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Genomic and Transgenic Approaches to Modified Plants: Disease Resistance in the *Brassica* as a Model System.

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ABSTRACT Molecular genetic techniques can now be applied to the development of advanced plant genotypes, either through genetic transformation or genomic approaches which allow researchers to transfer specific traits using molecular markers. In this paper, we discuss the use of these techniques towards understanding the genetics of blackleg resistance in *Brassica*. In a comparative mapping study between *Arabidopsis thaliana* and *Brassica napus*, 6 R-ESTs, 7 *B. napus* RFLP markers and a *B. napus* EST were located in a collinear region of N7 (*B. napus*) and chromosome 1 (*A. thaliana*). One of the *A. thaliana* R-ESTs and 4 of the *B. napus* RFLPs co-segregated and mapped to the LmR1 locus for blackleg resistance. Introgression of blackleg resistance from wild relatives is also investigated with the possibility of accelerating the introgression process via marker assisted selection.

Key words: Blackleg, *Brassica*, disease resistance, genetic mapping, introgression, transgenics.

Introduction

One of the greatest challenges and successes in plant breeding has been the introduction of durable disease resistance into crop plants. Sources for crop disease resistance occur naturally in different genotypes and in wild relatives of many crop species. Plant breeders have normally introduced these resistances into crop species via conventional methods involving crossing and backcrossing. Since these methods involve selection of desired genotypes based on expressed phenotypes, the process is often lengthy and labor intensive, especially if the resistance is controlled by many genes. With the introduction of molecular marker technology to plant breeding it is now possible to create combined genetic and physical maps of a particular plant and flank the respective R gene(s) with molecular markers. The exploitation of these markers in backcrossing programs can then be used to shorten the time necessary for the development of disease resistant elite cultivars. This strategy has been coined iMarker Assisted Selectioni (Tanksley and Hewitt 1988). Knowledge of the exact location of R genes on the chromosomes is also the first step in a map-based approach to clone these genes which allows the introduction of resistance into crops using transformation techniques. The possibility of pyramiding multiple resistance genes in elite backgrounds becomes a possibility for durable resistance in future cultivars, either by using molecular markers or when different cloned genes for the same resistance are available.

Canola (*Brassica napus* and *Brassica rapa*) is an important oilseed crop species grown in North America, Europe, Australia and China. It is prone to attack by a number of pathogens, including *Sclerotinia sclerotiorum*, *Albugo sativa* and *Leptosphaeria maculans*, which causes blackleg (Gugel and Petrie 1992). Blackleg is the major disease of canola worldwide. In western Canada the virulent form of *L. maculans* has spread rapidly over the last 15 years, resulting in yield losses in excess of $100 million dollars annually (D. Klaffke, personal communication). *L. maculans* consists of several strains or pathotypes that are morphologically similar but may be different species. Two of these pathotypes are termed ihighly virulenti and iweakly virulenti based on whether or not they can form stem cankers on *B. napus*

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(Williams, 1992). In Australia, blackleg isolates are more virulent than those found in other parts of the world (Purwantara et al. 1998). While the optimal climatic conditions for the development of large numbers of pseudotheca may occur in Australia, resulting in heavy losses to the crop, the differences in virulence of isolates found in some parts of the world, is also genetic as has been shown by Purwantara et al. (1998). Blackleg can be controlled by the application of fungicides, though it is much more cost effective and environmentally friendly to utilize disease resistant cultivars.

A number of blackleg resistance sources have been identified from *B. napus, B. juncea, B. nigra and B. carinata* , as well as wild relatives and some have been genetically positioned within the respective genomes (Dion et al. 1995; Ferreira et al. 1995; Mayerhofer et al. 1997), however none have been cloned in Brassicas. This is probably due to the technical difficulties imposed by a relatively large and highly duplicated genome. In contrast, in *Arabidopsis thaliana*, also a crucifer, many disease resistance genes have been cloned and characterized (Kunkel 1996; Warren et al. 1999). *A. thaliana* and *B. napus* have been shown to share regions of conserved gene content and gene order (Cavell et al. 1998; Scheffler et al. 1997). Based on the level of colinearity between the two species, cloned genes from *A. thaliana* may have similar functions in *B. napus* or in the least be useful as probes for isolating homologues in *B. napus* (Fray et al. 1997; Lagercrantz et al. 1996; Sillito et al. 2000). It has been shown that disease resistance genes cloned from different organisms share structural and amino acid motifs, such as leucine zippers (LZ), leucine rich repeat regions (LRR), protein kinase domains or nucleotide binding sites (NBS) (Bent, 1996). Conservation across different organisms and species of specific sequences or protein structures involved in disease resistance responses suggest that the different pathways may share a common origin.

In this paper, we present an overview of the results we obtained from the fine mapping and map-based cloning approach to isolate a blackleg resistance gene from *B. napus*. A comparative mapping effort of resistance gene homologues in *B. napus* and *A. thaliana*, and strategies for introgression of disease resistance from related crop species into *B. napus* are also presented.

Materials and Methods

Parental plant material

For the mapping of the blackleg resistance genes from the cultivars Shiralee and Maluka, 164 and 34 doubled haploid (DH) lines, respectively, were used. The susceptible parents in both crosses were advanced breeding lines from the University of Alberta breeding program. For the comparative mapping study, 30 doubled haploid (DH) *B. napus* lines from the highly polymorphic N-fo-61-9 population (Parkin et al. 1995) and 30 recombinant inbred (RI) lines of *A. thaliana* derived from a cross between the Columbia and Landsberg ecotypes (Lister and Dean, 1993) were used.

RFLP, RAPD and AFLP marker analysis

DNA extractions, Southern hybridizations, RFLP probes, RAPD and AFLP primer combinations and linkage analyses of these populations are described in Sillito et al. (2000) and Mayerhofer et al. (1997).

Interspecific hibridization between B. carinata and B. napus

A blackleg resistant line of *B. carinata*, was crossed with a susceptible *B. napus* cv. Topas. Successful crosses were ovule rescued according to the protocol of Quazi (1988) and eventually transferred to soil. The hybrids formed were in turn backcrossed to Topas and the BC_1 and BC_2 progeny derived analyzed for introgression of blackleg resistance using the protocols of Bansal et al. (1994) and Purwantara et al. (1998).

Results and Discussion

Mapping of blackleg disease resistance in Brassica napus

Bulked segregant analysis was used to select RFLP, RAPD and AFLP markers that were linked to the blackleg resistance locus of Shiralee and Maluka, respectively (Michelmore et al. 1991). In total 920 RAPDs, 192 RFLPs and 32 AFLPs were tested and linked markers consequently mapped in the segregating populations. Out of

these, 7 RFLPs, 12 RAPDs and one AFLP marker were linked to blackleg resistance. Figure 1 illustrates a fine scale map of the N7 linkage group. RAPD4 and RAPD654 align this fine scale map with the larger linkage map of Mayerhofer et al. (1997). In addition to random screening with the DNA markers described above, we made use of our data from the comparative mapping of disease resistance homologues in *A. thaliana* and *B. napus* (Figure 3). Six R-ESTs from *A. thaliana* and one from *B. napus* as well as the seven RFLPs were located in a collinear region of N7 (*B. napus*) and chromosome 1 (*A. thaliana*) and consequently mapped in relation to the blackleg resistance phenotype (Figure 1). It was found that one of the R-ESTs and four of the RFLPs co-segregated with the resistance gene, the remaining R-ESTs were not polymorphic and could not be mapped. The apparent co-segregation of these five markers with the resistance gene has to be viewed with caution since we have detected a translocation and consequently lack of recombination events in this region. Markers could therefore be physically further apart than the genetic mapping suggests. This problem is currently being addressed, with the analysis of a new mapping population lacking this translocation, in our laboratory.

Our results confirm the presence of a single major locus responsible for blackleg resistance in Shiralee and Maluka, which has been determined by genetic analysis in previous studies (Stringam et al. 1992; Mayerhofer et al. 1997). The location of this locus, which we call LmR1 (Mayerhofer et al. 1997), coincides with blackleg resistance loci from the cultivars Major (on linkage group LG6, Ferreira et al. 1995), Cresor and RB 87-62 (Rimmer et al. 1999; on linkage group N7). The LG6 linkage group corresponds to N7 on published *B. napus* maps of Parkin et al. (1995) and Sharpe et al. (1995) (Mayerhofer et al. 1999). It has yet to be shown whether these genes are allelic or part of a tightly linked cluster of resistance genes. However since the R-ESTis linked to LmR1 are not homologous to each other, it seems likely that the region containing LmR1 may include a cluster of resistance genes, although only one or a few of those genes may be functional. It is interesting that the blackleg resistance genes identified in the different cultivars studied thus far, map to the same region of the N7 linkage group. This suggests that the N7 chromosome plays a major role in A-genome derived resistance.

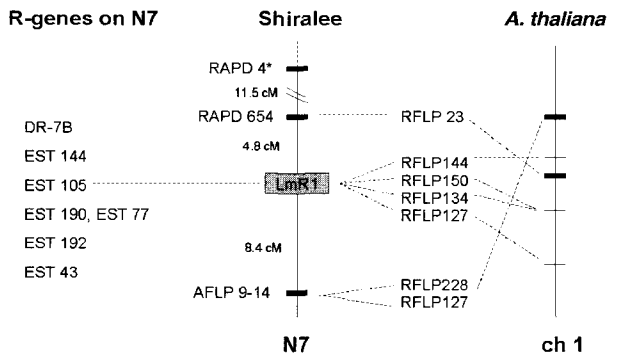

Figure 1. Fine scale mapping of the LmR1 gene on the N7 linkage group of *B. napus* cv (Shiralee) and comparative mapping of the R-EST's (R-genes) and RFLPs from N7 to the *Arabidopsis* linkage map. Thin diagonal lines denote colinear locations of R-EST's and RFLP between the two genomes.

One of the major goals of marker-assisted selection with resistance genes is the pyramiding of multiple resistance specificities using linked DNA markers into one elite cultivar with more durable resistance. However, the fact that the blackleg resistance genes mapped so far seem to be tightly clustered around the same locus will make this approach extremely difficult, if not impossible. It will require a large number of segregating offspring to detect recombination events between the individual resistance genes after crossing of the two resistant parents. A more promising way of pyramiding the different resistance genes in this case would be to clone the individual genes and introduce them into an elite cultivar via transformation, thereby circumventing the need for lengthy backcrossing schemes. The cloning of the resistance gene from Shiralee is currently underway and is being accelerated by comparative mapping in *A. thaliana* and the resources provided by the Arabidopsis Genome Project.

A comparison of traditional introgression of a resistance gene versus the cloning and transformation of the gene into a susceptible cultivar is presented in figure 2. The integration of cloned genes into different genotypes usually occurs by the integration of the left and right border of the T-DNA. However, recent developments in the area of homologous recombination now point to the possibility of using homologous replacement as a method to integrate genes into the plant genome. One of the interesting features of homologous recombination is that the resulting ìtransgenicî plants would be genetically identical to plants derived from traditional crossing.

Genetic mapping of Arabidopsis R-ESTs and Brassica R-gene homologues

In addition to random screening with the DNA markers described above we made use of our data from the comparative mapping of disease resistance homologues in *A. thaliana* and *B. napus* (Figure 3). Resistance homologues can be used not only to identify candidate genes for blackleg resistance, but can also be used to identify candidate genes for other resistance sources, once they have been mapped phenotypically.

Arabidopsis R-ESTs have been mapped in the segregating *B. napus* population Nfo-61-9 (Sillito et al. 2000). R-ESTs were mapped to every linkage group except N16, and ranged in number from 3 (N1, N2, N8, and N17) to 11 (N3) loci per linkage group. In total, 103 loci detected by the R-ESTs were placed on the *B. napus* genetic linkage map (Sillito et al. 2000). Figure 3 illustrates a comparative map between chromosome 4 of *A. thaliana* and the N8, N1 and N11 linkage groups of *B. napus*. As can be seen, N1 and N11 are homologous chromosomes originally derived from the A and C genomes respectively (Sharpe et al. 1995). While N8 has 2 of 7 R-EST's which map to Chromosome 4 of *Arabidopsis*, one of these probes (T14233) also detects polymorphic loci on chromosomes N1 and N11 of *B. napus*.

The benefit of studies such as these lies in the fact that conservation of gene order and content (Cavell et al. 1998; Scheffler et al. 1997) between *A. thaliana* and *B. napus* allows established genetic pathways and molecular tools in *Arabidopsis* to be used in teasing apart and understanding the more complex amphidiploid crop species *B. napus* and by extension other *Brassica* crop species. It has been suggested that DNA sequences identified via homology to conserved R gene motifs are likely to represent disease resistant genes (Aarts et al. 1998; Spielmeyer et al. 1998; Botella et al. 1997; Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). The *Arabidopsis* R-ESTs provide a good source for directly identifying novel disease resistance candidates in crop *Brassica* species and where information is available on the phenotypic location of disease resistance genes, these R-EST's can be used to retrieve potential disease resistance genes using the R-EST's as DNA probes.

Introgression of blackleg resistance from B. carinata into B. napus.

The value of introducing traits from wild relatives of crop species has been demonstrated in a number of crop species. In tomatoes (*Lycopersicum esculentum*), genes of interest responsible for several desirable traits have been introgressed from related wild relatives with great success. A case in point is the successful intro-

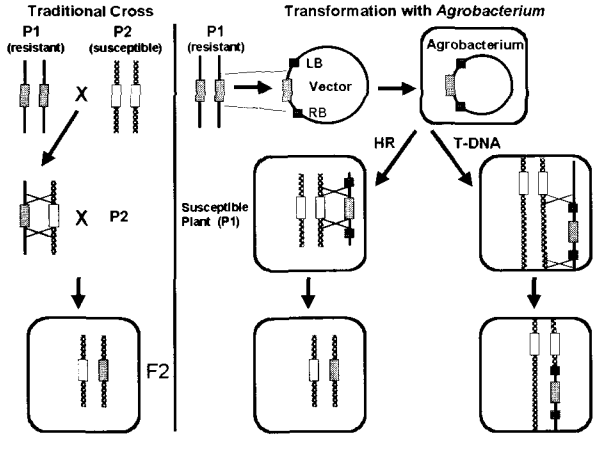

Figure 2. Comparison between conventional introgression of a resistance gene and cloning and transformation of the resistance gene into a plant. Insertion of the cloned gene can occur by homologous recombination (HR) or integration of the T-DNA into the chromosome. The F2 plant derived from traditional crossing is genetically identical to the transgenic plant where the cloned gene has integrated by homologous recombination.

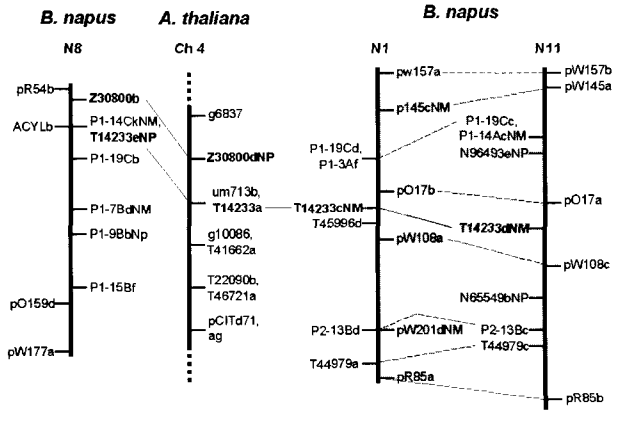

Figure 3. Comparative mapping of RFLP markers and disease resistance EST's from Chromosome 1 of *Arabidopsis thaliana* and linkage groups LG4, LG1 and LG11 of *Brassica napus*. LG1 and LG4 are from the A genome while LG11 is from the C genome of *B. napus*. The shaded region between N1 and N11 indicates colinearity between these two linkage groups.

gression of the Mi gene for resistance against nematodes from the wild tomato *Lycopersicum peruviana* into *Lycopersicum esculentum* (Milligan et al. 1998). Nematodes causes losses of over $80 billion annually in a wide range of crops species (Agios, 1997) and the introduction of this single gene has been estimated to have an economic value to the tomato industry of $1 to 3 billion annually (Valerie Williamson, personal communication).

In the *Brassica*, the introgression of the Ogura male sterility and restorer genes from *Raphanus sativus* and numerous other related and unrelated wild species into elite germplasm of canola (*B. napus*) has been widely used to establish pollination control via male sterility in hybrid seed production (Paulman and Robbelen 1988).

The apparent limitation of different resistance loci as well as the partial nature of blackleg resistance in *B. napus* will make it necessary to look at the *Brassica* B genome (*B. juncea*, *B. carinata*, and *B. nigra*), as well as other wild relatives such as *Eruca sativa*, as a source of new resistance genes. The process of interspecies transfer of resistance genes has met with mixed success in the past and the main limiting factors have been linkage drag and genetic instability. The root cause of both these problems is interspecies variation in genome structure, which sometimes make it difficult to obtain canola plants with resistance genes from related species inserted into the appropriate locations on resident chromosomes. With the development of RFLP probes effective across a wide range of cruciferous species (Sharpe et al. 1995), it is now possible to design and select interspecies hybrids containing desirable hybrid chromosomes and to use marker assisted selection to rapidly transfer such chromosomes into otherwise pure canola genotypes. These hybrid chromosomes should carry the targeted resistance genes and will have structures that will allow them to pair and recombine effectively with one of the normal resident chromosomes of *B. napus* and *B. rapa*.

B. carinata carries excellent resistance to blackleg and will form fertile hybrids with *B. napus* and *B. rapa*. The resistance has been shown to be located on the B genome and introgression of *B. carinata* traits into *B. napus* follows the general scheme outlined in figure 4. One of the advantages of working with the allotetraploid Brassica species is that the transfer of traits from the A, B or C genome can be achieved by using a bridging genome as outlined by Rick et al. (1986) and Mukhopadhyay et al. (1994). In this example (Figure 4), the C genome acts as the bridging genome to allow the plant to function normally, allowing pairing between the A and B homologous chromosomes. Nagpal et al. (1996) have successfully used this strategy to transfer genes from *Brassica tournefortii* to the allotetraploid *Brassica* species. A number of *B. carinata* lines have been identified that show absolute resistance even to the highly virulent *B. juncea* attacking strains of *L. maculans* (Purwantara et al. 1998). One such resistant line was crossed to the susceptible canola cultivar Topas and BC_1 and BC_2 populations have been developed. These are being analyzed for blackleg resistance and preliminary data indicates that the blackleg resistance from the B genome has been transferred into *B. napus*.

In conclusion, we have presented an overview of the fine maping and map-based coning approach to the isolation of a blackleg resistance gene from *B. napus*. The cloning and analysis of disease resistance genes in Brassica will allow us to begin to understand the signal transduction cascade the triggers the plants defense response. From a practical wiewpoint, an understanding of how plant's respond to pathogens will allow us to begin to engineer novel resistance genes which can protect a plant against different strains of the pathogen, or different pathogenic organisms.

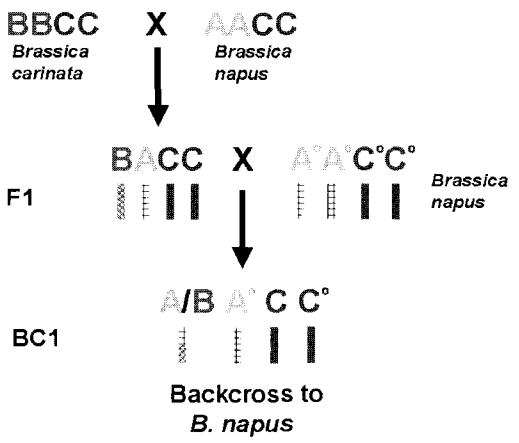

Figure 4. Introgression of B genome chromosomes from *Brassica carinata* to *B. napus*. *B. carinata* were crossed to *B. napus* and F1 hybrids developed through ovule rescue. Subsequent backcrosses were performed using normal sexual crossing. The segregation of the A, B and C genomes is illustrated in this Figure. In the F1 and BC1 plants, the C genome acts as the bridging genome, while pairing can occur between homeologous chromosomes from the A and B genome.

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