Association of single-nucleotide polymorphisms in resistance gene sequences with blackleg disease in oilseed rape (*Brassica napus*)

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Abstract

A broad variation for resistance to blackleg disease caused by *Leptosphaeria maculans* (anamorph *Phoma lingam*) is present in the *Brassica napus* gene pool, however due to the quantitative nature of the resistance it is difficult to develop effective selection markers. This project aims at detection and exploitation of genetic diversity of resistance gene candidates associated with blackleg resistance in *B. napus* via association studies between resistance data and single nucleotide polymorphism (SNP) haplotypes in a core set of 50 diverse oilseed rape genotypes. Resistance gene analogue (RGA) sequences have been amplified in resistant and non-resistant *B. napus* genotypes using degenerate primers for resistance gene motifs (TIR, NBS, LRR). In addition to these anonymous RGA sequences, further primer combinations were developed using potential resistance gene candidates (RGCs) selected from publicly available cDNAs derived from the *B. napus* defense reaction to inoculation with *L. maculans*. Sequence-independent SNP-detection using specific primers was performed in the genotype core set via BESS-T (base-excision sequence scanning), and the SNP data were used for association studies with *L. maculans* resistance/susceptibility data from field and greenhouse tests. Cloning of RGA and RGC sequences with significant associations to resistance data allowed development of locus-specific PCR primers, so that sequences of the relevant loci could be compared in the genotype set and verified in independent materials. The resulting SNP markers for haplotypes associated with quantitative resistance loci will be useful not only for marker-assisted selection, but also for identification of new resistance sources in the broader *B. napus* gene pool.

Key words: Rapeseed, Brassica napus, blackleg, Leptosphaeria maculans, SNP, association study, resistance genes

Introduction

Blackleg disease caused by the fungal pathogen *Leptosphaeria maculans* (anamorph *Phoma lingam*) is one of the most important oilseed rape (*Brassica napus*) diseases and can cause heavy yield loss of up to 50% in epidemic years. The pathogen attacks leaves, stems, roots, cotyledons and pods of oilseed rape. A broad variation for *L. maculans* resistance is present in the *Brassica napus* gene pool, however due to the quantitative nature of the resistance it is difficult to develop effective selection markers. In the last decades the genetics of blackleg resistance in *B.napus* has been examined in classical QTL analyses. Dion et al. (1995) examined a DH population with RFLP markers and identified an individual major Locus (LmFr1) for field resistance. Mayerhofer et al. (1997) identified a further Locus (LmR1) and Ferreia et al. (1995) identified an individual major locus (LEM1) responsible for cotyledon resistance. Pilet et al. (1998) documented quantitative variation of field resistance. The Canola cultivar "Surpass 400" possesses a dominant A-genome resistance against *L maculans* in cotyledon and adult plants (Li & Cowling 2003), however this resistance from *B. rapa ssp. sylvestris* was broken within three years in the commercial cultivation in Australia by virulent pathotypes (Li et al. 2003). This example illustrates the substantial advantage of quantitative, polygenic resistances, which can contribute to the more durable resistance compared with monogenic resistance. For the use of the existing allelic diversity for polygenic resistances it is very difficult to identify the genes involved in resistance and to develop effective selection markers for all relevant genes.

This study aims at detection and exploitation of genetic diversity of resistance gene candidates associated with blackleg resistance in *B. napus* via association studies between resistance data and single nucleotide polymorphism (SNP) haplotypes in a core set of 54 genetically diverse winter oilseed rape genotypes. Single nucleotide polymorphisms (SNPs) are single-base substitutions in genomic DNA. They are the most abundant form of genome variation and can be used as simple genetic markers and for associations between the allelic form and the phenotype. Therefore SNPs represent the ultimate tool for extremely fine genetic mapping, detection and exploitation of genetic diversity and offer the opportunity to uncover allelic variation directly within sequences of candidate genes. In recent decades SNP-technology has grown in importance for plant genetic research.

Allelic variation in resistance gene candidates was examined in a genome wide approach using resistance gene analogs (RGAs). These are anonymous sequences with conserved motifs like the nucleotide binding site (NBS), leucine rich repeat (LRR) or the Toll-Interleukin-1 like motif (Baker et al. 1997, Hammond-Kosack & Jones 1997, Meyers et al. 1999) As additional potential candidates a further set of 277 EST sequences were considered, which showed an over-expression in the defence reaction against *L. maculans* in *B. napus* after inoculation with the pathogen (Fristensky et al. 1999) These ESTs showed homologies to defence genes, resistance genes and stress genes, and to genes associated with other functions like photosynthesis and metabolism. Some of the resistance genes identified in this study showed no homologies to the conserved regions of RGA sequences, hence they represent an interesting alternative source for potential candidate genes (RGCs) for the

defence reaction against *L. maculans* with *B. napus*.

Material and methods

A genotype core-set of 54 genetically diverse winter oilseed rape varieties was used for the association studies. Blackleg resistance data were provided by the participating breeding companies. Field data were evaluated in two years on eight locations. Greenhaus and cotyledonen tests were provided in each case in one year at one location.

Genomic DNA was extracted from fresh leaf material of all genotypes using DNeasy DNA Extraction Kits (Qiagen). A 25 µl PCR-Reaction contained 20 ng template DNA, 0,2 µM each primer 200 µM each dNTP and 16 µM dUTP (BESS T-Scan™ kit;Epicentre Technologies), 1x reaction buffer, a final MgCl2-concentration of 2,5 mM and 1 u Taq polymerase. The PCR profile consisted of an initial denaturation step at 94 °C for 5 min; followed by 35 cycles of 45 sec at 94°C 1 min; variable annealing temperature 1 min und 72°C 1 min; finale extension 5 min.

SNP-detection using specific primers was performed in the genotype core set via BESS T (base-excision sequence scanning: Figure 1)) according to the protocol of the BESS T-Scan™ kit;Epicentre Technologies, Madison. The Fragments were separated on PAA gels with the Licor-system. Statistical analyses between SNP data and blackleg resistance data were performed with the freely available statistic software package *Powermarker* (http://statgen.ncsu.edu/powermarker/) and the genetic analysis package (gap) in the software package R (http://www.r-project.org).

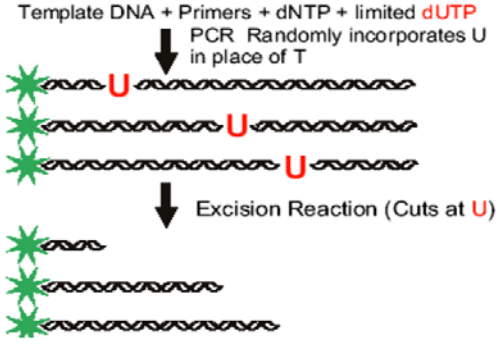

Figure 1: Principle of base-excision sequence scanning (BESS-T) for sequence-independent SNP detection.

Results & discussion

Resistance gene analogue (RGA) sequences were amplified in resistant and non-resistant *B. napus* genotypes using degenerate primers for resistance gene motifs (TIR, NBS, LRR). In addition to these anonymous RGA sequences, further primer combinations were developed using potential resistance gene candidates (RGCs) selected from publicly available cDNAs derived from the *B. napus* defence reaction to inoculation with *L. maculans*. Altogether 100 sequences were examined. Thirteen primer combinations showed more than one PCR fragment and eight primer combinations produced no PCR product. The remaining 79 primer combinations were screened for SNPs in the core set via BESS-T sequence scanning (Figure 2). Altogether 58 polymorphisms were detected in 32 sequences. In addition to SNPs and indels, differences in PCR fragment length among homoeologous loci were also scored. The SNP data were evaluated with the freely available statistic software package *Powermarker* (http://statgen.ncsu.edu/powermarker/). Associations between resistance gene sequences and blackleg resistance data were calculated based on average values from all years and locations in the field trials, along with data from the greenhouse and cotelydonen data. Single locus F-tests (simultaneous test of several loci associated with the phenotype data - similar to the analysis of variance) were used in the first step. Six sequences proved to be significantly associated with field data. Positive associations with greenhouse resistance data and for resistance data of the cotelydone were also found with this analysis. As expected the significantly associated sequences for cotyledon were different from the significantly associated sequences for field and greenhouse resistance data. In cases where more than one polymorphism was found within the significantly associated sequences these sequences were analysed in a second step via haplotype trend regression (examination of associations between haplotypes of several SNPs within a gene and/or a sequence). Here we used the genetic analysis package (gap) in the software package *R* (http://www.r-project.org) for statictical analysis. In this second step the *Powermarker* software supplied the frequency and the mean score of blackleg resistance data for individual haplotypes, while the F-statistics and significance of associations were computed with *R*.

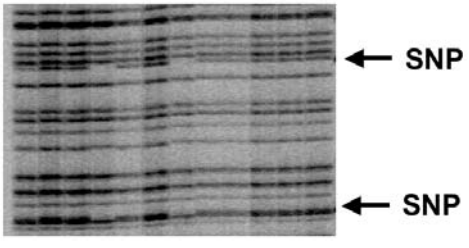

Figure 2: Example of base-excision sequence scanning (BESS-T) for SNP detection in a *B. napus* RGA sequence.

Conclusions

A broad variation for *L. maculans* resistance is present in the *Brassica napus* gene pool. The results of this study showed that sequence-independent SNP identification in anonymous resistance-related sequences can be successfully used to identify sequences with positive associations to blackleg resistance in *B. napus*. Of particular interest is the ability to identify SNPs between genotypes in homoeologous loci.

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