Towards low sinapine oilseed rape

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Abstract

Seeds of oilseed rape (*Brassica napus*) accumulate high amounts of antinutritive sinapate esters (SE) with sinapoylcholine (sinapine) as major component. These phenolic compounds are regarded as antinutritive, thus compromising the use of rapeseed meal as animal feed and excluding the valuable rapeseed protein from the human food market. Hence, the reduction of SE content is a substantial requirement for establishing oilseed rape as a protein crop. Sinapine is formed via a two-step biosynthesis that involves conversion of sinapate to sinapoylglucose by the enzyme UDP-glucose:sinapate glucosyltransferase (BnSGT1; UGT84A9) and a subsequent transacylation catalyzed by sinapoylglucose:choline sinapoyltransferase (BnSCT). Silencing of BnSGT1 by double-stranded RNA interference (dsRNAi) resulted in a marked reduction of SE content in seeds. Likewise, the seed-specific silencing of SCT and PAL (phenylalanine ammonia lyase), the enzyme marking the entry point into plant phenylpropanoid metabolism, led to low sinapine phenotypes in *B. napus*. To assess the number of BnSGT1 loci in the B. napus genome, a BAC library was screened. BAC analyses revealed that the haplotype of B. napus cv. Express is characterized by four BnSGT1 loci. This should be considered in designing TILLING approaches.

Key words: *B. napus*, sinapine, sinapate esters, dsRNAi, *BnSGT1* alleles

Introduction

Sinapoylcholine (sinapine) is the major phenolic seed component in *Brassica napus* that accumulates to values of up to 1% of dry weight. It is regarded as a major antinutritive factor preventing the well-balanced seed protein from being used as a possible source for food-grade supplement (Kozlowska et al. 1990; Shahidi and Naczk 1992). Hence, by reducing the sinapine content, oilseed rape could be established as a protein crop. Biosynthesis of sinapine proceeds via sinapic acid that is provided by the general phenylpropanoid pathway which starts with the conversion of the amino acid phenylalanine to cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL). Sinapic acid is activated by the enzyme UDP-glucose:sinapic acid glucosyltransferase (BnSGT1; UGT84A9) and then re-conjugated with choline by sinapoylglucose:choline sinapoyltransferase (BnSCT).

Our work is aimed at identifying target genes for sinapine reduction. For this reason, *B. napus* is transformed with hairpin constructs mediating seed-specific dsRNAi suppression of key biosynthetic genes of the sinapine metabolic pathway. Seed-specificity of this approach is based on expression of the hairpin cassette from a promoter that drives the expression of the seed storage protein napin (*napC*; Kridl et al. 1991). This approach allows for testing candidate genes whose constitutive silencing would potentially provoke deleterious effects on the pants. Here we describe the seed-specific silencing of genes involved in sinapine biosynthesis by stable transformation of *B. napus* with dsRNAi-mediating hairpin constructs. We used constructs designed to silence *BnPAL*, *BnSGT1* and *BnSCT*. Since suppression of *BnSGT1* leads to a substantial decrease of total sinapate ester content (Hüsken et al. 2004; Baumert et al. 2005), this gene is regarded as a promising target for the TILLING approach. To provide information for TILLING strategies we isolated genomic *BnSGT1* fragments from a *B. napus* BAC library and characterized alleles and loci found in the amphidiploid genome of *B. napus*.

Material and Methods

Plant material

Spring oilseed rape (*Brassica napus* L.) cv. Drakkar and Lisora was used. Plants were cultivated in the greenhouse at 20 °C under a 16 h light regiment with a photon flux density of 200-900 µmol/m2/s.

Vector construction

To generate the dsRNAi constructs (Fig. 1), cDNA subfragments ranging from 200 to 400 bp were PCR-amplified and successively cloned in antisense and sense orientation resulting in a suppression cassette which consists of the *napC* promoter (Kridl et al. 1991), the target sequence in antisense and sense orientation interrupted by a subfragment of the bacterial *uidA* gene (’GUS; Chuang and Meyerowitz 2000) as spacer element and the *nos* terminator. The suppression cassettes were transferred to the binary vector pLH7000 (Hausmann and Töpfer 1999).

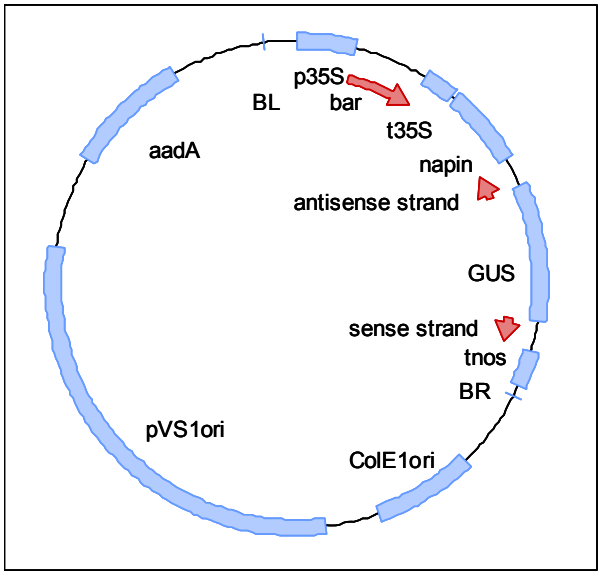

Fig. 1 Schematic map of dsRNAi vectors used for *Agrobacterium*-mediated transformation of *B. napus*. T-DNA elements are BL, left border; p35S, cauliflower mosaic virus 35S promoter; bar, *bar* gene encoding phosphinothricin acetyltransferase; t35S, cauliflower mosaic virus 35S terminator; napin, *napC* promoter; GUS, partial sequence of *E. coli uidA* gene; antisense and sense strand, target sequences of genes to be silenced in both orientations; tnos, terminator of *Agrobacterium tumefaciens* nopaline synthase gene; BR, right border.

Transformation

Agrobacterium-mediated transformation and plant regeneration were done as described previously (De Block et al. 1989). Transgenic plants were selected by phosphinothricin (PPT) resistance mediated by the *bar* gene of the inserted T-DNA. The copy number of T-DNA insertions was determined by Southern Blot analysis of genomic DNA from T1 plants with the 'GUS fragment as probe and by T2 segregation for PPT resistance. T3 segregation was used to isolate homozygous lines (Hüsken et al. 2005).

Analysis of sinapate esters

The two major sinapate esters from seeds, sinapoylglucose and sinapine, were identified by HPLC and spectroscopic comparison with standard compounds (Hüsken et al. 2005).

BAC library screening

A genomic BAC library of *B. napus* cv. Express was screened by hybridization with α-^{33}P-dATP labeled cDNA probes of *BnSGT.*

Southern Blot analysis

DNA restriction, electrophoretic separation of fragments and transfer to Hybond-NX membranes (Amersham Biosciences, Freiburg, Germany) was done according to standard protocols (Sambrook et al. 1989). Hybridization was performed with DIG-labeled probes. Labeling was done by PCR using the DIG-Hy-Prime Kit (Roche, Mannheim, Germany). For signal detection, the DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) was used.

Results and Discussion

Suppression of genes involved in sinapine biosynthesis

From *B. napus* transformants harboring dsRNAi suppression cassettes to silence *BnSGT1*, homozygous lines with single copy insertions have been developed (Hüsken et al. 2005). Seed-specific silencing of *BnSGT1* resulted in seed sinapate ester (SE) contents of 24% to 42% of the non-transformed control plants (Tab. 1). This low-sinapine phenotype was shown to be stable up to the fifth transgenic generation (T5).

Table 1. Total SE (mg/g) content in T5 seeds of *BnSGT*i and control plants

Line	n	mean	SD
3	48	2.5	0.35
8	49	3.5	0.97
13	42	2.2	0.50
14	48	3.0	0.38
23	49	2.8	0.89
44	45	2.0	0.33
WT	94	8.3	0.31

With a double suppression construct designed to silence *BnSGT1* and *BnSCT* simultaneously, transgenic lines with single copy insertions were generated and subject to SE analysis in T2 seeds. Three lines displaying SE reduction of up to 70% in single T2 seeds were selected for further propagation and segregation analysis to develop homozygous material.

Transformation of *B. napus* with a construct for silencing *BnSCT* led to the regeneration of 20 transgenic T1 plants. Of these, five (25%) had T2 seed SE contents below the levels found in control plants (Fig. 2). *BnSCT*-suppressing plants were characterized by accumulating moderate amounts of SE. Strongest suppression was indicated by a SE content accounting for about 70% of the amount accumulated by control plants.

Seed-specific suppression of *BnPAL* resulted in a recovery rate of 96% of silenced plants displaying SE contents below the control levels in T2 seeds. The best performing line was shown to accumulate about 44% of the SE content found in the lowest control plant.

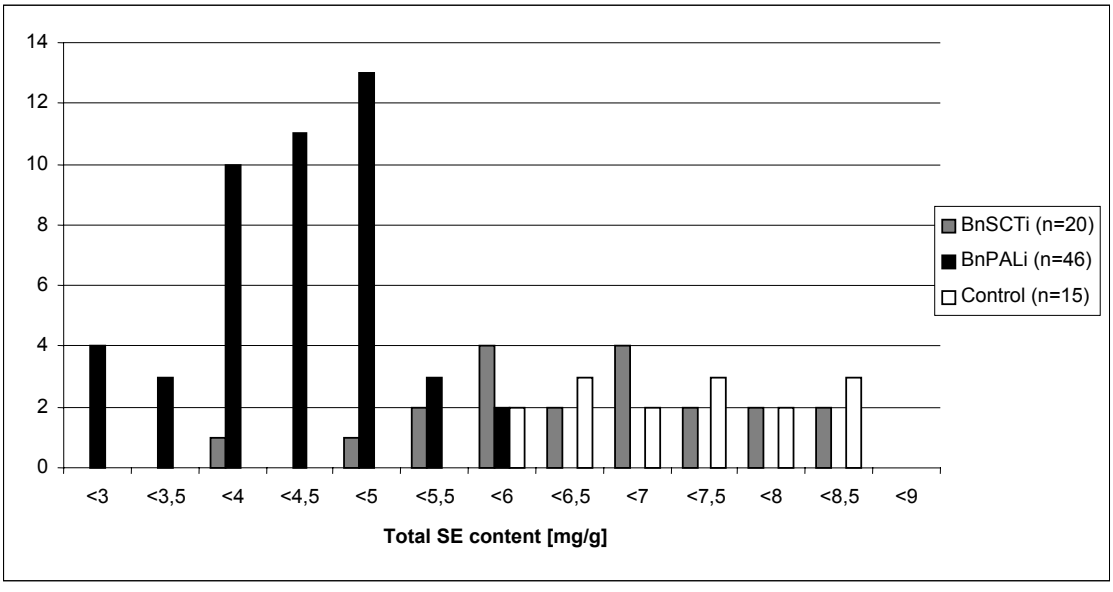

Fig. 2: Frequency distribution for the SE content in T2 seeds of *BnSCT*i, *BnPAL*i and control plants

Genomic organization of BnSGT1

Hybridization screening of a genomic BAC library from *B. napus* cv. Express with the *BnSGT1*-cDNA as probe resulted in the isolation of 53 BAC clones. A PCR approach designed to amplify the full length *BnSGT1* reading frame revealed 38 BAC clones carrying the entire *BnSGT1* coding sequence. By sequence analysis, the PCR products could be assigned to two subgroups differing in 11% of sequence positions. Southern Blot analyses of *BnSGT1*-BAC clones indicated two different hybridization patterns for each of these *BnSGT1* sequence variants. These results reveal that the haplotype of *B. napus* cv. Express is characterized by four *BnSGT1* loci.

Conclusions and perspectives

Seed-specific dsRNAi silencing via stable integration of suppression cassettes encoding hairpin RNA has proven an effective means to reduce the content of antinutritive SE in *B. napus* seeds. From transgenic plants, homozygous lines were developed with stable low amounts of SE. Driven by the *napC* promoter, the suppression state was restricted to developing seeds and the early seedling stages. Hence, dsRNAi suppression is regarded a useful tool to introduce the low sinapine trait into oilseed rape. On the other hand, the suppression approach has proven useful to define target genes for reduction of seed SE content. Based on sequence information from Arabidopsis we started a systematic approach to amplify seed ESTs from *B. napus* for enzymes of the phenylpropanoid pathway like cinnamate-4-hydroxylase (*REF3*), coumaroyl-CoA:shikimate coumaroyltransferase (*CST*), coumaroylshikimate-3-hydroxylase (*REF8*) and sinapaldehyde dehydrogenase (*REF1*) (Ruegger and Chapple 2001; Schoch et al. 2001; Franke etal. 2002; Nair et al. 2004). These sequences are used to develop seed-specific dsRNAi suppression cassettes to be applied for stable transformation of *B. napus*. This strategy will lead to transgenic oilseed rape lines suppressing defined steps of SE biosynthesis in a seed-specific manner. These lines allow evaluation of target genes to be applied in breeding of low sinapine oilseed rape cultivars. To increase the efficiency of the dsRNAi suppression approach that is calculated as recovery rate of silenced plants in the T1 generation, an intron in splicing orientation should be used as spacer element in the constructs instead of the '*GUS* fragment (Smith et al. 2000; Stoutjesdijk et al. 2002).

TILLING approaches in the amphidiploid oilseed rape will benefit from *a priori* information on alleles and genomic loci of the gene of interest. Since the suppression approach has defined *BnSGT1* as major target gene for breeding of low sinapine lines, future work will include allele characterization with regard to sequence polymorphisms, number of genomic loci and expression regulation.

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