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# High throughput screening for detecting EMS mutations in oilseed rape (*Brassica napus* L.)

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## Abstract

We have developed two EMS (ethyl methanesulfonate) mutant populations of oilseed rape (*Brassica napus* L.), one from the spring type line 'YN01-429' and the second from the winter type cultivar 'Express 617'. We established a high throughput TILLING (Targeting Induced Local Lesions IN Genomes) protocol to detect mutations in two sinapine synthesis genes with the aim to select low sinapine content rapeseed mutants. Sinapine is an important antinutritive compound and prevents an extended use of the protein-rich extraction meal as animal feed or in human nutrition. We detected 135 missense and 13 non-sense mutations in the two seed-expressed copies of the *BnaX.SGT* gene and 162 missense, 3 nonsense and 7 splice site mutations in the two *BnaX.REF1* gene copies. The mutation frequencies ranged from 1/12 kb to 1/22 kb in the Express 617 population and from 1/27 kb to 1/60 kb in the YN01-429 population, respectively. Due to the presence of multiple paralogs, single non-sense mutations did not result in lower seed sinapine content. Crossing experiments between mutants are on the way to produce double mutants in which both paralogs are mutated.

*Keywords*

EMS mutagenesis, mutant population, point mutations, TILLING

## Introduction

Oilseed rape (*Brassica napus* L.) as the most important oil crop in temperate regions is grown for the production of biodiesel, animal feed and vegetable oil for human consumption. Sinapoylcholine (sinapine) is the major phenolic compound of *B. napus* seeds typically varying from 3 to 12 mg/g (ZUM FELDE et al. 2007). Due to this limited variation within the rapeseed gene pool, genetic modification or mutation induction targeting the sinapine metabolic pathway genes are methods of choice to breed low sinapine rapeseed. The biosynthesis of sinapine in *Brassicaceae* is well known and starts via the phenylalanine/hydroxycinnamate pathway (MILKOWSKI et al. 2004). We focused on two genes encoding key enzymes of the pathway, *SGT* (UDP-glucose:sinapic acid glucosyltransferase) and *REF1* (sinapaldehyde dehydrogenase/coniferaldehyde dehydrogenase) (HARLOFF et al. 2012). Unfortunately, due to the amphidiploid nature of rapeseed, most *Arabidopsis* genes have 2 to 8 paralogs thus complicating any gene knock-down strategy. Because seed sinapine is the target of our project we selected two seed expressed paralogs of either *BnaX.SGT* (MITTASCH et al. 2010) and *BnaX.REF1* (MITTASCH et al. 2013). In previous experiments using an RNAi approach reductions of 76 and 45% in the sinapine content were obtained by downregulating *BnaX.SGT* and *BnaX.REF1*, respectively (HÜSKEN et al. 2005, MITTASCH et al. 2013). As genetically engineered rapeseed is not accepted in the EU, we started a project to identify mutants with a loss of function in the above mentioned genes using TILLING (Targeting Induced Local Lesions IN Genomes) in chemically mutagenized rapeseed populations (*Figure 1*). This technique has been used before in *Arabidopsis*, maize, rice, oat and wheat (CHAWADE et al. 2010, GREENE et al. 2003, SLADE et al. 2005, WEIL and MONDE 2007).

## Material and methods

### *Plant material and EMS mutagenesis*

A Canadian yellow-seeded spring type inbred line 'YN01-429' ($F_8$) kindly provided by Prof. G. Rakow (AAFC Saskatoon, Canada) and a winter type inbred line 'Express 617' ($F_{11}$) derived from the German cultivar 'Express' were used in this study. Seeds were soaked in tap water for 12 h prior to 12 h EMS treatment. Winter type rapeseed plants with six leaves were vernalized at 4°C for 12 weeks.

### *DNA extraction and pooling strategy*

Leaf samples from $M_2$ plants were harvested for DNA extraction. Leaves from spring type plants were sampled in 2 ml Eppendorf tubes whereas the winter type leaves were sampled in 96 well plates. Genomic DNA was isolated from freeze-dried material (sample dry weights 20-50 mg spring and 10-20 mg winter type material) in a 96 microtiterplate format using a NucleoSpin® 96 Plant I Kit (Macherey and Nagel, Düren, Germany) and the TECAN Freedom Evo 200 Liquid Handling Robot (4×96 samples/day; TECAN GmbH, Crailsheim, Germany). DNA concentrations were measured in a Genios Microplate Reader (TECAN GmbH, Crailsheim, Germany) using Quant-It-Picogreen dsDNA Reagent (Invitrogen, Karlsruhe, Germany). Average DNA yields were 10.3 ('YN01-429') and 5.8 ('Express 617') µg DNA/sample which is sufficient for screening 50 and 24 amplicons, respectively. As the same kit was used for DNA isolation, differences in DNA yield were due to different amounts of leaf material.

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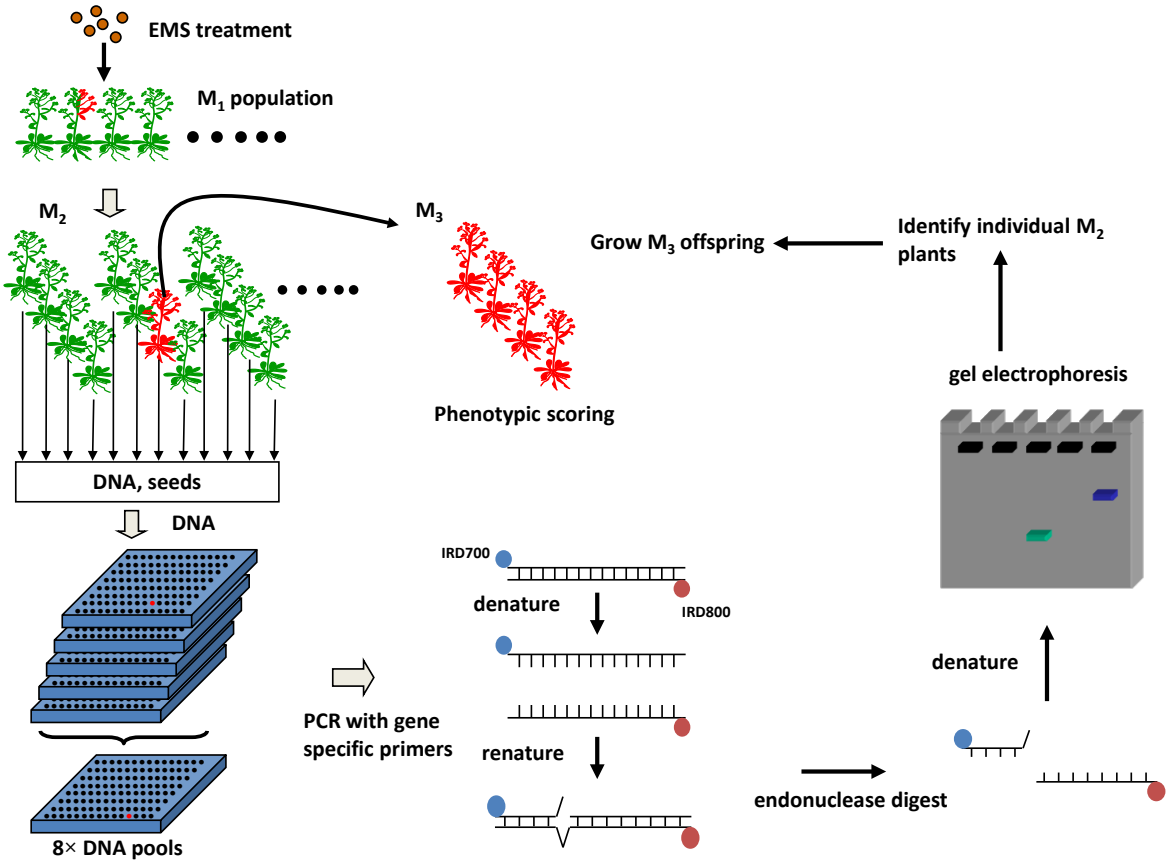


*Figure 1:* **Mutation screening by genotype: High throughput TILLING**

For normalization, DNA aliquots were diluted to a PCR-ready final concentration of 5 ng/µl and arranged in one-dimensional 4× pools for 'YN01-429' and two-dimensional (2D) 8× pools for 'Express 617'. In the 2D pools 4 microtiter plates with normalized DNA samples were combined in one pool plate with columns 1-6 containing the 8× column pool DNA and columns 7-12 containing the 8× row pool DNA. Due to this arrangement, every single sample is represented twice on a 96 lane LI-COR gel and can be directly identified by assignment of the lanes. As leaf sampling and DNA extraction were performed according to family-number with subsequent column-wise storage in the microtiter plate and 4× pools were built up by combining equivalent positions of 4 plates, 4× pools never contained sibling $M_2$ samples whereas in the 8× pools the column pools contained samples from 8 families (8×1), whereas the row pools contained samples from only 2 families (4×2). In the case of 1D-4× pools, we sequenced the respective amplicons of all four plants for mutant identification, whereas in the 2D-8× pool the mutant plant could be directly identified and only one amplicon had to be sequenced which greatly facilitated the mutant detection procedure.

## *Sequence information of the BnaX.SGT and BnaX.REF1 genes*

The coding sequence of *BnaX.SGT* (UGT84A9) is 1,494 bp in size organized in one exon, whereas both *BnaX.REF1* genes have 9 exons and 8 introns. Their genomic sequences are 3,977 and 3,973 bp in size with a coding sequence of 1,503 bp. Sequence data and copy numbers are based on BAC library screening, expression studies and Southern hybridization. The GenBank accession numbers for *BnaA.SGT.a* (UGT84A9b), *BnaC.SGT.a* (UGT84A9a), *BnaA.REF.a* and *BnaC.REF.a* are FM872285, FM872284, FN995990, and FN995991, respectively. In addition to BLAST analysis the software CLC Main Workbench (CLC bio, Aarhus, Denmark) was used for *in silico* sequence evaluation.

## *Primer design and PCR conditions*

Locus specific primers for the PCR amplification of the coding regions of these genes were designed with the program FastPCR© (KALENDAR et al. 2009) and tested with unlabeled and 5' labeled primers (IRD labels Dy-681 in the forward and Dy-781 in the reverse primers, Biomers, Ulm, Germany) according to the protocol of TILL et al. (2006). For PCR, we used a DYAD thermal cycler (MJ Research Inc., Waltham, MA, USA).

## *Heteroduplex analysis, fragment detection and calculation of mutation frequencies*

The CEL I enzyme was extracted from celery as described by TILL et al. (2006). Heteroduplex formation of the PCR product and digestion with CEL I were performed according to the same reference. Prior to loading to the gel, 2 µl of the digestion product were mixed with 2 µl formamide loading

dye and denatured for 3 min at 95°C. Aliquots of 0.3 to 0.5µl were applied to a 6.5% polyacrylamide gel (KB$^{Plus}$ Gel Matrix, LI-COR®, Bad Homburg, Germany) and separated on a LI-COR 4300 DNA Analyzer with double laser detection system for IR-labeled primers. The gel was run for 4:15 hours at 1,500 V, 40 mA and 40W. The fragments were analysed with the GelBuddy software (ZERR and HENIKOFF 2005). After the sample assignment of the fragments, mutations were identified by Sanger sequencing of the corresponding PCR products. Sequence analysis was performed using Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA) on a 3730xL DNA Analyzer (Applied Biosystems). Mutation frequencies F [1/kb] were calculated using amplicon sizes corrected by 100 bp for LI-COR gel border effects according to the formula:

$$F\,[1/kb] = 1 / \left( \frac{(amplicon\ size\ [bp] - 100) \times (number\ of\ M_1\ plants)}{(number\ of\ mutations) \times 1{,}000} \right)$$

## *Determination of sinapic acid metabolites*

The $M_3$ plants were grown in the greenhouse under 16 h light. $M_4$ seeds were harvested after bag isolation. Sinapine and sinapoylglucose were determined by HPLC as described in MILKOWSKI et al. (2004). If not otherwise indicated, single $M_4$ seeds were analysed. Sinapic acid ester equivalents (SAE) were determined in single seed extracts after alkaline hydrolysis for 3 h at 50°C in 5 N KOH (WOLFRAM et al. 2010).

# Results and discussion

## *EMS mutagenesis and development of TILLING populations*

The purpose of our study was to establish a mutant screening protocol for rapeseed by selecting mutations within two major genes of the sinapine biosynthesis pathway. Two different rapeseed lines were employed in this experiment. First we produced an EMS mutant population with the spring type rapeseed line 'YN01-429'. We used different concentrations of 0.5, 0.8, 1.0 and 1.2% EMS (*Table 1*). Survival rates in the $M_1$ generation dropped from 80% (0.5% EMS) to 50% (1.2% EMS). Therefore, higher EMS concentrations were avoided and concentrations between 0.5 and 1.2% were chosen for further studies.

According to different EMS treatments, the 'YN01-429' population was subdivided into two subpopulations. The 1$^{st}$ subpopulation consisted of 500 vigorous 'YN01-429' derived $M_1$ plants derived from 2400 EMS treated seeds (0.5 and 1.0% EMS). They were self-pollinated by bag isolation and $M_2$ seeds were harvested. Of each $M_2$ family, 4 plants were grown to avoid loss of mutant alleles due to the chimeric character of the $M_1$ plants. After bag isolation $M_3$ seeds were harvested from 1724 $M_2$ plants grown in the greenhouse. The 2$^{nd}$ subpopulation derived from 0.8 and 1.2% EMS treatments, consisted of 2833 vigorous $M_1$ plants. Bag isolation resulted in seeds from 2833 $M_2$ families. Three plants of each $M_2$ family were grown in the field and $M_3$ seeds of 3629 plants were harvested without bag isolation. Leaf material was taken and DNA was isolated from all 5361 plants of the 'YN01-429' spring rapeseed $M_2$ population. DNA samples of all treatments were later jointly investigated by TILLING.

For the production of a winter type rapeseed TILLING population, the 'Express 617' inbred line was treated with 1% EMS, resulting in an $M_1$ lethality rate of ~40%. A total of 2103 viable $M_1$ plants were obtained and seeds were harvested from 1902 $M_1$ plants. A total of 7608 $M_2$ plants (4 plants/family) were grown in the greenhouse and $M_3$ seeds were harvested from 6775 $M_2$ plants. DNA was isolated from 3488 $M_2$ plants representing 940 $M_1$ plants. Leaf samples of another 3732 $M_2$ plants were frieze-dried and stored for further use (*Table 1*).

## *Primer design and CEL I digest*

TILLING in *B. napus* is hampered by the fact that many sequences exist as paralogs and orthologs with high sequence similarity among each other. Therefore, we designed locus specific primers which cover gene regions coding for functional domains of the polypeptide. The amplicon size should not exceed 1500 bp due to resolution and background of the LI-COR gels (TILL et al. 2006). Primers were carefully tested prior to TILLING: first, a so-called PCR crash test was carried out with primer pairs and single primers from paralog and ortholog loci. Suitable primer combinations should not

*Table 1:* **Features of the rapeseed TILLING platform at the University of Kiel (HARLOFF et al. 2012)**

| Population | YN01-429 | | Express 617 |
|---|---|---|---|
| Number of seeds | 2400 | 5000 | 3120 |
| EMS concentration | 0.5%, 1% | 0.8%, 1.2% | 1% |
| Number of $M_1$ plants | 2000 | 3980 | 2103 |
| Number of $M_1$ plants with seed set | 500 | 2833 | 1902 |
| Number of seeds per $M_2$ family | 4 | 3[a] | 4 |
| Number of $M_2$ plants | 2000 | 3860 | 7608 |
| Number of selfed $M_2$ plants | 2000 | 0 | 7608 |
| Open pollinated $M_2$ plants | 0 | 3860 | 0 |
| Leaf samples, DNA extraction | 1905 | 3456 | 3488[c] |
| $M_1$ plants represented | 500[b] | 1500[b] | 940 |
| DNA samples in $M_2$ population | 5361 | | 3488 |

[a] 8499 seeds were sown in the field, only 3860 $M_2$ plants survived
[b] estimated values of represented $M_1$ plants due to combination of different subsets
[c] only half of the leaf material was extracted, leaf material of 3732 additional $M_2$ plants is available (originating from another 950 $M_1$ plants)

give any non-specific amplicons visible as additional bands or smear after gel electrophoresis. Second, the obtained PCR products were sequenced to confirm locus specificity and third, the PCR was repeated with IRD labeled primers. *Figure 2* shows the genomic structure of the target genes and the location of the amplicons (HARLOFF et al. 2012). All primers were 20 to 30 nucleotides in size with melting temperatures of 60-65°C to avoid interaction between the two IRD labels during the PCR reaction. It was known that four *BnaX.SGT* loci are present in the *B. napus* genome, but only two of them (*BnaA.SGT.a* and *BnaC.SGT.a*) are expressed in ripening seeds (MITTASCH et al. 2010). Therefore, we designed three locus specific primer combinations for *BnaA.SGT.a* and *BnaC.SGT.a* which gave rise to amplicons in a range between 1270 and 1420 bp covering between 85 and 95% of the coding sequence (HARLOFF et al. 2012).

Two *REF1* homologues (*BnaA.REF1.a* and *BnaC.REF1.a*) had been discovered in the rapeseed genome (MITTASCH et al. 2013). For each locus, primers were designed for two amplicons with sizes between 943 and 1361 bp. Together, they cover 84% of the coding sequence including 7 out of 9 exons.

Apart from primer design an optimized CEL I digest in combination with a refined pooling strategy is critical for successful mutant detection. We performed a number of heteroduplex digestion experiments with varying amounts of CEL I enzyme to determine the optimal signal-to-noise ratio after LI-COR gel electrophoresis (data not shown). An existing SNP within *BnaC.SGT.b* between 'YN01-429' and 'Express 617' served as a positive control as two fragments became visible after CEL I digestion of the mixed amplicons.

## *Detection and characterization of EMS mutations*

We used 4× and 2D-8× pooling strategies to screen the spring type and winter type populations, respectively. Our protocol enabled the detection of TILLING fragments, even in the case of a low signal-to-noise ratio. Fifty to 80% of the polymorphic fragments identified after gel electrophoresis indicated real point mutations as verified by Sanger sequencing. The position of the SNP matched the position of the CEL I cleavage with a precision of 5-20 bp. In the 8× pools no effect of increased effective concentrations of mutant alleles in the 2D row pools containing sibling samples was observed.

TILLING of four sinapine genes resulted in a total of 683 mutations which were later verified by Sanger sequencing (*Table 2*). As in some cases the same mutation was found in more than one plant of the same $M_2$ family we corrected the number of heritable germ line mutations by counting those mutations only once in each family. This resulted in a total of 570 different transitions by subtracting 113 mutations within one and the same family (HARLOFF et al. 2012). The mutation frequencies varied between the diffe-

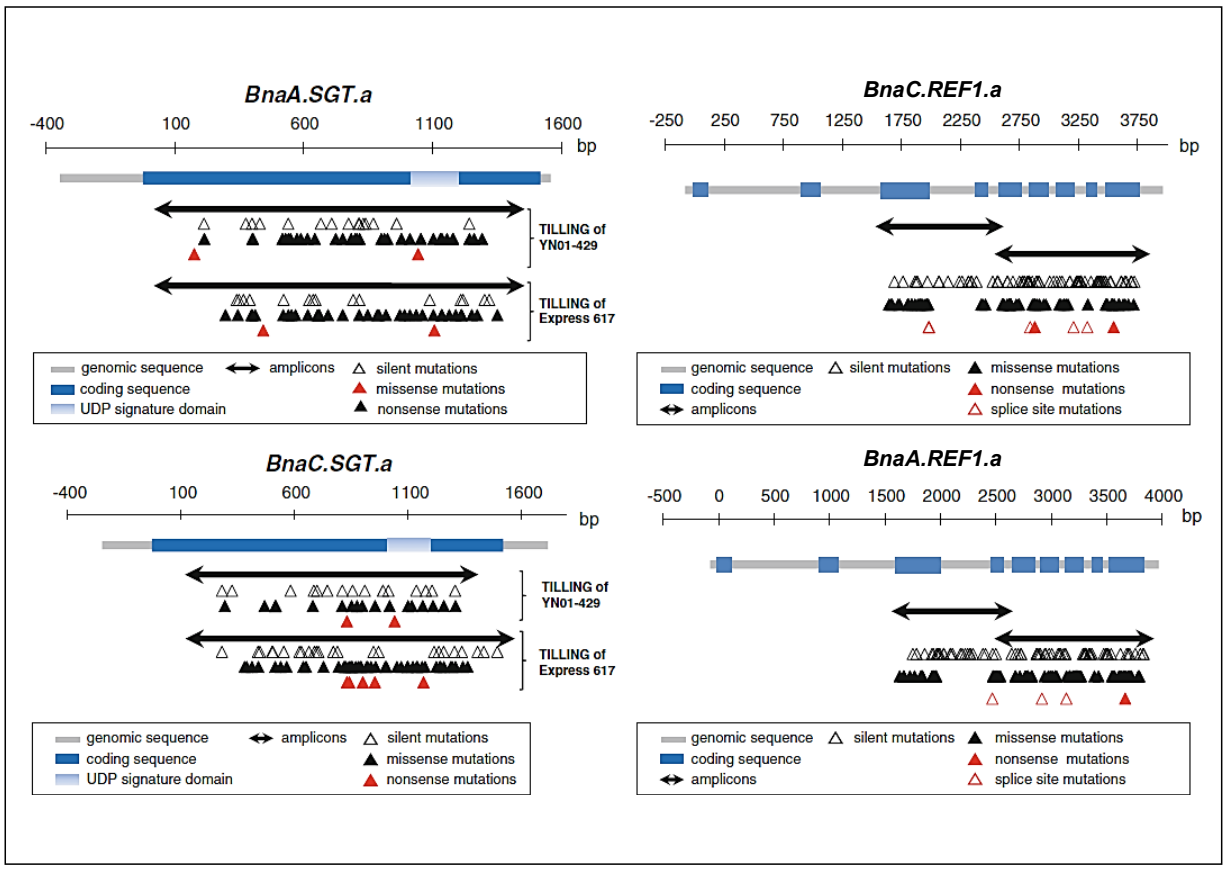


*Figure 2:* **Gene structure of *BnaX.SGT* and *BnaX.REF1* genes and mutations detected by TILLING (HARLOFF et al. 2012)**

*Table 2:* **TILLING with sinapine biosynthesis gene sequences. For *BnaX.REF1* values of both amplicons were added. Mutation frequencies were calculated as number of mutations/$M_1$ plant which was determined by analyzing the $M_2$ families (HARLOFF et al. 2012)**

| Gene | ORF (bp) | % covered by TILLING | LI-COR fragments detected | Mutations verified by sequencing | Mutations verified by $M_3$ analysis | $M_3$ phenotypes | Mutation in $M_1$ | Mutations frequency [1/kb][a] | Mutations /1000 $M_1$/1000 bp |
|---|---|---|---|---|---|---|---|---|---|
| YN01-429 | | | | | | | | | |
| *BnaA.SGT.a* | 1494 | 94 | 95 | 61 | 14 | 2 | 55 | 1/27[b] | 20 |
| *BnaC.SGT.a* | 1494 | 85 | 82 | 39 | 13 | 3 | 39 | 1/60[c] | 15 |
| Express 617 | | | | | | | | | |
| *BnaA.SGT.a* | 1494 | 94 | 107 | 60 | 20 | 1 | 56 | 1/22 | 42 |
| *BnaC.SGT.a* | 1494 | 95 | 127 | 90 | 6 | 1 | 79 | 1/16 | 59 |
| *BnaX.REF1.a* | 1503 | 84 | 250 | 205 | 4 | 0 | 164 | 1/12 | 77 |
| *BnaX.REF1.b* | 1503 | 84 | 291 | 228 | 3 | 0 | 177 | 1/12 | 83 |

[a] amplicon correction for LI-COR gel border effects by 100 bp
[b] screening of 1140 $M_1$
[c] screening of 2000 $M_1$

rent sequences investigated. We found the lowest mutation frequency within the spring rapeseed population (*BnaC.SGT.a*, 1/60 kb) and the highest frequency within the winter rapeseed population (*BnaX.REF1.a*, 1/12 kb). Average mutation frequencies within the *BnaX.SGT* and *BnaX.REF1* genes were calculated as 1/31 kb and 1/12 kb, respectively. Differences were also found between both populations with lower frequencies in the spring population.

Then we grouped the mutation events into distinct classes to address the question whether EMS mutations are randomly distributed across the different genes. It has been reported that only a limited number of nucleotide triplets can be changed by EMS treatment, with guanine being the predominant target of ethylation (STEPHENSON et al. 2010). Thus, the frequency of mutagenic events should be correlated to the frequency of G on both strands or with the frequency of G/C (G+C) on the coding strand. Accordingly, 99.3% of our mutations were G/C→A/T transitions while only 0.7% (4 among all 570 mutations) were non-G/C→A/T transitions. We classified the mutations in relation to the number of G/C residues (i.e. the maximum number of EMS targets) excluding the 4 non-G/C→A/T transitions. The frequency of mutated G/C residues ranged between 6 % (*BnaX.SGT*) and 20% (*BnaX.REF1*). Moreover, no apparent strand selectivity could be found, with the ratio of G→A to C→T transitions in most cases almost equaling the G/C ratios in the codon strand.

We further calculated the frequency of multiple mutations within one gene (>1 mutation/kb/$M_2$ plant) with regard to the total number of mutations. It ranged between 3-4% for *BnaC.SGT.a* (disregarding double/triple mutations for *BnaA.SGT.a*) and 2-5% for *BnaX.REF*. We also calculated the average number of mutations per single plant by multiplying mutation frequencies by genome size (2258 Mbp/2C; ARUMUGANATHAN and EARLE 1991) and corrected for an estimated average G/C content in *B. napus* of 35.7% (as a mean of 36.0% in *B. oleracea* (TOWN et al. 2006) and 35.4% in *B. rapa* (TRICK et al. 2009). As a result, the number of mutations/plant in the 'YN01-429' and in the 'Express 617' EMS population were 40000 and 130000, respectively.

## *Missense and non-sense mutations within sinapine genes*

We found a number of putative loss-of-function mutations that cause amino acid changes (missense), stop codons within coding regions (non-sense) or splice site mutations at intron borders. We detected 16 stop codon mutants (2.8%) and 8 splice site mutations (2.3%) with G→A exchanges at the 5' and 3' ends of the introns. Those mutations should result in non-functional enzymes which are expected to have an impact on sinapine content.

We harvested $M_3$ seeds from all $M_2$ mutant plants. In a first step, we selected 14 *BnaA.SGT.a* and 13 *BnaC.SGT.a* mutants from the spring rapeseed population and 4 *BnaA.SGT.a* and 5 *BnaC.SGT.a* mutants as well as 4 *BnaX.REF1.a* and 3 *BnaX.REF1.b* mutants from the winter rapeseed population with promising base pair transitions as described above. We aim to select homozygous $M_4$ offspring for crossing and phenotyping experiments. Homozygous plants were found for all stop codon mutations clearly demonstrating that loss of one gene alone did not seem to have a deleterious or even lethal effect because those plants showed a normal growth habit.

We did first experiments with $M_4$ seeds to analyze the contents of sinapine, sinapoylglucose and sinapic acid equivalents by HPLC. For these measurements we chose two segregating families (winter types 101612 and 101650), indicating that the parents were heterozygous. No significant reductions could be observed and clearly demonstrate that the knock-down of only one of two seed-expressed genes was not sufficient to produce a measurable effect.

As we expect drastic reductions of sinapine contents after down regulation of both seed-specific *BnaX.SGT* or *BnaX.REF1* genes, crossings of homozygous stop codon and splice site mutants in the *BnaX.SGT* and *BnaX.REF1* genes from the spring and winter rapeseed population have been performed in order to combine two loss-of-function mutations in one plant. First results with double mutants point at drastic reductions of sinapine contents in seeds.

## *Comparisons between different TILLING platforms*

TILLING platforms have been established for a number of plants. The main features of TILLING platforms are the number of $M_2$ families represented by their DNA samples and the availability of $M_{2:3}$ seeds. Their efficiency relies mainly on (1) the mutation frequency, (2) the number of $M_2$ plants jointly tested in an experiment (pooling strategy), and (3) the costs for DNA extraction, enzyme reactions and fragment analysis. Here, we will address these questions comparing our results with previously published TILLING protocols. The spring and winter type rapeseed TILLING platforms presented here are open for scientists to screen their sequences in our institute.

The $M_1$ mutation frequency is a critical parameter for TILLING. It depends on the species and the target tissue, the mutagen, the developmental stage of the mutagenic treatment and the mutagen concentration. Typically, mutation frequencies are measured in the $M_2$ generation which is derived from selfed $M_1$ plants. Further generations can be produced by single seed descent with an increased number of families or, to avoid loss of mutations, small sized $M_2$ families are grown (SUZUKI et al. 2008, RIGOLA et al. 2009, STEPHENSON et al. 2010).

In our winter rapeseed population, we measured an average mutation frequency of 1/15 kb, which is higher as reported for most *Brassicaceae* like *Arabidopsis* (1/170 kb, GREENE et al. 2003), *B. napus* (1/130 kb and 1/42 kb, WANG et al. 2008), *B. oleracea* (1/447 kb, HIMELBLAU et al. 2009) or *B. rapa* (1/30 kb, STEPHENSON et al. 2010). Our mutation frequency is comparable to hexaploid (*Triticum aestivum*, 1/24 kb) or tetraploid (*T. turgidum* subsp. *durum*, 1/40 kb) wheat (SLADE et al. 2005) or oat (1/20 and 1/40 kb, CHAWADE et al. 2010) suggesting that polyploids can tolerate a higher mutation load due to gene redundancy. This is also a reason to use the comparatively high EMS concentration of 1% for mutagenesis resulting in 130000 mutations/plant in our 'Express' population. The number of mutations was substantially lower in the 'YN01-429' population, however, with different EMS concentrations (0.5-1.2% EMS; 40000 mutations/plant). Likewise, much lower mutation frequencies have been reported for EMS treated populations of *B. rapa* 'R-o-18' (2C=2n) (0.3/0.4% EMS; 20000 mutations/plant, STEPHENSON et al. 2010) and *B. napus* 'Ningyou7' (0.6% EMS; 29000 mutations/plant, WANG et al. 2008).

Three *Brassica* TILLING platforms have been published so far. They differ substantially from our TILLING platform with regard to size and screening efficiency. Mutant populations of the diploid species *B. rapa* and *B. oleracea* were screened by a standard 4× or 5× pooling strategy (HIMELBLAU et al. 2009, STEPHENSON et al. 2010). To avoid the selection of locus specific primer combinations, WANG et al. (2008) screened single DNA samples of a *B. napus* $M_2$ population subtracting natural SNPs for mutant detection. In contrast, we applied a 2D-8× pooling strategy in combination with locus specific primers. This protocol is much more efficient for gel based mutant detection, as it drastically reduces the scoring of false positive fragments due to background and *Taq* polymerase error rate and it enables mutation detection of all orthologous or paralog sequences of a polyploid genome.

The method of choice for accurate measurement of mutation frequencies throughout the whole genome seems to be re-sequencing of EMS mutants and wild type. Here, we were able to show by indirect means, i.e. correlation of mutation events to G/C residues, that there is strong evidence for a random distribution of mutations within genes. We did not find any evidence for individual hotspots for EMS mutations in the *B. napus* genome.

Some recent publications describe alternative techniques for detecting mutations in large populations of tomato (*Solanum lycopersicum*) like Conformation Sensitive Capillary Electrophoresis (CSCE), High Resolution DNA Melting Analysis (HRM) (GADY et al. 2009), and Next Generation Sequencing (NGS) (RIGOLA et al. 2009, TSAI et al. 2011). All these technical alternatives have in common that they avoid the laborious CEL I digestion and LI-COR gel electrophoresis and offer a higher and faster sample throughput. However, with one exception (TSAI et al. 2011) the sequences to be analyzed were much smaller (<400-600 bp) which requires the development of a two to three times higher number of locus specific TILLING amplicons to attain the same gene coverage in a candidate gene. GADY et al. (2009) found an average mutation frequency of 1/737 kb after screening an $M_2$ population (1% EMS) of *Solanum lycopersicum* by CSCE and HRM. However, they detected a high percentage of false positives which required much proof reading and re-screening activity. In conclusion, they regarded the 'classical' LI-COR method to be more sensitive. This was in line with the NGS technique using the GS FLX 454 (RIGOLA et al. 2009) where a lower mutation rate (1/431 kb) was found as compared to the classical approach (1/322 kb, MINOIA et al. 2010). Another technical improvement has been described recently by TSAI et al. (2011) who used TILLING amplicons <1500 bp for DNA library construction followed by Illumina sequencing based mutation detection. Their results were in line with 'classical' screening methods. However this study was suffering from a very small population size of only 768 plants tested, an underrepresentation of GC rich regions after Illumina sequencing and a loss of rare heterozygous mutants due to statistical noise. The advantage of a faster screening procedure by NGS contrasts to an increased statistical and bioinformatics input to analyze the sequence reads. In addition, our TILLING method is still very cost-effective allowing the screening of 3500 plant DNA samples (1500 bp amplicon) for ~1000 €. Notwithstanding, further technical improvement is needed in the future to facilitate mutant detection in large populations.

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***Note:*** *The online version of the proceedings includes figures in colour. It can be either downloaded from the homepage of the meeting (http://www.raumberg-gumpenstein.at/ - Downloads - Fachveranstaltungen/Tagungen - Saatzüchtertagung - Saatzüchtertagung 2013) or requested from the corresponding author.*