An EMS population of *Brassica napus* L. for TILLING

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Abstract

Major constraints for large rapeseed meal exploitation for food and feed industry are seed colour, lignin content and the presence of antinutritional compounds like sinapine. Classical breeding attempts towards a better meal quality in *Brassica napus* have been started, but should be considered as a midterm goal in rapeseed breeding. In the meantime transgenic approaches to reduce the sinapine content have been successful, but restrictive legal regulations hinder the commercialisation in Europe. Therefore acceleration of breeding processes by additional sources for genetic variation like mutation breeding is necessary. TILLING (Targeted Induced Lesions INto Genomes) is a further development of traditional mutation breeding. Here we present data on a *Brassica napus* TILLING platform, which has been set up as a joint venture between a private breeding company and a public research institute. The target genes *BnSGT1 (UGT84A9*; UDP-glucose:sinapate glucosyltransferase) and *BnSCT* (sinapoylglucose:choline sinapoyltransferase) from the sinapine biosynthesis pathway are used for this strategy to enable breeding for rapeseed varieties with low sinapine content by selection for knock off mutants for the genes. M3-seeds representing 5000 M2 lines from two independent mutation treatments of the same yellow seeded spring rapeseed parent (YN01-429) have been produced. In a first population all generations were selfed, while the second M2 population was grown in the field and the M2 populations was left open pollinated to also ensure seed set on male sterile plants. For screening experiments specific primers for at least two relevant genes are under development.

Key words: Oilseed rape, *Brassica napus*, EMS (Ethylmethanesulfonate), TILLING, *BnSGT1*, UDP-glucose:sinapate glucosyltransferase, *BnSCT*, sinapoylglucose:choline sinapoyltransferase, Sinapine content

Introduction

TILLING procedures have been established for a large number of plants (Arabidopsis, Barley, Maize, Lotus, Wheat and others) and animals (Zebrafish, Drosophila). Although TILLING approaches within the basic genomes (*B. oleracea* and *B. rapa*) have been successfully established, efficient TILLING in *Brassica napus* has not been reported, so far. TILLING is based on the production of EMS (ethylmethanesulfonate) mutagenised plant collections and rapid systematic identification of mutations in target sequences (McCallum et al., 2000, Henikoff & Comai, 2003). The advantage of this method is the identification of mutations in target genes without production of genetically modified organisms. Furthermore, the PCR based detection procedure can be easily scaled up to a high throughput system. However, most importantly, the approach represents an efficient way to identify diverse mutants in a specific gene with a higher potential agronomic value.

On the other hand, despite the tight relation of *Brassica napus* to *Arabidopsis thaliana*, its genome has a complex structure (Parkin et al., 2005). In deed chromosomal segments are duplicated and rearranged and single copies of genes often share high sequence identities with related copies in different locations of the genome. Furthermore, non-functional copies of these genes might also be present, which might complicate the development of useful primer pairs for the amplification of single alleles and the identification of the respective single lesions.

We are aiming to identify mutations in key genes of the phenylpropanoid pathway (Dixon *et al.*, 2002) to prevent sinapine accumulation in seeds of *B. napus*. Sinapine is the most common of all phenolics in *B. napus* seeds. It contributes to the bitter taste and astringency of rapeseed products and may form complexes with proteins thus preventing a broad application of rapeseed protein extracted from the meal. Two main genes (*BnSGT1*, UDP-glucose: sinapate glucosyltransferase and *BnSCT*, sinapoylglucose: choline sinapoyltransferase) are responsible for the accumulation of sinapine in the seed. Transgenic plants carrying RNAi constructs designed to silence these two genes have proven the feasibility to dramatically reduce the amount of sinapine in seeds of *B. napus* (Milkowski *et al*., 2004, Hüsken *et al.*, 2005). Thus *BnSGT1* and *BnSCT* are two candidate genes for the TILLING approach presented here.

Materials and Methods

Plant material, mutagenesis and plant cultivation

All plant material is based on a yellow seeded spring rapeseed line (YN01-429, kindly provided by Dr. G. Rakow, AAFC, Canada; Rakow 2005). A first population (population 1) consisting of 2000 M1 plants has been generated by combining different treatments: pre-incubation time (6h and 12h), EMS concentrations (0.5 % (v/v) and 1.0% (v/v)) and EMS incubation time (8h, 10h, 12h). All M1 plants were self-pollinated. To rise a M2 population, 500 M1 plants were identified, which have been treated with high stringency mutagenesis (1% EMS, 10h and 12h of EMS incubation). Six seeds harvested from each M1 plant were propagated in the greenhouse; dead seedlings were subsequently replaced to gain a M2 population of at least

2000 plants. M3 seeds per plant were generated by self-pollination of M2 plants. Further 5000 seeds of the same genotype were treated with two different concentrations of EMS (0.8 % and 1.2% (v/v)) at two different incubation times (10h and 12h). The seeds were directly placed into multipot plates. M1 plants were cultivated and selfed in the greenhouse until harvest of M2-seeds. Three M2 seeds per M1 plant (N=8000) were directly sown in a field nursery in the northern of Germany and cultivated according to conventional cultivation conditions (population 2).

To enable the identification of potential phenotypic mutants, each third row in the nursery consisted of wild type individuals. The plant material was screened for deviating phenotypes repeatedly during the whole developmental period until harvest of the seeds. All plants were subjected to open pollination to ensure development of M3 seeds on male sterile plants too. The relative fertility of the mutant plants was determined by dividing the amount of seeds harvested per single plant and by the mean amount of seeds harvested on open pollinated standard plants.

DNA extraction

Duplicate leaf samples from all M2 plants (population 1 and population 2) were harvested in the 4 to 6 leaves stage. Leaves were subsequently freeze dried and stored for further processing. DNA extraction was either manually monitored using Nucleospin(R) 96 Plant-Kits (Macherey&Nagel, Germany) or using an automated extraction robot (Tecan FREEDOM EVO), especially adapted to these needs. DNA concentrations from both populations 1 and 2 were adjusted manually to 5ng/µL.

Bioinformatics and Primer design

Primary target gene for the presently described TILLING approach is the gene BnSGT1 (UDP-glucose:sinapate glucosyltransferase). Based on the available cDNA sequence information of the BnSGT1 gene (Acc Nr. AF287143.1) (Milkowski et al., 2000) a genomic sequence of the BnSGT1 locus was isolated from a BAC library using the variety „Express“. The sequence was then subjected to analysis via the web-based software package CODDLE (http://www.proweb.org/coddle/) to identify the optimal regions for primer development. PCR amplicons of the entire region from the genotype YN01-429 were also cloned and subjected to multiple sequencing to identify assumedly deviating sequences within the amplicon. All sequences were analysed using software packages DNASTAR (Lasergene, DNASTAR Inc.) and CloneManager (Sci Ed Software). Identified sequence regions were than used to design primers able to amplify products of 800 to 1500 bp in size, surrounding the potentially mutated site identified via CODDLE.

Results

To generate a large number of mutants and introduce potential mutations in the sinapine genes we applied different mutagenesis conditions and concentrations of EMS. With increasing EMS concentrations germination rates were slightly reduced and the survival rate of M1 plants in both populations also decreased. A concentration between 0.8 % and 1.2 % EMS (v/v) per application seems to be suitable for inducing a high number of mutations in rapeseed as indicated by the number of plants displaying segmental leaf chlorosis or reduced viability as well as a reduction in general fertility. In addition, mutant plants showed a reduced seed set, and a number of phenotypic variations were observed. These effects were inherited in the M2 generation and the mutation effect was more drastically visible.

Varying amounts of seeds have been harvested from 1889 plants of population 1. From 8000 M2 seeds of population 2 sown directly in a field trial, 3860 single M2 plants germinated and survived until leaf harvest. However, 153 plants did not set any seeds. Within the remaining 3707 plants the seed set varied to a great extend from one M3 seed per plant to more than 80 g seeds and an average of 14 g seeds/plant. The mutant plants showed variation in relative fertility, with the majority of plants showing nearly normal fertility (75% up to more than 300%). Only less than 40 % of plants showed a degree of fertility lower than at least 75% of the standards (**Fig. 1**).

DNA probes were extracted from the different mutant populations as indicated in Material and Methods and used for specific PCR amplification of mutant alleles. Design of specific primers for TILLING was based on the specific amplification of single gene copies. To that end using a DNA probe specific for the *BnSGT1* gene, screening of a BAC-library derived from the variety “Express” identified 53 BAC clones, which were subsequently sequenced. Analysis of the sequences indicated that the rapeseed genome might have at least 4 copies of the *BnSGT1* gene, with respective locations on A and C genomes. On the other hand sequencing of 20 single clones derived from genomic amplicons of the *BnSGT1* gene from the genotype YN01-429 confirmed this assumption. Three to four subgroups have been established within the resulting sequences excluding those with single base exchanges appearing only once. These results indicate that in contrary to previous assumptions four different copies of the *BnSGT1* gene might be present in the rapeseed genome.

Further bioinformatics analysis of these sequences revealed specific regions, where induced mutations would lead to silencing of the gene. On this basis, specific primer pairs were designed to amplify regions of the four different *BnSGT1* alleles to be used in TILLING approaches. This work is now ongoing.

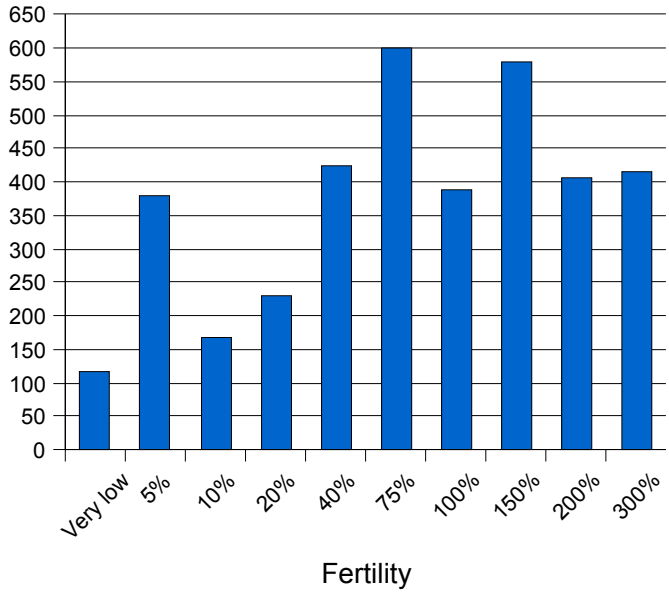

Fig. 1: Distribution of general fertility within M2 population 2. Fertility was scored as amount of seeds harvested per single M2 plant in relation to the amount of seeds harvested on single wild type plants in the same environment. All plants were subjected to open pollination.

Discussion

The aim of this study is to generate a TILLING population for the discovery of new mutants in the sinapine pathway and subsequent breeding for revalorisation of rapeseed meal. A first breakthrough was the identification of the key biosynthetic genes *BnSGT1*and *BnSCT* and their use in dsRNAi-mediated suppression strategies in rapeseed (Milkowski et al., 2004; Hüsken et al., 2005). Seed-specific silencing of the respective genes demonstrated the role of the encoded enzymes in the sinapine pathway and their usefulness as target genes for the TILLING strategy. We therefore generated M3 seeds from more than 5000 M2 plants. Our results indicate that rapeseed is very tolerant regarding the intensity of mutation induction. EMS concentrations between 0.8% to 1.2 % (v/v) did not have unexpected negative effects on the viability of plants. This was corroborated by higher LD50 levels, which could imply a high rate of mutations within the rapeseed genome. A comparable effect is known from the hexaploid wheat genome, where in comparison to diploid barley, much higher EMS concentrations can be used (Slade et al., 2004). These results indicate that lethal mutations can be reduced, due to gene duplications in poly- or amphidiploid genomes.

However, the redundancy of sequences might in turn increase difficulties in detecting mutants for specific genes using the TILLING protocol. As TILLING does not rely on analysing single DNA but pools of DNA, largely differing DNA sequences within one pool would engender target locations for the restriction enzyme resulting in a large fraction of small DNA fragments, most of them not visible on a LICOR sequencer because of label lacking. This indicates that a thorough optimisation of the protocols is necessary.

On the other hand, expression profiling of the gene copies can serve to select active gene copies, which then can be used as target for TILLING and minimise time of detection. To this aim, we have set up a cAFLP approach to monitor the expression of different *BnSGT1* alleles in seeds.

Morphological deviations within the population have been scored. The influence of the open pollinated production of M3 seeds on the late efficiency of the whole TILLING approach has not yet been determined. But it is assumed, that the mutants will be preserved within the M3 seeds at least in heterozygous state. It should be noted, that due to the fact that rapeseed tends to self fertilize to high extent (Becker et al., 1992) and a large proportion of seed mutants will be homozygous for the mutation, recovering these mutated plants for future breeding tasks will be simplified, because of the availability of mutation-linked markers that enable the screening of larger proportions of the M3 plants. This will reduce the number of necessary back crosses for introgression of the mutation into advanced materials.

Conclusion

The set up of plant material as a TILLING resource, based on a the yellow seeded genotype presented in this study was successful. Due to complex homologies between the A- and the C-genome, and further high sequence homologies between the gene copies present in one genome, the development of specific primers still is ongoing. This will afford further optimisation of the TILLING protocol to speed up the detection process of mutants in the target genes and assess mutations in multiple genes within the same assay.

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