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Structure, function and evolution of oleosins in plants

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Oleosins are major protein components of all oil-bearing seed tissues examined to date. These proteins are localized exclusively on the surface of the storage oil bodies, i.e. at the lipid-water interface[1]. To date, our laboratory has sequenced 5 oleosin genes/proteins from *Brassica napus*, one from *Arabidopsis*, one from radish and two from sunflower[2,3]. Sequence data from carrot, maize and soybean oleosins have also been published. All oleosins contain an unusual but highly conserved 70-residue central β-strand hydrophobic domain, flanked by more polar C- and N-terminal domains. The function of oleosins is unclear, but it has been proposed that they are involved in stabilizing oil bodies during seed desiccation and possibly facilitating their mobilization after germination. A direct involvement in oil body assembly is unlikely, since data from protein abundance, Northern blots and *in situ* hybridization studies all show that oleosin genes are expressed and oleosin proteins accumulate much later than oil body formation during seed development in rapeseed[4]. Rather, oleosins accumulate in a temporal pattern similar to that of late embryo abundant (LEA) gene products, which are also involved in seed desiccation.

These findings were reinforced by recent studies in olive and avocado oleogenic tissues. Olive and avocado both contain large quantities of storage oil in the mesocarp tissues of their fruit or drupe. Olive also stores oil in the endosperm and embryo tissues of its seed, although avocado does not. It was found that the oil bodies of olive and avocado mesocarp were about 1000 times the volume of those found in seeds and were entirely devoid of oleosins[5]. In contrast, olive seed tissues, both endosperm and embryo, contained small oil bodies and abundant oleosins. Unlike seeds, the mesocarp tissue does not undergo desiccation, nor is the stored oil used subsequently (e.g. after gemination) by the plant or its progeny. Rather, this fatty tissue serves as a bait to attract animal vectors to aid seed dispersal. The presence of oleosins is, therefore, correlated with a requirement for desiccation tolerance and oil body mobilization and with the presence of relatively small oil bodies.

A quite different but analogous structure to the seed is the pollen grain. Mature pollen grains from *Brassica napus* contain up to 25% $^{w}/_{w}$ storage oil which is laid down as a reserve during microsporogenesis and subsequently mobilized during germination of the pollen tube. We have very recently cloned and sequenced two cDNAs encoding oleosin-like sequences from an anther-specific library[6]. One of these was identical to a partial amino acid sequence determined directly from an oleosin protein, purified from oil bodies isolated from pollen grains. These results show that there may be a gametophyte-specific lipid storage

pathway in *Brassica* microspores in addition to the well-characterized sporophytic pathway found in developing seeds. Examination of the gametophytic and sporophytic oleosin protein sequences using dendrograms shows that they constitute two independent but related lineages (Fig.1). It is hoped that future studies will elucidate further the evolution of these proteins, particularly during the transition from gametophyte-dominant genera (such as bryophytes) to the more recent spermatophytic genera (including angiosperms).

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Gene dispersal from transgenic plants.

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Oilseed rape will be one of the first crop species to have transgenic varieties in widespread use. Throughout the world, genes have been inserted for resistance to the herbicides glufosinate and glyphosate, resistance to insects using the *bt* or the trypsin inhibitor gene and modification of oil quality using the stearoyl-ACP gene. Within the Brassica and Oilseeds Research Department, transformation programmes are in progress to modify oil composition, to alter self incompatibility, to introduce transposable elements for gene isolation and to incorporate marker genes to study pollen dispersal.

Before transgenic plants can be grown outside a containment glasshouse it is necessary to carry out a risk assessment. This assessment is overseen and subsequently approved in the UK by the Advisory Committee on Release to the Environment (administered by a secretariat from the Department of Environment and the Health and Safety Executive). Procedures are now being harmonized across Europe in response to EEC Directive 90/220.

An important part of risk assessment is the provision of information on the likelihood and consequences of the transfer of introduced genes to related plant species (especially weeds) and to adjacent crops. The principal aim of our experiments with *Brassica napus* has been to determine the frequency of pollen transfer over various distances. The project has been supported by the PROSAMO programme (Planned Release of Selected and Modified Organisms) and the data generated will be used to determine isolation distances and monitoring procedures for the field evaluation of novel transgenic *B. napus* plants in the future.

The first experiment in 1990 consisted of a 9m diameter circle of transgenic plants carrying the dominantly expressed *bar* gene (phosphinothricin acetyltransferase), which confers resistance to the herbicide glufosinate. These transgenic plants were surrounded by one hectare (105m x 105m) of non-transgenic herbicide sensitive plants. All plants were the spring oilseed rape cultivar Westar. Six honeybee hives were included in the experiment to optimize the opportunity for cross pollination. Seeds were harvested at various distances from the transgenic central circle and screened for hybrids resulting from pollination by the transgenic plants. The hybrids could be detected easily because of their resistance to glufosinate.

During 1991 the harvested seeds were grown in the glasshouse or in a 2.2ha field test and sprayed with the herbicide glufosinate. A sample of plants surviving spray treatment were taken to a containment glasshouse for seed production. Their progeny were again sprayed with glufosinate to confirm resistance. The presence of the *bar* gene was confirmed by analyzing a sample of survivors using Southern blot analysis with the *bar* gene as the probe.

The estimated percentage of cross pollination for the samples of 1m and 3m from the edge of the transgenic circle was 1.4% and 0.4%, respectively. The frequency decreased sharply up to 12m (0.02%). At 47m it was 0.00033% or 3 hybrids per million seeds.

Isolation distances commonly used for production of breeders seed are 200m and 400m using plot sizes of approximately 20m x 20m. In 1992 our experiments were based on parameters used for genetic isolation of breeders plots, and again we used glufosinate resistant transgenic plants as the pollen source. The screening of this experiment is in progress and the data will be available in 1993.

The data generated in these gene dispersal experiments are being used by the Regulatory Authorities to determine isolation distances and monitoring procedures for the release of transgenic *Brassica napus* plants in the United Kingdom.

Acknowledgements

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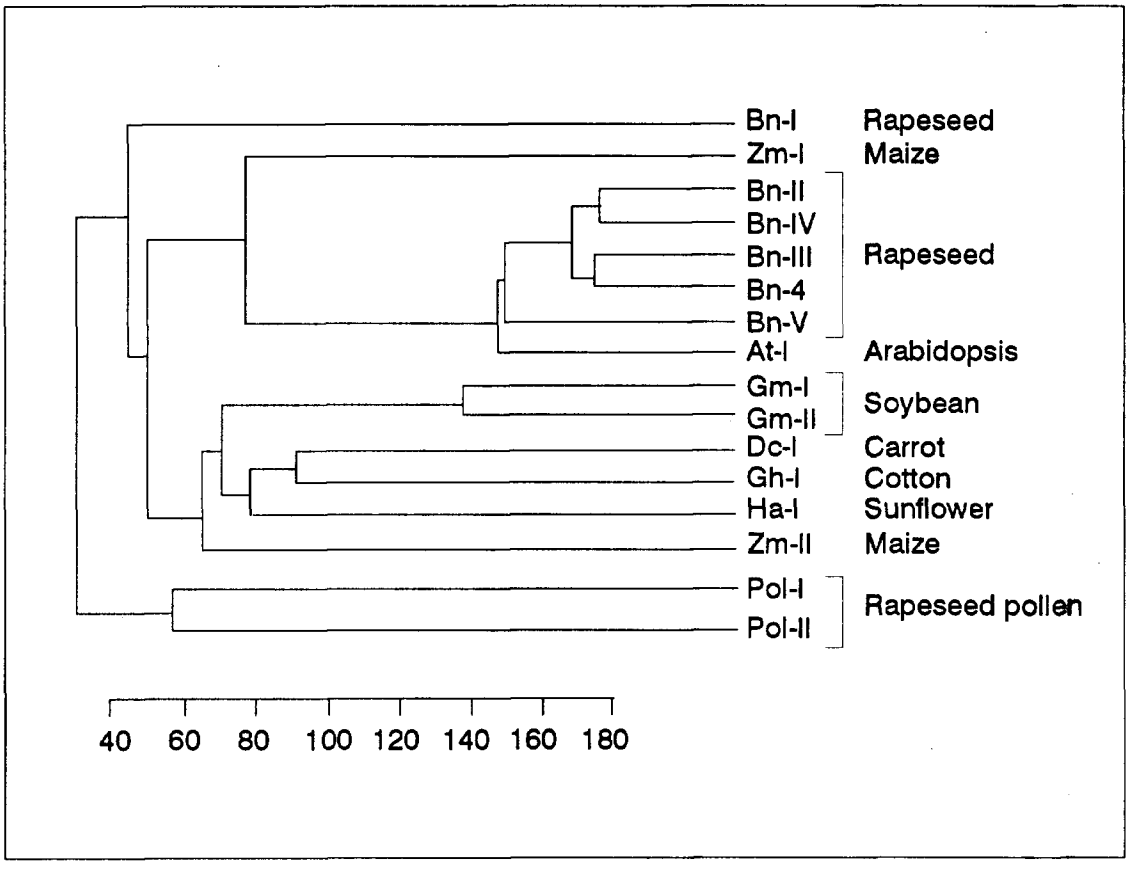
Fig. 1. Dendrogram showing amino acid sequence homologies of 14 oleosins from the seeds of 7 species of angiosperm and 2 oleosins from the pollen of rapeseed. Data were compiled using the CLUSTAL program.



Progress in the study of *Brassica* self-incompatibility

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Self-incompatibility (SI) mechanisms[1] have evolved polyphyletically in angiosperms and may represent the elaborated forms of discrete steps in cellular recognition and signalling. We are studying the molecular genetics of the SI system of *Brassica* species, which is controlled by a single, multi-allelic *S* locus, through a combination of gene and RFLP analysis and plant transformation experiments.

Much of our work so far has centred on the DNA sequence and expression of alleles of the *S* locus glycoprotein gene (*SLG*) which may mediate the stigmatic SI response. In collaboration with Dr Lydiate's group we have used an *SLG* cDNA clone from *B. oleracea*[2] to "fingerprint" *Brassica* lines which carry different *S* alleles. The probe reveals extraordinary polymorphism at the *S* locus which underlies its unusual mode of evolution and is of utility in analysing crosses in which SI is to be manipulated. Interestingly, the probe detects expressed *SLG* sequences in self-compatible (SC) *B. napus* which we have cloned and sequenced. Genetic evidence from Dr Lydiate's group suggests that the SC trait may be effected by activity of an unlinked suppressor locus.

We have also cloned and sequenced allelic variants of an unlinked, *S* locus-related gene (*SLR1*)[2,3] which is structurally homologous to and co-expressed with *SLG* in both incompatible and compatible plants. This gene was found to be expressed in styles of transgenic tobacco and its promoter also directs low level GUS expression in bombarded tobacco pollen. To define its role we are now transforming *SLR1* constructs into *Brassica.* Additional sense copies did not perturb endogenous *SLR1* expression in transgenic *B. napus* and did not affect its compatible phenotype. The consequences of antisense *SLR1* expression both in *napus* and in incompatible *oleracea* are being investigated.

It is now known that the *S* locus is likely to be a complex one which may evolve as a set, or haplotype, of coadapted genes. One of these is the *S* receptor kinase (*SRK*) which is extremely closely linked to the *SLG*[4]. It encodes a putative serine/threonine protein kinase whose extracellular receptor domain is very similar to the *SLG*. We have cloned six different *SRK*-like genes from an S_{29} *Brassica* line and have compared them over a defined region in the catalytic domain containing exons and introns. One of these sequences shows 99.7% sequence identity to an *SRK* cloned from an S_{63} plant and yet their receptor domains do not cross-hybridise. This may indicate some type of rearrangement mechanism active in the evolution of the gene family.

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Dense genetic linkage maps of *Brassica napus* derived from two wide crosses identify the ancestral *B. oleracea* and *B. rapa* genomes of oilseed rape

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Microspore culture has been used to produce two large *Brassica napus* populations of recombinant doubled-haploid lines from contrasting wide crosses. Two F_1 plants from the first cross, which involved a doubled-haploid Winter oilseed rape cultivar and a doubled-haploid Spring oilseed rape cultivar, have yielded a total of 241 doubled-haploid lines. Three F_1 plants from the second cross, which involved the same doubled-haploid Winter oilseed rape cultivar and a synthetic *B. napus* line, have yielded a total of 186 doubled-haploid lines. A high proportion of the microspore-derived lines have been subjected to a thorough RFLP analysis using 120 probes of *Brassica* genomic DNA. This has identified 393 segregating loci of which 335 have been assembled onto 20 linkage groups (Fig. 1.).

B. napus (n=19) is an amphidiploid species formed from the hybridization of two diploid ancestors, *B. oleracea* (n=9) and *B. rapa* (n=10). The synthetic *B. napus* line was formed from a new hybridization of *B. oleracea* with *B. rapa*. The close similarity of the linkage groups derived from the two crosses demonstrates that all (or most) oilseed rape chromosomes are able to pair normally with their corresponding *B. oleracea* or *B. rapa* chromosomes. This suggests that the *B. napus* genome has remained essentially unchanged since the initial hybridization of the two ancestral species. Ordered pairing between the homologous chromosomes of oilseed rape and synthetic *B. napus* will facilitate the introduction of useful genetic traits into oilseed rape from its more diverse diploid ancestors. However, this analysis of *B. napus* has also revealed that recombination between homoeologous chromosomes (resulting in chromosomal translocations) occurs at a low frequency even in oilseed rape cultivars. This homoeologous recombination is probably a major factor in the genetic instability sometimes observed in doubled-haploid lines of *B. napus*.

The populations of doubled-haploid lines which have been used to generate these genetic linkage maps have also been the subject of extensively replicated field trials in the 1991-1992 growing season. A number of quantitative traits have been scored in these trials. The scoring data is currently being analysed and it is expected that a number of quantitative trait loci will be identified when the scoring data and mapping data are correlated.

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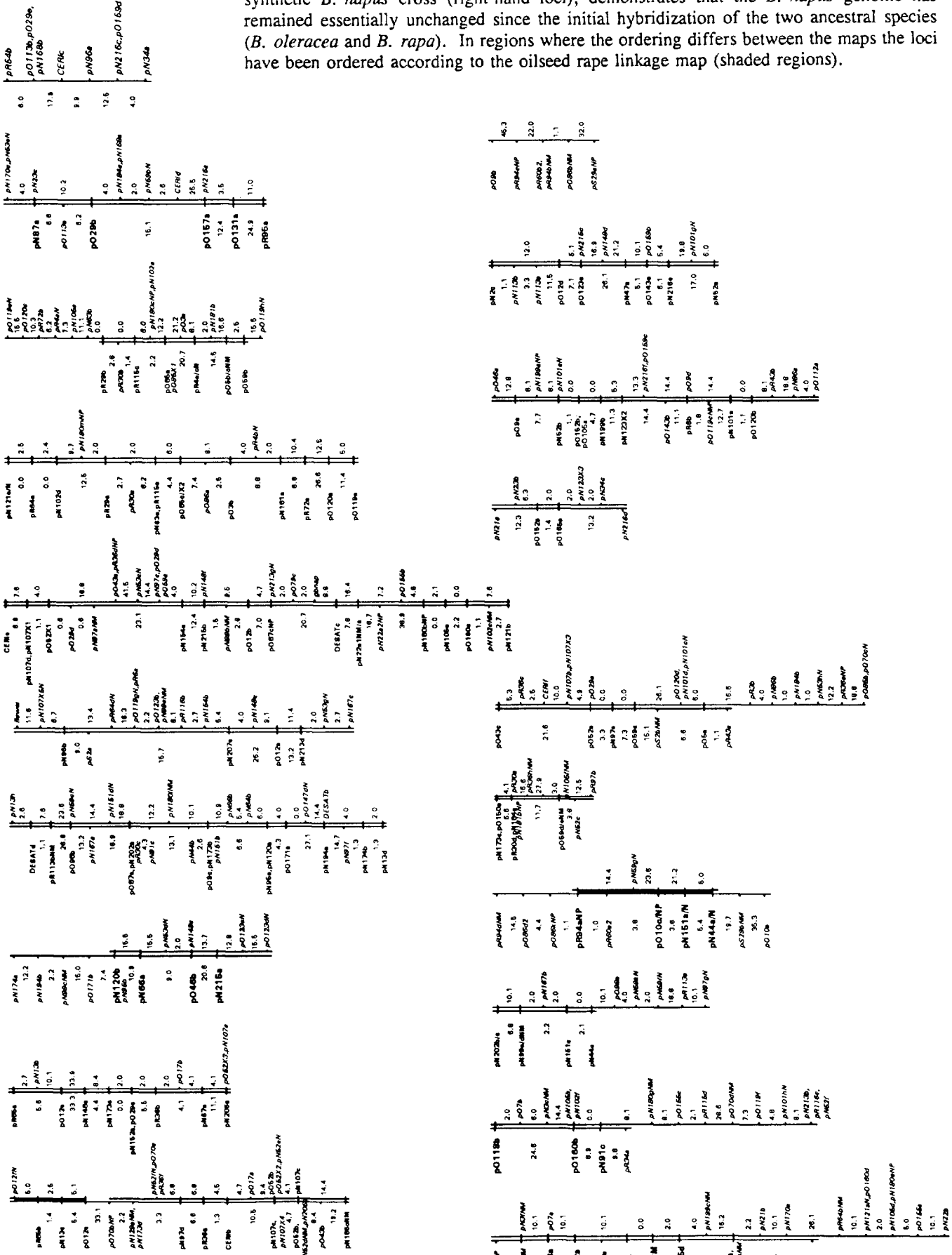
Fig. 1. A comparison of two *B. napus* genetic linkage maps, one derived from an oilseed rape × oilseed rape cross (left-hand loci) and the other derived from an oilseed rape × synthetic *B. napus* cross (right-hand loci), demonstrates that the *B. napus* genome has remained essentially unchanged since the initial hybridization of the two ancestral species (*B. oleracea* and *B. rapa*). In regions where the ordering differs between the maps the loci have been ordered according to the oilseed rape linkage map (shaded regions).



Manipulating glucosinolates in *Brassica napus*

R Magrath, A Giamoustaris, D Toroser and R Mithen

Glucosinolates represent the major class of secondary metabolites in Cruciferous plants. The glucosinolate molecule consists of two parts: a glycosyl moiety and a variable side chain which is derived from amino acids. *Brassica* species contain three classes of glucosinolates: aliphatics derived from methionine, indolyls derived from tryptophan and aralkyls derived from phenylalanine. The glucosinolates themselves have little biological activity but following tissue damage they undergo hydrolysis to produce a complex array of products which have important biological activities. Of these the isothiocyanates determine the flavour of vegetable and condiment crops and affect the quality of rapeseed meal as a livestock feed by reducing its palatability. Glucosinolate hydrolysis also produces goitrogenic compounds which affect the feed quality of rapeseed meal. Many hydrolysis products have also been implicated in pest/pathogen interactions with *Brassica* crops. For example, isothiocyanates are toxic to pathogens and are involved in both the attraction and repulsion of invertebrate and vertebrate pests. As part of our objectives to enhance the seed quality and pest and disease resistance of oilseed rape we are seeking to alter the individual aliphatic glucosinolates which occur in the vegetative and seed tissues of the crop.

The aliphatic glucosinolates comprise methylthioalkyl, methylsulphinylalkyl, alkenyl and hydroxyalkenyl homologues of propyl, butyl and pentyl glucosinolates. However, in oilseed rape, only butenyl and pentenyl glucosinolates and their hydroxylated analogues occur. A range of synthetic *B.napus* lines have been developed from wild accessions of *B.oleracea* and *B.rapa*, the diploid progenitors of oilseed rape. These synthetic lines have contrasting glucosinolate profiles to natural forms of *B.napus* and crosses between them and oilseed rape cultivars has enabled the genetic basis of aliphatic glucosinolate biosynthesis to be elucidated. We found that eight loci regulated the aliphatic glucosinolate profile of oilseed rape[1], and manipulation of the alleles at these loci has been used to alter the glucosinolate profile of oilseed rape lines. In collaboration with the *Brassica* genetics groups these loci have been mapped onto an RFLP map of *B.napus*. *Arabidopsis thaliana* synthesizes a similar range of aliphatic glucosinolates and, in collaboration with the Department of Molecular Genetics, a homologous set of loci have been located onto an RFLP map of this species.

As a results of these genetic studies, it has been possible to develop breeding lines of oilseed rape with altered leaf and seed glucosinolate profiles. Preliminary results from field trials suggest that increasing the proportion of butenyl glucosinolates by elimination of pentenyl and hydroxyalkenyl glucosinolates decreases the palatability of leaf tissue to unspecialized *Brassica* pests such as pigeons and rabbits, but increases susceptibility to specialized insect pests. However, insects pests such as flea beetles can be repelled by increasing the concentration of methylsulphinylalkyl glucosinolates. Further studies are in progress in collaboration with a commercial plant breeding company and IARC-Rothamsted.

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Disease resistance in *Brassica napus*

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Research in the *Brassica* pathology group is concerned with understanding the genetical and physiological basis to disease resistance in *Brassica*, and, in collaboration with commercial plant breeding companies, developing resistant *Brassica* lines for use in breeding programmes. Three economically important fungal pathogens of *Brassica* are studied; *Leptopshaeria maculans*, which causes leaf spot and stem canker disease, *Pyrenopeziza brassicae* which causes light leaf spot disease and *Plasmodiophora brassicae* which causes clubroot.

Stem canker disease has resulted in severe crop losses but has become less important since the introduction of resistant French cultivars. It is however, one of the major diseases of rapeseed in eastern Europe, Canada and Australia, and continues to threaten rapeseed production in western Europe. An extensive survey of wild and cultivated relatives of oilseed rape identified two wild accessions of *B.rapa* which had high levels of resistance to highly aggressive isolates. Using interspecific hybridization and embryo rescue techniques the resistance genes have been transferred into *B.napus* lines. Preliminary field trials suggested that one of these sources of resistance is highly effective against a genetically diverse pathogen population and further trials are in progress in the UK, France and Australia. Genes determining this novel form of resistance are being located onto a *Brassica* RFLP map.

Clubroot is the most important disease of horticultural *Brassica* crops and is also important on oilseed rape crops in northern Europe. A series of single dominant resistance genes have been introduced into oilseed rape from *B.rapa* and these are currently being located on a *B.napus* RFLP map. In addition, the interaction between *Plasmodiophora* and *Arabidopsis* is being investigated. A survey of wild type and mutant *Arabidopsis* lines did not reveal any sources of resistance. However, these studies have increased our understanding of the life history of this pathogen by identifying a motile amoeboid phase and a stage in the infection process in which the host nuclei becomes completed surrounded by plasmodia[1]. Reports of the possible uptake of host DNA by *Plasmodiophora* are being investigated and its implication for risk assessment for the release of transgenic crops assessed through a research programme funded by MAFF.

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