



## Botanical files 2003

An exploration of the scientific literature on two aspects of GM crop biosafety:

- (1) The application of molecular markers to the detection of gene flow between crops and wild relatives
- (2) The possibilities of the use of gene function knowledge from *Arabidopsis* to assessing environmental safety of transgenes in GM crops

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# Summary

This report addresses two aspects of biosafety assessment of possible outcrossing of transgenic crops to wild relatives: (1) the feasibility of using molecular markers for identification of hybrids between crops and wild relatives, including subsequent generations and (2) to what extent the quickly growing body of knowledge on gene function in *Arabidopsis thaliana* can be put to use for predicting impact of specific types of transgenes on the environment after outcrossing to wild relatives.

The first issue, the use of molecular markers for hybrid identification, was explored in oilseed rape (*Brassica napus*), strawberry (*Fragaria grandiflora*) and lettuce (*Lactuca sativa*) and their respective wild relatives. The effectiveness of molecular markers was shown in hybrid detection in the *Brassica* spp. Transgenic markers, in particular genes conferring herbicide tolerance, could be applied more efficiently in an experimental set-up, but can not be used in the Dutch context, as long as the environmental effects of transgenic herbicide tolerance (trHT) are under discussion. Molecular markers then are the most efficient universally applicable method: in principle, they are providing information from over the whole genome and they are capable of tracing hybridisation in agro-ecological settings that have not been manipulated by the researcher. The studies of gene flow in oilseed rape were relatively straightforward: one identified either direct escapes from specific cultivars or inter-specific hybridisation. In the inter-specific hybridisation between allopolyploid *B. napus* and diploid rapeseed *B. rapa*, one can reliably identify species-specific markers. However, in the detection of intra-specific gene flow into natural populations in e.g. strawberry and lettuce, the occurrence and direction of gene flow is more difficult to assess using crop-specific markers. There is a problem of how to distinguish between two alternative causes for finding markers in common between crop and wild populations: recent introgression or common ancestry from the time of domestication. Thus, in these cases, there is a need for detailed analysis of the distribution of genetic variation between crop and wild forms, including its distribution over the genome.

For the second issue, the use of gene function knowledge in *Arabidopsis* for predicting transgene impact on natural populations, a exploratory literature survey was performed. This focussed on genes expected to be having an impact on fitness, in particular those influencing dispersal capacity of plant species. With the availability of the full genome sequence, all genes could be putatively inferred for *Arabidopsis* in principle. For many genes, functions have already been described, and a picture is beginning to emerge of the pathways and networks in which their protein products interact in plant development and signal transduction. In addition, a start has been made with the study of effects of genes and their allelic variation under natural conditions. As far as could be ascertained, this has not yet resulted in *Arabidopsis* studies directly addressing gene functions in relation to dispersal. Diverse patterns of selection were shown to act on *Arabidopsis* genes studied: both purifying (adaptive) selection and diversifying (balancing) selection, that is, selection maintaining variability in the occurrence of genes/alleles in populations, could be inferred. Also, pleiotropic and epistatic interactions between genes were indicated. All these interactions imply that effects of transgenes introduced into natural settings will not necessarily be straightforward. For instance, the diversifying selection shown for some disease resistance genes implicates that an introduced R-gene might not be able to simply 'take over' complete populations. One also has to take into account that in natural circumstances completely different pathogens may be limiting for population fitness than in the crop situation. Moreover, inferring selection processes from field data is fraught with difficulties, because often alternative explanations may be offered by demographical history of the study populations. Comparison to rice, for which the genome sequence also has been recently elucidated, indicates that homologous genes do not always have similar functions in different genetic backgrounds. Even within *Arabidopsis* itself, alleles can have different effects in different genetic backgrounds. Thus, knowledge from *Arabidopsis* studies will for certain provide clues about possible gene effects, and generalisations will increasingly become possible when more comparisons to other plant species have been made. However, at the present state of knowledge, information from *Arabidopsis* only will most likely not be conclusive, and there will remain a need for collecting additional information on the specific crop/gene combination itself.





# 1. Introduction

In the ongoing debate on the biosafety of transgenic crops, the likelihood of transgene escape through outcrossing to natural populations is regarded as important in relation to possible impacts on the environment. Examples of impacts that are often suggested in the literature are: (1) the possibility of enhanced weediness of crop wild relatives by gaining traits enhancing fitness in the field or (2) the possibility of effects on non-target organisms of disease resistance traits such as Bt (*Bacillus thuringiensis* toxin). Risks are often expressed as the product Hazard x Exposure, so, in this case, the product of the magnitude of the effect imposed by the transgene and the probabilities of spread of the transgene by outcrossing. These probabilities and effects will depend on the crop, the transgene and the specific environmental setting. Therefore, a case-by-case approach has often been advocated.

For authorities involved in biosafety of genetically modified crops, such as the GMO office (BGGO) enforcing regulations and the Advisory Commission on Genetic Modification (COGEM) providing advice to the government in the Netherlands, it is imperative to be able to make estimations of the risks following from outcrossing between crop and wild relatives. Biosafety assessment by any case-by-case approach would gain in efficiency by the availability of generalised knowledge about gene flow between crop and wild relatives. Outcrossing from crop to wild relatives must have been going on since the onset of agriculture (Ellstrand *et al.*, 1999) and as such has usually not been perceived as a hazard. Nevertheless, there might already have been effects of gene flow from crops on wild relatives. Thus, in order to put any risk from transgenes into perspective, it is essential to have baseline knowledge of any effects already imposed by outcrossing in conventional agriculture.

As a first approach to assessing the likelihood of gene flow, De Vries *et al.* (1992) made an inventory in the Dutch flora based on herbarium material and floristic literature. This established the so-called Botanical files (De Vries *et al.*, 1992). An additional morphological field study was performed for the lettuce crop/wild species pair, *Lactuca sativa* and *L. serriola*, to explore the full potential of the Botanical files approach. The study concluded that they were most likely conspecific in view of the continuum in morphological characters between them and their interfertility. This implied that, in principle, gene flow could occur from crop to wild forms in lettuce (Frietema de Vries *et al.*, 1994).

The Botanical files studies can be criticised on the grounds that floristic data might not be representative for assessing the actual occurrence of gene flow between crops and wild relatives. They have been gathered for other purposes. Moreover, morphological characters need not be sufficient for detecting hybrids between crop and wild relatives. However, any possible bias in floristic data is more likely to be in a direction favourable to the detection of gene flow between a crop and wild relatives. Since florists tend to collect unusual specimens, there will be an over-representation of hybrids in herbaria. Therefore, herbaria will show a sort of worst-case scenario of the chances of hybrid formation to occur in nature. On the other hand, morphological characters might indeed fall short for the detection of hybrids. For instance, domestication traits often show recessive inheritance (Small 1984), leading to first generation hybrids being most similar to the wild form and thus the possibility of hybrids going undetected in the field.

In the last decade, there has been an enormous development in molecular markers for the identification of genotypes, increasing the efficiency of plant breeding and the study of natural populations. These markers could also be helpful in the identification of hybridisation between crop and wild relatives in the field and might overcome the limitations of morphological markers. For instance, in the example of the potential of gene flow hypothesised for the lettuce pair *L. sativa*/*L. serriola*, virtually nothing is known about its actual occurrence. Gene flow might be small in view of the inbreeding nature of the crop, but this situation might be different in the field, depending on the availability of pollinating agents or other circumstances promoting outbreeding.

With regard to estimating the impact of outcrossing, there is also an urgent wish for generalised knowledge. It would be desirable to be able to predict from the type of trait used in crop transformation what its potential effects are on wild forms in the field. Traits that will have a significant influence on fitness will be the more relevant. With the recent availability of the complete sequence of the *Arabidopsis* genome (Arabidopsis Genome Initiative 2000), this plant model species has become even more important in establishing functions of genes. This opens the possibility for an

attempt to mine this fast developing source of knowledge on gene function for predicting potential effects of particular genes used in crop transformation on wild relatives.

This desk study consists of two parts. The first part addresses the potential of molecular markers for the detection of hybrids between crop and wild relatives, including subsequent generations/back-crosses, in the field. Within the available time, only two examples have been studied in detail, (1) oilseed rape (*Brassica napus*) and its wild relatives, which represent one of the most studied examples, and (2) lettuce, for which an EU project addressing gene flow was recently started by the author and a consortium. In addition, a study in strawberry will be briefly discussed to illustrate potential obstacles in implementing molecular markers for detecting gene flow. The second part of this desk study describes a limited survey of the quickly expanding body of literature on the elucidation of gene function in *Arabidopsis* for traits to be expected relevant for plant fitness. The focus was on genes that might be expected to influence plant dispersal and/or dispersion, since these traits are expected to be important to the weediness of a plant species.

## 2. Detection of hybridisation using molecular-genetic markers

In the following, *Brassica* and *Lactuca* will be discussed separately, with a short sideline on strawberry to illustrate specific obstacles in hybrid detection. Details of the molecular marker systems discussed are described in Box 1 (for a recent overview, see Schlötterer, 2004).

### 2.1 Oilseed rape and wild relatives

Oilseed rape (*B. napus*) is the most intensively studied crop with regard to the risks of transgene escape to the environment. *B. napus* is a facultative outcrosser, showing on average about 30% outcrossing within the crop. There are two ways of gene flow open to this crop: (1) the occurrence of crop volunteers, that is, populations derived from seeds remaining on the field after harvest or seed spillage during transport of harvests, and (2) outcrossing to both feral populations of the species and to wild relatives from the genus *Brassica* and related genera. The species studied in this regard are presented in Table 1. Extensive studies have been performed in the UK, France, Denmark, Canada and Australia. Most of these studies concern spring oilseed rape, whereas in the Netherlands practically only winter oilseed rape is cultivated, for the most part in the northern province of Groningen (approximately 600 ha in 2002, Kempenaar *et al.*, 2003). This may limit direct applicability of results from foreign studies to the Dutch situation. For instance, the long-term survival of volunteers of winter oilseed rape has been questioned for the Netherlands and the spectrum of wild relatives available for outcrossing, that is, flowering concurrently with the crop in early summer, will be limited. However, descriptions of the technical possibilities of assessing gene flow are relevant to this study.

Table 1. Species studied for their ability to hybridise to oilseed rape (*B. napus*) in sequence of declining chances of hybrid formation

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<i>B. napus</i>
<i>B. rapa</i> (syn. <i>B. campestris</i> )
<i>B. juncea</i>
<i>B. oleracea</i>
<i>B. nigra</i>
<i>Hirschfeldia incana</i> ( <i>B. adpressa</i> )
<i>Raphanus raphanistrum</i>
<i>Erucastrum gallicum</i>
<i>Sinapis arvensis</i>

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Eighty-two original publications on gene flow issues in *Brassica* spp. have been analysed for the use of molecular marker techniques in tracing spontaneous hybridisation under agro-ecological conditions (listed in Annex 1). From these 82, 29 mainly dealt with other aspects, such as hybrid fitness or modelling approaches. Another six discussed exclusively controlled crosses to establish the basic possibility of hybrid formation. In 37 publications, spontaneous hybridisation in controlled field experiments was described; for detection of hybrids 16 of these 37 publications used molecular markers. This also counts the use of isozymes, although strictly speaking these represent a biochemical rather than a genetic marker system. The remaining 21 publications used other types of markers for hybrid detection: (1) in most cases, transgenic herbicide tolerance (trHT), (2) morphological characters, (3) cytogenetic markers, both direct chromosome counts or by flow cytometry, or (4) secondary metabolites (erucic acid and glucosinolates). Methods (2), (3) and (4) were often used in combination. Cytogenetics, when making use of flow cytometry, and secondary metabolites can be applied with reasonable efficiency, but are not universally applicable.

Erucic acid and glucosinolates are limited to *Brassica* crops and cytogenetics can be easily applied because oilseed rape is the only polyploid among the species studied.

In the end, ten publications addressed the application of molecular markers to tracing hybrids in natural populations. Conclusions from these publications will be discussed more extensively in the following sections.

### 2.1.1 Gene flow detection within oilseed rape *B. napus*

Two European publications used molecular markers for studying the dynamics of feral populations, one in the context of a landscape study in the Tayside area in Scotland using ISSR (Inter-Simple Sequence Repeat PCR) (Squire *et al.*, 1999) and the other focussing upon a specific site in France using isozymes (plus secondary metabolites) (in Loir-et-Cher, Pessel *et al.*, 2001). In spite of the popular notion that seed spillage only leads to short-lived populations, both studies showed that volunteer populations could persist for several years, in the French case up to at least eight. This followed from the molecular marker-based identification of cultivars that were not longer grown in the areas studied. In addition, both studies indicate that additional hybridisation within the feral populations and between these and nearby crops had occurred, which explained heterogeneity found within some feral populations. Hybridisation between cultivars/volunteers is specifically addressed in a Canadian study on the occurrence of multiple herbicide-resistant volunteers. In Alberta, volunteers were found containing various combinations of both transgenic glufosinate and glyphosate tolerance, and classically selected imidazolinone resistance. Such multiple herbicide-resistant volunteers were most likely produced by spontaneous hybridisation between varieties containing the respective single herbicide-resistances. Hall *et al.* (2000) were able to show that these volunteers were indeed hybrids between the different mono-resistant varieties grown in the area, using cultivar-specific RFLP markers.

### 2.1.2 Gene flow detection between *B. napus* and related species

About hybridisation between oilseed rape and other species, studies using molecular markers were found only for rapeseed (*B. rapa*) and wild radish (*Raphanus raphanistrum*). They will be discussed separately.

#### 2.1.2.1 *B. rapa*

Two European research groups performed studies on outcrossing from crop *B. napus* to *B. rapa* under agro-ecological conditions using molecular markers: in Denmark and in England. In Denmark, seeds from weedy *B. rapa* plants within fields of *B. napus* cultivation were tested for hybrid nature using, a.o., isozymes and RAPDs (Jørgensen & Andersen 1994, Landbo *et al.*, 1996). Hybrid progeny could be assigned using markers that were previously identified as characteristic for *B. napus*. They also compared winter and spring oilseed rape: not unexpectedly because of more overlapping flowering periods, hybrids were only found in the spring type (Landbo *et al.*, 1996). In a later study on a mixed weedy population of *B. napus* and *B. rapa* in an organically cultivated field, Hansen *et al.* (2001) used species-specific AFLP markers. By comparison to marker results from controlled crosses, they were able to identify not only first generation hybrids but also introgressed individuals; the introgressed individuals were most similar to the BC<sub>2</sub> (second backcross) generation to *B. rapa*.

In England, Scott and Wilkinson (1998) were able to identify hybrid progeny in seeds collected from *B. rapa* plants using, a.o. molecular markers, in this case ISSR. Instead of weedy mixed populations, they studied wild populations growing in the vicinity of oilseed rape cultivation along the river Thames. In a more extensive follow-up study, Wilkinson *et al.* (2000) first located sites of possible sympatry between the two *Brassica* species (and also *B. oleracea*) by remote sensing, that is, by identification of *B. napus* cultivation in the immediate neighbourhood of potential wild *B. rapa* habitat along river edges. Subsequently, sites were explored for presence of *B. rapa* populations and newly recruited plants all populations found were tested for hybrid nature by ISSR and SSR (Simple Sequence Repeats or microsatellites). In this way, one hybrid was found among 505 plants from two populations (none was identified in *B. oleracea*). In a recent publication, Wilkinson *et al.* (2003) used a large body of information gathered in this way to make estimates of the total probabilities of hybridisation in the UK.

### 2.1.2.2 *R. raphanistrum*

Thalmann *et al.* (2001) analysed plants from natural populations of *R. raphanistrum* growing alongside fields with oilseed rape cultivation in the previous year in Switzerland. In the first instance, potential hybrids were detected by flow cytometry. Individuals that could not be reliably assigned by flow cytometry were subsequently tested using RAPD markers. In this way, no hybrids were found among the about 750 plants tested.

### 2.1.3 Discussion

In the oilseed rape publications discussed, it proved possible to trace hybridisation under agro-ecologically relevant conditions by using molecular marker techniques. The specific hybridisation results reported in these publications already indicate a need for additions to the Botanical files as published by De Vries *et al.* (1992): (1) the persistence of *B. napus* volunteer populations, at least under Scottish and French conditions (reported as uncertain in Botanical files), (2) a sometimes high occurrence of outcrossing from *B. napus* to *B. rapa*, e.g. in Danish field studies (mere possibility of hybridisation between *B. napus* and cultivated *B. rapa* reported in Botanical files) and (3) the possibility of outcrossing between *B. napus* and *R. raphanistrum* is not addressed in Botanical files. Hybridisation in the combination of *B. napus* and *R. raphanistrum* was not found in the study by Thalmann *et al.* (2001) on a limited number of plants. However, experimental studies using trHT markers on higher numbers under agro-ecological conditions reported low, though widely varying hybridisation rates: from none (Rieger *et al.*, 2001, Australia), to between  $10^{-7}$  and  $3 \cdot 10^{-5}$  (Chèvre *et al.*, 2000, France) or up to between  $6 \cdot 10^{-5}$  and  $2 \cdot 10^{-3}$  (Darmency *et al.*, 1998, France) of seedlings tested. In principle, this transgenic type of marker is the most efficient method for tracing outcrossing, since it only involves assessment of surviving progeny after spraying the appropriate herbicide. This can be simply performed on huge numbers of seedlings, but for doing so, the transgenic line first has to be experimentally introduced into the field. Therefore, it can only be used where field release of the particular GMO is allowed, and, by definition, it can not be used in tracing evidence for past introgression from crops to wild relatives. The next efficient, universally applicable method for tracing outcrossing then is molecular marker techniques.

With the use of molecular markers for detecting hybridisation, it should be noted, however, that the publications discussed address relatively clear-cut cases. Within *B. napus*, either volunteers could be identified as belonging to specific cultivars, or multi-herbicide resistant plants could be confirmed as hybrids by reference to the cultivars known to have been cultivated in the study area. In the species combination of *B. napus* and *B. rapa*, hybridisation has been shown to be possible at a rather high rate, depending on the circumstances. They are rather closely related; according to the triangle of U, *B. napus* is an amphidiploid species hybrid with *B. rapa* (together with *B. oleracea*) as parental species (see Fig. 1). Thus, *B. napus* is a polyploid containing both a '*B. rapa*' and a '*B. oleracea*' genome. Moreover, *B. rapa* as an obligate outcrosser will show a high incidence of hybridisation when occurring isolated from other *B. rapa* within an oilseed rape field (cf. Jørgensen *et al.* 1998). In the examples discussed, the likely parental *B. napus* genotypes of the hybrid/introgressed plants detected were present at the same site as references. In other cases, such as the mostly selfing lettuce where outcrossing is expected to be rare, or where potential parental genotypes are less well known, one may be faced with more difficulties in tracing hybridisation by molecular marker methods.

Before turning to the lettuce example, another crop/wild pair, the strawberry *Fragaria grandiflora* (syn. *F. xananassa*) and wild *F. virginiana* will be briefly discussed as an illustration of possible complications.

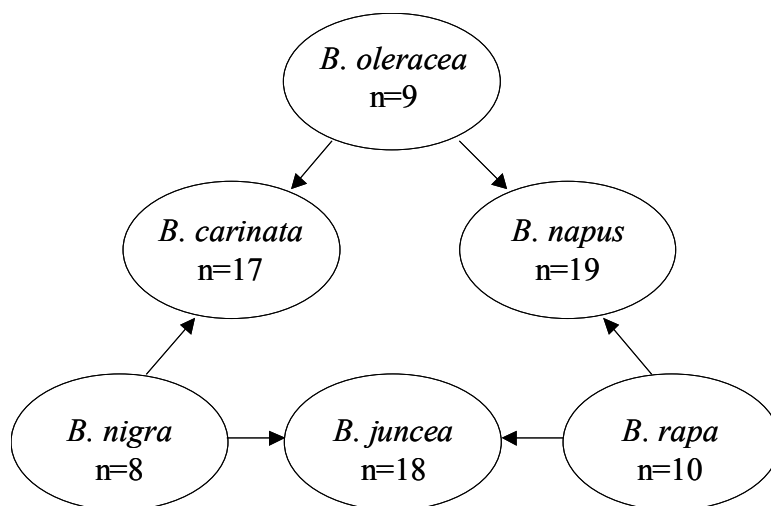


Figure 1. Triangle of U, showing the relationships between three diploid *Brassica* species with haploid chromosome numbers of 8, 9 and 10, respectively, and the allopolyploid (amphidiploid) hybrids between them resulting in haploid chromosome numbers of 17, 18 and 19, respectively.

## 2.2 Gene flow detection in strawberry

Westman *et al.* (2001) studied natural populations of native *F. virginiana* in the surroundings of strawberry cultivation in the south-eastern part of the USA. Cultivated strawberry is an octoploid species hybrid of *F. virginiana* and *F. chiloensis* and is cross-compatible with both parental species. Westman *et al.* (2001) consider introgression from the crop into wild *F. virginiana* highly likely in view of the combined possibilities of outcrossing, vegetative spread and fruit dispersal by animals. AFLP markers specific for the cultivated species, or very rare in the wild form, were selected, and also markers specific for cultivars not longer grown in the study area could be found. Both types of markers could be found in the wild populations. There was no relationship evident with geographical distance. However, the results were based on a rather small number of populations and markers, among which markers also occurring rarely in the wild species. Thus, it appears difficult to ensure the crop-specific nature of the markers used. Even though outcrossing appears not unlikely in these circumstances, it is hard to establish the significance of finding such markers in the wild populations of these field studies. If possible at all, it would have been preferable to use crop-specific markers specifically derived from the *F. chiloensis* wild progenitor, which does not occur in the study region.

Problems with the use of crop-specific markers will be even more severe in species where outcrossing is expected to be rare, such as in the lettuce species pair *L. sativa/serriola*. This will be discussed in the next section.

## 2.3 Gene flow detection in lettuce

Because of the serious lack of knowledge -in comparison to *Brassica* spp.- and related genera on gene flow in Compositae crops, an EU project was recently started, titled 'Analysis of gene flow from crop to wild forms in lettuce and chicory and its consequences in the context of GM-crop biosafety' (acronym 'ANGEL', QLK3\_CT-2001-01657, website <http://www.plant.wageningen-ur.nl/projects/angel>). Seven partners are involved, coming from five European countries: the Netherlands, the UK, Denmark, Switzerland and Italy (cf Annex 2). The Danish and Swiss partners were already encountered in the discussion of the *Brassica* spp. above. One of the project's objectives is testing the possibilities of molecular markers to trace introgression from cultivated to wild forms and this will be discussed here for lettuce.

When tracing evidence for past introgression from crops to wild relatives by molecular methods, several obstacles need to be overcome. As already implicated in the discussion of the strawberry study in the USA by Westman *et al.* (2001), there is the problem of ascertaining that the markers to be used are indeed crop-specific. Even though markers can be selected that are specific for a panel of varieties of the cultivated form, rare occurrences of these seemingly crop-specific markers in the wild form may still be attributable to common ancestry at a considerably earlier stage of domestication, and not to recent introgression. On the other hand, wild relatives are widely used in breeding, e.g. for the introduction of disease resistance genes, for example, resistance genes against the downy mildew *Bremia lactucae*, a particularly troublesome pathogen in lettuce cultivation. Thus, specific genomic segments from wild accessions of *L. serriola* have been introgressed into cultivated lettuce during the past century of breeding. Therefore, crop and wild forms may be found to have genomic segments containing disease resistance genes in common, however, not because of outcrossing from the crop to the wild form, but because of breeding activities the other way round. On the other hand, these genomic segments from specific wild accessions could in turn end up by outcrossing in other wild populations elsewhere in *L. serriola*'s distribution area with introgressed cultivated lettuce as intermediary.

To tackle the problems of assessing evidence for recent introgression, we have aimed at increasing detail in the analysis. In the first place, three different molecular marker approaches are tested: two multi-locus marker systems (AFLP and the retrotransposon-based SSAP= Sequence-Specific Amplified Polymorphism) that are considered to be 'neutral' systems, implying that they sample the genome more or less randomly, and the relatively new system of NBS-profiling. The NBS-profiling is also a multi-locus marker system, but it does not aim at random sampling of the genome. Instead, NBS-profiling is screening for variation in and around disease resistance genes containing the conserved NBS (Nucleotide Binding Site) region. This NBS region is present in the great majority of resistance genes described so far (Van Tienderen *et al.*, 2002; Van der Linden *et al.*, submitted). At least one of the lettuce resistance factors against *Bremia*, Dm3, has recently been shown to be encoded by a gene belonging to this NBS-LRR type of resistance genes (Shen *et al.*, 2002). Therefore, the NBS profiling should provide data about parts of the genome that will have been introgressed from wild *L. serriola* to the crop in the breeding process and thus could confuse the detection of spontaneous introgression the other way round. Resistance genes in general will be more extensively discussed in the *Arabidopsis* part below.

From the 'neutral' marker systems, AFLP is well established (Vos *et al.*, 1995) and its use has already been mentioned in the discussion of the *Brassica* ssp. The more recently developed SSAP is based on retrotransposons occurring ubiquitously in eukaryotic genomes. The use of retrotransposons has the advantage of, in principle, providing markers of which the direction and order of change can be inferred from the way they move through the genome. Contrary to the classical transposons described e.g. for maize, retrotransposons will stay in their place, for new insertions elsewhere in the genome are created by an RNA intermediate transcribed from the original retrotransposon. This intermediate gives rise to new insertions at unique sites in the genome through retrotranscription, similar to what happens with retrovirus multiplication. Thus, shared insertions at the same site in the genome can be taken as strong evidence for a common origin of the chromosomal parts of the plants/populations in which they are found (for review Kumar & Hirochika, 2001).

In the second place, as many markers as possible will be located on the genome by linkage mapping. In this way, one can check whether representative coverage of the genome has been achieved. Moreover, it becomes possible to see how differences in genetic diversity between crop and wild are distributed over the genome. This should provide background knowledge, from which possible recent introgression of specific chromosomal segments could be better inferred than from random marker data alone.

The SSAP and NBS-profiling techniques have been adapted successfully to use in lettuce, but no complete datasets have become available yet (Van de Wiel *et al.*, 2003). A pilot study has been performed with an AFLP dataset combined with existing mapping knowledge from an integrated lettuce map. This indicated that it might not be straightforward to distinguish recently introgressed fragments in this way. However, as yet not enough markers could be traced back on the genomic map to reach sufficient detail for definitive conclusions and more markers are being mapped at the moment (Van de Wiel *et al.*, 2003).

## 2.4 Conclusions

The strength of molecular markers was shown in hybrid detection in the *Brassica* spp. Transgenic markers, such as genes conferring herbicide tolerance, could be applied more efficiently in an experimental set-up, but can not be used in the Dutch context. Molecular markers then are the optimal universally applicable method, in principle providing information over the whole genome and capable of tracing hybridisation in agro-ecological settings that have not been manipulated by the researcher. The studies of gene flow in *B. napus* were relatively straightforward: one either identified direct escapes from specific cultivars or inter-specific hybridisation. In the inter-specific hybridisation between allopolyploid *B. napus* and diploid *B. rapa*, one can identify reliably species-specific markers from the 'oleracea' genome of *B. napus* (cf. triangle of U, Fig. 1). In the detection of relatively rare intra-specific gene flow in e.g. strawberry and lettuce, it is more difficult to assess direction of gene flow. Moreover, there is a problem of how to distinguish between markers in common because of recent introgression or because of common ancestry. Thus, there is a need for detailed analysis of the distribution of genetic variation between crop and wild lettuce, including distribution over the genome. This is still being studied in the EU project 'ANGEL'.

### Box 1: Molecular marker methods

Molecular marker methods screen genetic variation by targeting directly the genomic DNA. In the following, the techniques mentioned in this report will be briefly described.

#### Isozymes

Isozymes are an exception in that variation is not assessed at the DNA level but at the protein level. Genetically based variation in amino acid sequence of enzymes is screened by separating isoforms varying in their electrical charge due to changes in amino acid composition. Separation of the isoforms is performed by electrophoresis whereupon the isoforms are visualised by staining reactions based on biochemical reactions specific for the respective enzyme.

#### RFLP

RFLP (=Restriction Fragment Length Polymorphism) was one of the first methods targeting variation in the genomic DNA directly. Total genomic DNA is digested (cut) by restriction enzymes that recognise a specific 4- or 6-basepair sequence. The DNA fragments are separated by electrophoresis through an agarose gel. The separated fragments are transferred to a filter (blotted). Subsequently, the blot is hybridised to a cloned genomic fragment (probe) selected for the detection of variability in size of specific fragment(s) recognised by the probe. Hybridisation of the probe is visualised using a radioactive label or another staining method and in this way bands of varying sizes can be seen. Mutations or deletions changing the recognition site of the restriction enzyme used are the major cause of this variability in size. The method thus scores one locus at a time by using a probe specific to the respective locus.

The RFLP method has become outdated because of its laborious nature and its use of relatively large quantities of DNA. It has been superseded by more efficient methods based on PCR (the Polymerase Chain Reaction). PCR enables to amplify specific DNA fragments by using oligonucleotides of 10 to 30 nucleotides long (so-called primers) that hybridise to the ends of the fragments. Amplification is performed by a series of rounds of separating DNA strands, annealing of the primers and DNA polymerisation starting from the primers. In each round, a doubling of the specific fragment occurs leading to an exponential amplification. The final product can then be easily visualised by staining or a radioactive or fluorescent label. In the following, the methods based on PCR are described.



## RAPD

One of the first and most easily applied methods was RAPD: Random Amplified Polymorphic DNA. It uses short, in the order of ten nucleotides, primers of random sequence in the PCR amplification. Primers are selected for the production of scorable band patterns after agarose gel electrophoresis. Thus, the method has a clear advantage in that no prior sequence knowledge is required for its performance. However, the use of short primers brings with it that primer annealing and subsequent duplication are not robust and strongly dependent on laboratory conditions. Consequently, the method is not simply exchangeable between labs (Jones *et al.*, 1997) and has run out of favour.

## AFLP

Like RAPD, AFLP is also a PCR method for which no prior sequence information is required. However, it is significantly more robust by some experimental refinements which make it a more demanding method in terms of laboratory equipment. Although sometimes translated as Amplified Fragment Length Polymorphism, AFLP was defined as a trademark by the original inventor, the biotech company, Keygene. Like RFLP, the method is based on restriction digestion of total genomic DNA and searching for variation in fragment sizes generated by mutations obliterating restriction enzyme recognition sites. Polymorphisms are generated as follows: small adapter DNAs (in the order of 20 base pairs long) are ligated to both ends of the genomic fragments. These adapters enable hybridisation of primers for PCR amplification of the genomic fragments. In order to obtain a

sizeable number of products in the final analysis, a subset of fragments is selected by using primers having an additional number of random nucleotides (usually three) at their end that are not specified by the adapters. Thus, a specific genomic fragment will only be successfully amplified if a number of nucleotides at its end match those at the end of the primers used. Upon polyacrylamide gel electrophoresis of the PCR products, multi-banding patterns appear in which polymorphisms are scorable as band presence/absence. Thus, like with RAPD, the method is efficient in that a series of loci are screened in one run. A disadvantage is that it represents a so-called dominant marker system, which means that the knowledge per locus is limited to the presence or absence of one allele; the presence of other alleles can not be established, and in general, it is also not possible to distinguish heterozygotes, that is individuals having only one version of the allele plus another unspecified one. An example of a co-dominant marker system, in which heterozygotes can be distinguished by positively identifying all different alleles at a specific locus is described in the following section describing microsatellites.

## Microsatellites

Microsatellites or SSRs (Simple Sequence Repeats) are repetitive stretches of 1 to 8 nucleotides occurring ubiquitously in genomic DNA. They are highly variable in length due to a high mutation rate in the number of repeats. This is attributed to polymerase slippage during DNA duplication (Schlötterer & Tautz, 1992). The variability in allele length is screened by PCR amplification using primers designed from sequences flanking the microsatellite. Individual allele sizes can then be determined by polyacrylamide gel electrophoresis. Advantage of this system is the possibilities of co-dominant scoring (heterozygote identification) and the construction of databases that can be used by several labs provided that proper standards are used by all participants. A disadvantage is the relatively high investment needed: each microsatellite locus has to be developed by establishing flanking sequences and designing effective primers on them, and this has often to be performed for each new species, since microsatellites are generally not well transferable, even between related species.

An alternative way of using microsatellites as marker systems is mentioned in the discussion of the *Brassica* spp.: ISSR (=Inter-Simple Sequence Repeats). Here primers are based on the microsatellites themselves and so, genomic fragments lying in between two microsatellites occurring in each other's vicinity are amplified. Multi-banding patterns are produced on agarose gels similar to those produced by RAPD. In the publications cited in this report, microsatellite primers are used with a 5' anchor (Charters *et al.*, 1996), that is, a few random nucleotides are attached to their 5' end. This should ensure that the primers anneal to one specific end of the microsatellite and not at random all over the microsatellite, in which case fragments of randomly differing sizes would be generated. Whether a 5' anchor would suffice for consistent annealing, however, is debatable, because the 3' end is the site conditional to proper amplification.

## Conclusions

From the methods described here, AFLP and microsatellites (SSR) have established themselves as the most efficient and robust techniques. Even though they are the easiest to apply, both RAPD and, most probably ISSR, fall short in reproducibility of their results. AFLP is capable of efficiently producing markers in species where a limited amount of sequence information is available. Thus, it can also efficiently generate markers from all over the genome. This makes AFLP useful for assessing introgression between populations when only limited parts of the genome would be involved. Obtaining a similar coverage of the genome with SSRs would mean a large investment in development and scoring of each locus, although present day semi-automatic sequencing machinery enables to combine several (3 to 8) loci into one multiplex. For monitoring of gene flow between highly interfertile populations, population-genetic techniques using heterozygote scores are the most desirable, which can best be produced by SSR.

All methods discussed here, including the more specialised methods of SSAP and NBS-profiling described in the lettuce section, suffer from the disadvantage of the relatively laborious use of gel electrophoresis (though, more recently, the more efficient capillary separation became available). More sophisticated methods circumventing electrophoresis, such as in the retrotransposon-based marker type or with SNPs (Single Nucleotide Polymorphism) are being developed now, but have not yet been tested in outcrossing of plants at a large scale.

### 3. *Arabidopsis* gene function analysis in relation to plant dispersal

For assessing environmental safety of transgenic crops, it would be useful not only to be able to monitor outcrossing *per se*, but also to have general insight in the effects that specific genes might have upon introduction into wild populations, in particular on their fitness. The Botanical files on gene flow could then be extended with gene files for reference for regulatory authorities (Conner *et al.*, 2003). With the recent completion of the total genome sequence of the already well-established model plant *Arabidopsis thaliana* (Arabidopsis Genome Initiative 2000), an enormous boost was given to elucidation of all sorts of gene functions in this species. Therefore, it appears to be worthwhile to explore whether this quickly expanding resource could be mined for the creation of gene files. Adding to this is the most recent possibility of comparison to rice from which the genome sequence has become available as well. The following is a first exploration of the field. First, a background will be given of the types of approaches developing in the *Arabidopsis* research field. Subsequently, in order to give an idea about the types of genes for which knowledge is quickly expanding now, an overview is given with the focus on genes expected to be involved in plant dispersal and/or dispersion. Such genes could have an influence on the amount of 'weediness' in a species.

#### 3.1 Background

*Arabidopsis* has become a popular plant model because of its small genome size and short life cycle. The species is native to Eurasia and North Africa, and has extended its range over the Western Hemisphere in the wake of European colonisation. A wealth of biological information has already been gathered (for reviews, e.g. Mitchell-Olds & Bergelson, 2000; Pigliucci, 2002; Borevitz & Nordborg, 2003). A well-characterised set of ecotypes/accessions is available for studying variation as well as a large amount of mutants for studying gene function (Alonso-Blanco & Koornneef, 2000; Feder & Mitchell-Olds, 2003). In addition, a vast array of molecular tools, such as transformation protocols and gene expression arrays, is available. For specific biological questions for which *Arabidopsis* appears less suitable, researchers could turn to wild relatives from the Brassicaceae (Crucifer) family (Mitchell-Olds, 2001). The plant types available within this family will of course limit this to a certain extent, e.g. no Crucifers with a tree growing habit are known.

An important role in elucidating the genes underlying specific traits is played by QTL (Quantitative Trait Loci) mapping, which is enabled by the fast generation of a large number of progeny from controlled crosses in *Arabidopsis*. Because of its selfing nature, subsequent generation of RILs (Recombinant Inbred Lines) or NILs (Near-Isogenic Lines) for further analysis is straightforward (Alonso-Blanco & Koornneef, 2000). For both evolutionary studies and gene cloning, a lot of effort has been put into the study of synteny (extent of colinearity of genes/markers on genomic maps of different species) with other well studied species (Schmidt, 2002). This encompasses mostly crops, both from the Brassicaceae family, such as *Brassica* and from other families, tomato (Solanaceae) and monocotyledons, such as cereals and rice. In addition, the availability of extensively characterised genetic resources enables the application of LD (Linkage Disequilibrium) or association mapping, that is, the localisation of traits by their combined occurrence in a large screen of individuals. The LD mapping approach has to be applied with care, because co-occurrence of markers and traits in the genotypes screened could also be strongly influenced by their demographical history, i.e. phylogenetic relationships of these genotypes, obscuring any effect from the real genetic linkage of the markers/traits (Borevitz & Nordborg, 2003).

Demographical history has been studied to some extent using different techniques, including morphology, quantitative traits, isozymes, RFLP, AFLP and SSR (microsatellites). By such studies, the highly selfing nature of *Arabidopsis* came to be questioned by the high intra-population variability sometimes found and the difficulty in reconstructing phylogeny of ecotypes: the relationship pattern was indicative of ongoing genetic recombination (Pigliucci, 2002; Sharbel *et al.*, 2000). In general, no clear relationship was found between genetic distance and geography of the populations. Explanations proposed were recent expansion or human-aided long-distance gene flow (Pigliucci, 2002). These observations show interesting parallels to the situation in prickley lettuce (*L. serriola*), like its selfing nature

and its being a recently expanding pioneer species, both aspects subject of the EU study discussed in the first part of this report. On the other hand, an AFLP study at a European scale by Sharbel *et al.* (2000) provided evidence for postglacial colonization from both Asian and Southern Europe refugia. Another study based on morphology and SSRs provided evidence that North-American populations were derived from Europe (cf. Pigliucci, 2002).

Many studies focused on the genetics underlying organ development, e.g. the regulatory pathways directing organ (calyx/crown/anthers/pistil) identity in flowers, mostly involving genes of the MADS-box type, a special class of homeotic genes (cf. De Bodt *et al.*, 2003). Homeotic genes are controlling complete developmental pathways leading to specific organ formation. Also popular in research are genes involved in signal transduction (e.g. Morris & Walker, 2003), e.g. in relation to abiotic stresses, such as drought, and biotic stresses, such as pathogens. In the following brief overview, the focus will be on genes thought to be relevant for dispersal and/or dispersion of a species and/or having received attention for agronomical considerations: seed dormancy, timing of flowering, pollen development and recognition, seed dispersal, apomixis and disease resistance genes.

## 3.2 Seed dormancy

Seed dormancy is actively studied in relation to crop improvement. It is also regarded as important to weed survival in agricultural and ruderal habitats, because it enables the establishment of a seed bank necessary for spreading risk in an unpredictable environment. As such, it has been studied in the context of GM crop environmental studies on *Brassicá's*. Crop forms have been selected for lack of dormancy, but could regain this trait by hybridisation to weedy forms. In this way, the persistence of hybrids containing a transgene would be increased. Seed dormancy is a complex trait under the control of high number of genes of varying types (Bentsink & Koornneef, 2002). Mutants of the following types have been described:

- Seed maturation mutants involving transcription factors
- Mutants in plant growth regulator biosynthesis or signalling
  - Abscisic acid (ABA)
  - Gibberellin (GA)
  - Ethylene
  - Brassinosteroids
- Light perception mutants, e.g. phytochrome (red/far-red sensitive)
- Seed coat mutants
- And others, including DOG (Delay Of Germination) QTLs described recently from a cross of two ecotypes differing largely in their seed dormancy behaviour (Alonso-Blanco *et al.*, 2003).

The hormones ABA and GA have counteracting roles: ABA is crucial in controlling seed dormancy and GA is needed for germination; the latter's synthesis is controlled by light through the phytochrome system. Many other factors, such as the strength of the mechanical barrier imposed by the seed coat, are evident from mutants, but have not been cloned and characterised yet (reviewed by Bentsink & Koornneef, 2002).

## 3.3 Flowering timing

Timing of flowering is also a complex trait in which photoperiodicity and temperature (vernalisation requirements: need for a cold period) play important roles. In *Arabidopsis*, there is important variation in this trait, leading to the occurrence of rapid-cycling ecotypes as well as ecotypes having a winter annual habit, thus bolting at favourable conditions in spring with a need for a cold period in winter (Alonso-Blanco & Koornneef, 2000). Determinants for vernalisation requirements are, for instance, *FRI* and *VRN1* and *2*, the latter two members of the polycomb group regulating gene expression through chromatin structure. Like in seed dormancy, photoreceptor genes are involved, not only phytochromes, but also cryptochromes 1 and 2 (blue-sensitive). Some eleven circadian clock genes have been implicated. Inputs from all these genes come together at the circadian clock mediator gene *CONSTANS (CO)*. The *CO* gene, in turn, controls the floral pathway integrator gene *FT* (flowering locus T). From this point, flowering is set in motion, involving inflorescence architecture gene *TF*, floral meristem identity and floral organ identity genes (mostly MADS-box genes) (Izawa *et al.*, 2003).

In rice, a homologue of the *CO* gene was identified, *Hd1*, which was shown to function at a similar position in the floral pathway. However, whereas *CO* in short day *Arabidopsis* promotes flowering, *Hd1* in long day rice exerts a negative control over flowering (Cremer & Coupland, 2003). So, gene effects clearly depend on the genetic background of the species in this example. The genes in this pathway also show varying patterns of selection: for instance, *TF* shows evidence of adaptive (purifying, directional) evolution limiting the amount of variation present in the gene, whereas its promoter region shows a high level of variation appearing to be maintained by diversifying (balancing) selection (Olsen *et al.*, 2002). Variation in flowering time through the *FRI* and *FLC* genes was shown to arise by the generation of various non-functional or weak alleles (Gazzani *et al.*, 2003). Moreover, strong variation in the contribution of specific QTLs involved in bolting was shown between controlled and natural conditions. Thus, photoperiodic QTLs having a strong effect in the growth chamber could not be detected in natural environments indicating that their effect was overruled by factors specific to the field (Weinig *et al.*, 2002). The direction of selection on a particular gene/QTL may also change with genetic background: e.g. epistatic interactions were shown for *TF* with two other loci in field experiments (Weinig *et al.*, 2003).

### 3.4 Pollen development and recognition

With regard to improving the breeding process, much work has been done on this aspect of plant reproduction, for instance male sterility for hybrid variety production. Thus, pollen incompatibility attracted interest, and the isolation of anther-specific gene promoters led to their application, coupled to a Barnase (extracellular ribonuclease) gene, as transgenically induced male sterility (Mariani *et al.*, 1990). *Arabidopsis* being a self-pollinator, however, could not be used for this purpose; much work therefore, has been performed on *Brassica* (for recent review, Kachroo *et al.*, 2002). Several other genes involved in pollination success have been identified in *Arabidopsis*, such as an oleosin-domain protein (GRP17) in the pollen coat (Mayfield & Preuss, 2000). The oleosin proteins were implied to be important to species-specific pollen recognition by the stigma, and therefore, they could be involved in plant speciation (Mitchell-Olds & Clauss, 2002). Diverse other genes involved in pollination have been described, among which a jasmonic acid (hormone) biosynthesis gene (*CYP74A*) (Park *et al.*, 2002), an LRR receptor protein kinase (*EMS1*) important for microsporocyte/tapetum development (Zhao *et al.*, 2002), and a K<sup>+</sup> channel (*SPK*) important for pollen tube growth (Mouline *et al.*, 2002).

### 3.5 Seed dispersal

By definition, seed dispersal is conditional to dispersal success of the species. In contrast, seed retention is one of the more obvious traits selected for in the domestication process. Nevertheless, in oilseed rape, still substantial yield losses are incurred by pod shatter. Thus, in *Arabidopsis*, genes have been characterised directly involved in silique (crucifer fruit) dehiscence, which sets free the seeds: two MADS-box genes, *SHATTERPROOF* (*SHP1* and *2*) involved in development and lignification of the dehiscence zone (Liljegren *et al.*, 2000) and the myc/bHLH transcription factor *ALCATRAZ* (*ALC*) directing formation of the non-lignified cell layer in the dehiscence zone (Rajani & Sundaresan, 2001). In addition, a negative regulator of *SHP* in fruit valve differentiation, *FRUITFULL* (*FUL*), also a MADS-box gene, was described (Ferrándiz *et al.*, 2000).

### 3.6 Apomixis

Apomixis is embryo development and concomitant seed set without fertilisation of the egg cell; however, often fertilisation of the central cell in the embryo sac is required in order to obtain the development of the endosperm necessary for successful seed maturation. Apomixis is an attractive trait to plant breeders, because in this way heterozygous elite genotypes could be propagated intact without loss of vigour. It has also been suggested as a method to attain inherent biosafety of GM crops, namely by preventing outcrossing when combined with male sterility (for review of engineered biosafety: Daniell, 2003). However, in many plant species apomixis is not an exclusive reproductive strategy and exists in combination with sexual propagation (Van Dijk, 2003). Therefore, it might be difficult to predict the effect of introducing apomixis in crop species originally not having this strategy. In

such crops, it has proven to be difficult to introduce an apomixis breeding system, even though in apomictic plant species, this trait has been localised to one or a few tightly linked loci (Richards, 2003; Spielman *et al.*, 2003). In *Arabidopsis*, not an apomictic species by nature, genes holding promise have been identified by mutation strategies (Spielman *et al.*, 2003): *fis* (fertilisation-independent seed) mutations leading to some endosperm development without fertilisation. In addition, genes have been described, such as *AtSERK1* and *BABY BOOM*, of which ectopic expression leads to embryo development in callus and on adult plant parts (leaf margin, shoot apex), respectively. Since epigenetic factors have been shown to be important in balancing the paternal and maternal genome combination for successful development of the endosperm, genes directing DNA methylation have also been implied as highly relevant (Spielman *et al.*, 2003).

### 3.7 Disease resistance genes

Disease resistance genes (R-genes) are an important focus of research and breeding programmes. The most easily targeted genes are the ones showing a gene for gene relationship with the pathogen, in which a specific plant host resistance gene product is hypothesised to interact with a specific avirulence gene product from the pathogen. A successful interaction of both gene products is followed by a hypersensitive response (HR) in the host leading to the formation of anti-pathogen substances and local cell death effectively stopping infection progress. Several types of such resistance genes have been described, encoding the following types of proteins (Jones, 2001; Lahaye, 2002):

- PK (protein kinases),
- TM (transmembrane) proteins with an intracellular CC (Coiled Coil) domain,
- TM proteins with an extracellular LRR (Leucine-Rich Repeat) with or without an intracellular PK domain, and
- intracellular NBS-LRR.

The NBS-LRR genes represent the most common family as already shortly mentioned under lettuce above. The LRR domain is highly variable and thought to be involved in pathogen recognition. The LRR domain's importance is evidenced by the disproportionate presence of sites implied to be under positive selection relative to the rest of the gene (Mondragón-Palomino, 2002). In the completely sequenced *Arabidopsis* ecotype Columbia, 166 NBS-LRR genes were found, occurring both as single-gene loci, and in clusters and superclusters (Richly *et al.*, 2002). Based on phylogenetic analysis, nine clades plus a few orphans were recognised that could be derived from just 17 progenitors. Most clusters were homogeneous, that is, contained genes from one clade. However, some 10 cluster were mixed, indicating that complex rearrangements have taken place in this gene family during Angiosperm evolution. Also more recent gene rearrangements could be inferred. In successful pathogen recognition, complex formation of several types of proteins is involved, of which only recently a more complete picture starts to arise (Shirasu & Schulze-Lefert, 2003; Rathjen & Moffett, 2003). Specificities in this complex formation might be one of the reasons for the restricted taxonomic functionality shown for the R-genes. On the other hand, the *Arabidopsis NPR1* gene, which is thought to be part of the signalling cascade between R-genes and final defence gene transcription, was shown to be effective in rice, indicating wide taxonomic functionality (McDowell & Wolfenden, 2003).

Several lines of evidence have indicated that the extreme variability in R-genes is maintained by frequency-dependent selection (Noël *et al.*, 1999). In contrast to classic purifying selection in which disadvantageous alleles are purged, genes and/or alleles become disadvantageous once they have reached a frequency at which the pressure on the pathogen to successfully adapt to the R-gene has become too high. This has been circumscribed as 'trench warfare', with advances and retreats of specific genes/alleles, leading to the maintenance of a dynamic variability in R-genes (Stahl *et al.*, 1999). In addition, another check on R-gene frequency could be a fitness cost which was shown to be incurred by the *Arabidopsis RPM1* gene in the absence of the pathogen using different transgenic plants derived from the same genotype (Tian *et al.*, 2003).

### 3.8 Discussion and conclusions

With the availability of the full genome sequence, all genes could be putatively inferred for *Arabidopsis* in principle. For many genes, functions have already been described, and a picture is beginning to emerge of the pathways and

networks in which their protein products interact in plant development and signal transduction. In addition, a start has been made with the study of effects of genes and their allelic variation under natural conditions. As far as could be ascertained, this has not yet led to *Arabidopsis* studies directly addressing gene functions and dispersal. Diverse patterns of selection were shown in the form of purifying as well as diversifying selection; also pleiotropic and epistatic interactions between genes were indicated. All these interactions imply that effects of transgenes introduced into natural settings will not always be straightforward. For instance, the diversifying selection shown for the R-genes implicates that an introduced R-gene might not be able to simply 'take over' complete populations, but little is known about this as yet. In this regard, one has to take into account that in natural circumstances completely different pathogens may be limiting in population fitness than in crops. Moreover, inferring selection processes from field data is fraught with difficulties, because often alternative explanations may be offered by demographical history of the study populations (Borevitz & Nordborg, 2003).

As to predicting gene functions from information in *Arabidopsis* only, the *CONSTANS* flowering pathway integrator is an interesting example: its counterpart rice functions in the same pathway, but with an opposite effect on the following homologous link in the signal transduction chain. Thus, gene function in a different genetic background is not always predictable. Also even within *Arabidopsis* itself, alleles can have different effects in different genetic backgrounds. Thus, knowledge from *Arabidopsis* studies will for certain provide clues about possible gene effects, and generalisations will increasingly become possible when more comparisons to other plant species have been made. However, at the present state of knowledge, information from *Arabidopsis* only will most likely not be conclusive, and there will remain a need for collecting additional information on the specific crop/gene combination itself.





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## Appendix I.

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## Appendix II.

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