence of hybrids already found in the initial DNA-analyses. Comparisons of the various *B. napus* and *B. rapa* derived plants, three DNA-fragments (marked as 1, 2 and 3 in figure 7) seemed to be *B. rapa* specific. Three of the four hybrids analysed contained this amplified fragment, while hybrid E3-29 did not.

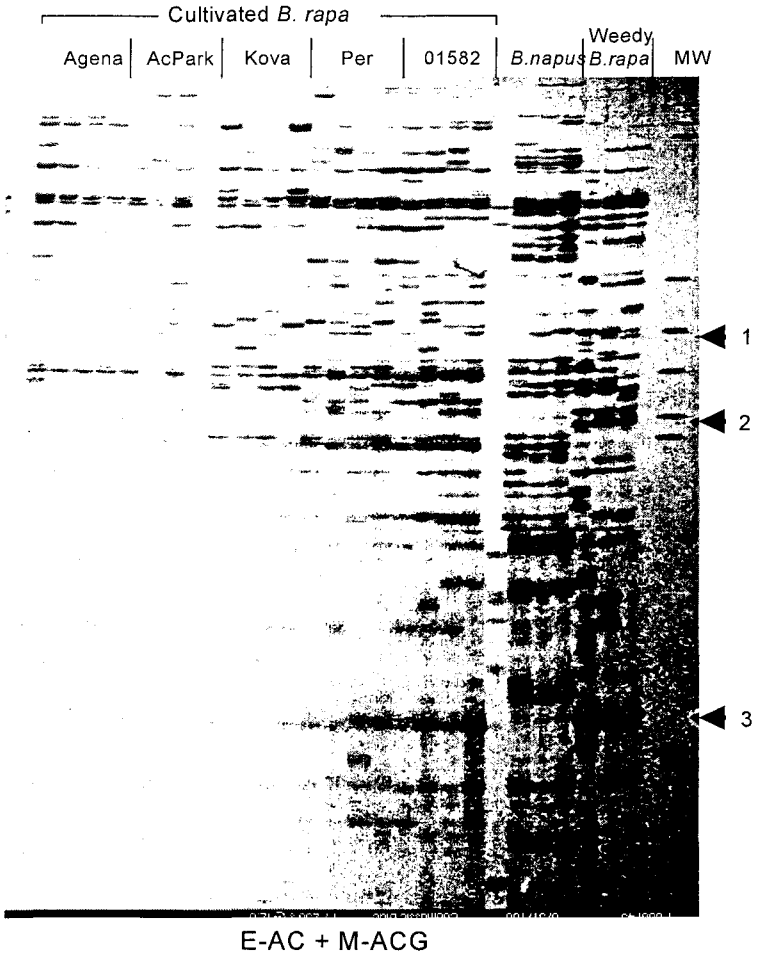

Figure 8
AFLP-analysis demonstrating inter-cultivar genetic diversity.
Banding-profiles of five B. rapa *cultivars, three B. napus and three* weedy *B. rapa individuals. The specific AFLP-amplification was performed with primers E-AC and M-ACG (listed in appendix). Number 1,2 and 3 indicate B. rapa specific bands.*

2.4 Discussion – Experimental part

To increase the knowledge on conditions having an effect on rates of spontaneous hybridisation events, *B. napus* and weedy *B. rapa* was planted in outdoor experimental plots. In these plots both ratios between the two species and density of plants varied. In the small investigation described in this project report, plants from seeds collected from some of the *B. napus* plants representing the various types of plots (high, intermediate and low density), were tested regarding the presence of *B. napus* / *B. rapa* hybrids. Six hybrids were found and the frequencies of hybrids discovered within each plot type were estimated. Due to the small number of samples tested here, the investigation is not statistically valid. Even so, the numbers obtained indicate that density of plants when the two species are organised in a 1:1 ratio positively affect the hybridisation frequencies. Here, low density gave no hybrids, intermediate density – 3 % hybrids, and high density – 6 % hybrids. Further characterisation of the field material may or may not reinforce this tendency. Presumably, the knowledge gained from this investigation will improve the ability to predict transgenic geneflow and chance of *B. napus* / *B. rapa* hybridisation depending on agricultural practices.

The initial markers that were used to screen for hybrids, were inter-SSR and one microsatellite. Six hybrids out of 281 samples tested were identified as hybrids using these systems. Some of the hybrids exhibited *B. rapa* specific ISSR-bands when compared to the *B. rapa* control, i.e. hybrid C7-9, while other hybrids did not show such *B. rapa* specific fragments very clearly, but did show different fingerprint patterns compared to the *B. napus* control. The microsatellite marker also distinguished hybrids from the original *B. napus* sample in some cases, although in some cases this marker seemed to fail in discriminating between *B. napus* and *B. rapa*. This may indicate that the inheritance patterns in these crossings are difficult to interpret due to the presence of several genomes within each individual plant and point to the importance of using several marker systems to verify the presence of hybrids.

The AFLP-technique is a fingerprint technique very well suited for studies of inter- and intraspecific polymorphisms. Here, the technique was used for two purposes: 1) to confirm the presence of some of the hybrids and to show inter-individual differences between these, 2) to investigate the technique's ability to demonstrate genetic differences within separate cultivars of *B. napus* and cultivated and weedy *B. rapa*. The AFLP-analysis verified the presence of hybrids among the samples that were tested. Furthermore, the hybrids contained a mixture of *B. napus* and weedy *B. rapa* specific DNA-fragments.

AFLP was performed on three individual plants within four different commercial *B. napus* cultivars and four individual samples within five different *B. rapa* cultivars. The experiment demonstrated genetic diversity within all the cultivars that were tested. The results indicated that cultivated *B. rapa* has an even higher genetic diversity than demonstrated for *B. napus*. This is in agreement with our expectations since *B. rapa* is known to cross-pollinate to a larger extent than *B. napus*, which is able to self-pollinate. The commercial *B. rapa* cultivars show genetic similarities both to *B. napus* and weedy *B. rapa*, and some of the samples contain the specific *B. rapa* fragments suggested as *B. rapa* specific on the basis of what was found in the hybrids and the weedy *B. rapa* samples. The inter-individual genetic differences that were demonstrated in both AFLP-experiments proclaim the importance of including several samples of the same cultivar when executing these kind of surveys.

2.5 General conclusion and further recommendations

Many applications of genetic engineering in agriculture and forestry will probably have neutral environmental consequences. However, commercial-scale production of a few types of transgenic plants could lead to undesirable consequences for natural and agricultural systems. A concern is that the experience with hundreds of small-scale field tests that have been carried out to evaluate the performance of genetically engineered crops, have not been designed to investigate and will not enable us to evaluate the ecological risks associated with widespread commercialisation (Snow & Palma 1997). Logically such investigations are very difficult to perform be-

cause of the unpredictability of dynamic and living systems such as the ecosystem. Even so, small-scale field experiments may help us to understand some of the underlying biological mechanisms. Furthermore, tools to assess and monitor possible consequences of large-scale releases have to be developed on a small-scale basis.

There are still few studies where investigations of potential negative consequences of releasing transgenic plants are in focus. This is especially striking seen from an ecological viewpoint. Clear conclusions from such investigations are not easily obtainable because of the nature of this research. However, small experimental studies may tell us something about tendencies of certain events. The research group at RISØ is trying to increase the knowledge on the potential of pollen-flow and hybridisation between oilseed rape and weedy *B. rapa* to occur. This is done by experimental approaches where parameters known to influence hybridisation rates are being varied. In these investigations both spontaneous and controlled hybridisation events are studied. In the study described in this report ratios and abundance of the two hybridisation-partners in focus, varied in different field plots. Morphological based distinction of hybrids and parental origin is difficult, therefore molecular tools are being used. Three marker-systems to estimate the presence of hybrids were used, inter-SSR, one microsatellite and AFLP. Two were used in the initial screening, and the latter used for verification and for higher resolution investigation of genetic variability. Even if the two hybridisation-partners were of two genotypes only, the hybrids that were found were quite genetic diverse. Some had obvious "fingerprints" common with the pollen-donor, *B. rapa*, while others seemed to be unique. This "diverse" inheritance pattern is assumed to be a result of the complex genomes that these plant species possess. Thus, this investigation demonstrated the importance of including several markers in the initial screening procedure.

Monitoring of transgenic organisms released into the environment involves a thoroughly planned strategy based on knowledge of the released organisms, the environment to which it will be released and the interaction between the two. The basis in all monitoring studies is the ability to detect potential changes as a result of an activity and to distinguish this from the changes that occur naturally. In the case of release of transgenic organisms, this means to be able to detect potential effects of the release on surrounding environment. Such detection will only be possible if appropriate base-line knowledge of both the release site and the introduced organism is present.

The following are some of the general questions that have to be addressed regarding indirect or direct damages to the environment caused by gene flow according to Hill (1999) and which is important to consider in future investigations and monitoring studies:

- What kind of gene flow do we want to monitor? To wild relatives or neighbouring crops surrounding the release site or both?
- Is the frequency of gene flow important or is the effect of gene flow better to focus on?
- If gene flow to wild relatives is an important issue, knowledge of the potential recipient will be of importance.
- What is the distance over which we want to monitor?
- What should be the size of sample of wild plants to ensure the detection of assumed frequency of hybridisation?
- Are there adequate techniques to identify hybrids, and how practical are these to carry out on a large scale?
- In considering the effects of gene flow, one have to consider potential enhances in fitness.
- In assessment of effects it is important to have baseline data about species diversity and levels of populations.
- Does introduced genes such as insect resistance have an effect on insect diversity or higher up in the food chain if their spread into wild species outside the agricultural environment.
- Questions relevant to volunteers will also be relevant to consider for neighbouring crops.
- A key issue regarding monitoring schemes is whether the time put aside enables temporal effects to be detected.

In the case of releasing transgenic *B. napus*, relevant knowledge related to the potential impact on the surrounding flora is: 1) hybridisation-partners in the region, such as nearby weedy *B. rapa*

populations 2) already present hybrids in the region, 3) of the plants that will be affected by the release, e.g. knowledge of genetic structures and population dynamics of these populations. Several of the points addressed above emphasise the importance of knowledge on the frequencies of introgression between oilseed rape and weedy *B. rapa* that has previously been going on in nearby regions of the release site. If such knowledge is not present, this will preclude the ability to detect the actual hybrid frequencies resulting directly from the release. Such information will also increase our knowledge of the frequencies of such events to occur in the future. Recent investigations done by Jørgensen and colleagues (pers. comm), showed pronounced introgression (45 out of 102 plants investigated in total) between oilseed rape and weedy *B. rapa* in a mixed, self maintained weedy population. This hybrid population was assumed to have maintained itself for eleven years, which was the last time oilseed rape was cultivated in this field in an organic farm manner. Frequency of introgressed plants found in conventionally grown oilseed rape fields was demonstrated to be much lower, which indicate the great importance of agricultural practice on potential hybridisation events. Without the use of molecular markers similar to the ones discussed above, knowledge of the sort demonstrated in Jørgensen and colleagues' investigation would not have been available. This emphasises the usefulness of including molecular marker systems in such studies.

From the studies performed by Jørgensen and her colleagues at RISØ National Laboratory, we will have better knowledge concerning factors related to ratios and abundance of *B. napus* and *B. rapa* that will influence hybridisation frequencies. These studies are still not finished, but the results will hopefully give indications of agricultural and also more general environmental conditions that will give the lowest possible number of hybrids. This will provide recommendations for agricultural practices, which might reduce the potential gene flow and cross hybridisation to a minimum.

An accurate estimate of the area in Norway of which *B. napus* and *B. rapa* are grown respectively is hard to get because these are pooled in the available statistics. However, to estimate potential gene flow from separate fields and potential impact of this on the surrounding environment, it is crucial to gain more detailed agricultural knowledge of the two cultivars. Information required is size and location of agricultural areas, density of fields in various regions of the country, agricultural practise (important for estimating gene flow), characterisation of the extent of potential weedy hybridising partners in the surrounding environment and a characterisation of already existing hybrids surrounding such agricultural fields.

In Norway, no genetically modified *Brassica* has ever been released. In fact, at present no genetically modified plants are grown outside green houses at all. Moreover, there are no plans of commercial growth in the near future. However, it would be interesting to perform a survey among farmers on the interest and the need for crop types improved by genetic engineering. The question is whether or not Norwegian farmers would reorganise their agricultural practise if they could see a great enough agricultural improvement potential using genetically engineered crops or had a great demand for such products. Which are the agricultural problems regarding the cultivation of *B. napus* and *B. rapa* at presently performed agricultural practise? Second generation GMPs comprising quality modifications rather than agricultural modifications, e.g. modification of oil content in *Brassica* species, may involve the demands of the consumer in a much greater extent than before. This has to be included in any future considerations of what genetically modified plants there might be a commercial interest for. Ideas of which transgenic plants that would be of interest would help us to focus on which risks it would be most urgent to increase our understanding on.

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4 Appendix

Primer sequences

ISSR-primers (Charters et al., 1996):
ISSR-888: 5'- BDB CAC ACA CAC ACA CA
5'-anchor consists of three variable base positions. B = C, G or T, similarly D = not C.

Microsatellite primers (Szewe-McFadden et al., 1996):
12A 5': 5'- GCC GTT CTA GGG TTT GTG GGA
12A 3': 5' – GAG GAA GTG AGA GCG GGA AAT CA

AFLP adapters (Maughan et al., 1996):
MseI adapter + strand: 5'- GAC GAT GAG TCC TGA G
MseI adapter – strand: 5'- TAC TCA GGA CTC AT
EcoRI adapter + strand: 5'- CTC GTA GAC TGC GTA CC
EcoRI adapter – strand: 5'- CTG ACG CAT GGT TAA

AFLP primers for preamplification (Maughan et al., 1996):
E-A: 5'- AGA CTG CGT ACC AAT TCA
M-A: 5'- GAC GAT GAG TCC TGA GTA AA

AFLP primers for amplification (Maughan et al., 1996):
E-AC: 5'- GAC TGC GTA CCA ATT CAG
E-AG: 5'- GAC TGC GTA CCA ATT CAG
M-ACG: 5'- GAT GAG TCC TGA GTA AAC G
M-AGG: 5'- GAT GAG TCC TGA