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Improvement in the quality of seed storage protein by transformation of *Brassica napus* with an antisense gene for cruciferin

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Abstract The levels of certain essential amino acids, in particular cysteine, lysine and methionine, in the seed storage protein of a commercial spring variety of rape, *Brassica napus*, have been increased by the introduction of an antisense gene for cruciferin, which is the most abundant storage protein in rapeseed. The antisense construct contained part of the *cruA* gene in an inverted orientation, and the gene was driven by the 5′ flanking region of the gene for napin such that antisense RNA was expressed in a seed-specific manner. The construct was introduced by *Agrobacterium*-mediated gene transfer. In self-pollinated seeds (T1 seeds) of transgenic plants there was a reduction in the levels of the $\alpha 1\beta 1$ and $\alpha 2/3\beta 2/3$ subunits of cruciferin, whereas the level of the $\alpha 4\beta 4$ subunit was unchanged. The total protein and lipid contents of transgenic seeds did not differ significantly from that of normal seeds. Seeds with reduced amounts of cruciferin accumulated higher amounts of napin than non-transformed seeds, but the level of oleosin was unaffected. Amino-acid analysis of the seed storage protein revealed that T1 seeds with reduced amounts of cruciferin contained higher relative levels of three essential amino acids, namely, lysine, methionine and cysteine, with increases of 10%, 8% and 32% over the respective levels in non-transgenic seeds (*B. napus* cv Westar).

Key words *Brassica napus* · Cruciferin · Antisense · Seed storage protein · Amino-acid composition

Introduction

A great deal of efforts has been devoted to attempts at improving the quality and quantity of seed storage proteins by conventional breeding in cereals (Payne 1983) and soybean (Kitamura 1993). In the case of oilseed rape, however, breeding for protein quality has been limited and most efforts have concentrated on reducing the levels of glucosinolates. The levels of certain essential amino acids, in particular methionine, cysteine and lysine, are frequently inadequate, not only in cereals but also in oilseed rape, for both human and animal nutrition (Krebbers 1990).

With the development of genetic engineering in plants, several reports have described improvements in the quality and quantity of seed storage proteins by genetic transformation (Radke et al. 1988; Kerckhove et al. 1989; Clercq et al. 1990; Guerche et al. 1990; Altenbach et al. 1992; Knutzon et al. 1992; Müller-Röber et al. 1992; Voelder et al. 1992; Iida et al. 1993; Shimada et al. 1993). For example, Altenbach et al.(1992) introduced the gene for the sulphur-rich 2*s* albumin of Brazil nut into *Brassica napus* in an attempt to modify the nutritional value of the seed protein, and the seed protein of transgenic plants contained up to 33% more methionine than the controls.

Recently, antisense genes for various proteins have been introduced into plants to investigate the biological functions of the products of the corresponding sense genes or to improve economically important characteristics (Krol et al. 1988; Mol et al. 1990). As compared to classical mutagenetic methods, one of the advantages of the antisense method is that expression of the target gene can be inhibited without any effect on other genes.

Seeds of *B. napus* accumulate seed storage proteins during development of the embryo and these proteins account for about 20% of the seed weight in the mature seed (Fowler and Downy 1970; Finlayson 1976). Cruciferin is a 12*s* seed storage protein that accounts for 60% of the total seed protein of *B. napus* (Crouch and Sussex 1981) and it contains lower levels of methionine and cysteine than the second most prominent seed storage protein, napin (Simon et al. 1985). Cruciferin is composed of six subunits and each subunit contains two chains, α and β, encoded by one gene (Schwenke et al.

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1981). Four different subunits exist, namely $\alpha1\beta1$, $\alpha2\beta2$, $\alpha3\beta3$ and $\alpha4\beta4$. The $\alpha2$ and $\alpha3$ chains do not differ from each other in terms of molecular weight, and $\alpha2\beta2$ and $\alpha3\beta3$ subunits are the most abundant in the cruciferin hexamer. The genes coding for each cruciferin subunit form a multigene family and the homology between the genes for the respective subunits is about 60% (Rödin and Rask 1990). Here, we report the effects of an antisense gene for cruciferin on the accumulation of cruciferin and other storage proteins. The cruciferin content of seeds was reduced by the introduction of the antisense gene and the reduction was balanced by an increase in napin content. This change resulted in an increase in the relative levels of essential amino acids in the seed storage proteins.

Materials and methods

Construction of plasmids and transformation of *B. napus* cv Westar

The promoter region of a gene for napin and the coding region of a gene for cruciferin, used for the antisense construction, were amplified by PCR. The amplified promoter was the region of the *napA* gene from position 1 to 1145 (Joseffson et al. 1987). The primers for amplification of the cruciferin coding region corresponded to nucleotides in the *cruA* gene (Ryan et al. 1989) from position 680 to 700 for the 5′ oligonucleotide, and from position 1261 to 1278 for the 3′ oligonucleotide. The amplified cruciferin-coding sequence contained a region from the first exon to part of second exon of the *cruA* gene. To make the antisense construct, the amplified cruciferin-coding region was fused to the napin promoter in inverse orientation and the fragment was ligated to the terminator of the gene for nopaline synthase (NOS) of pBI221 (Clontech Lab. Inc., Palo Alto, Calif.). The expression cassette was integrated into pLAN411 (Uematsu et al. 1991) to produce pNACR (see Fig. 1). *Agrobacterium*-mediated transformation was performed by the methods reported elsewhere (Kohno-Murase et al. 1994).

Analysis of seed proteins by SDS-PAGE

A single grain, or half of a single grain, was homogenized with a mortar and a pestle in 50–100 µl of a protein-extraction buffer that contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.0001% bromo-phenol blue, the left for 10 min on ice. After centrifugation (17000 g, for 10 min), 1 µl of the supernatant was used for determination of total protein content with a Protein Assay Kit (Bio-Rad Laboratories, Richmond, Calif). Twenty micrograms of protein were fractionated by SDS-PAGE in the discontinuous buffer system of Laemmli (1970), on slab gels that contained 12% and 20% acrylamids, for analysis of the levels of cruciferin and napin, respectively. After electrophoresis, each gel was stained with Coomassie Brilliant Blue R-250.

Survey by PCR of the introduced antisense gene for cruciferin

A seed was cut into two halves. DNA was extracted from the seedlings germinated from the half seed by the method of Walbot and Warren (1988). The extracted DNA was used as a template for amplification by PCR of the introduced antisense gene. PCR was performed for 25 cycles in 25 µl of enzyme buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatin) that contained 1 mM of each dNTP, 1 pmol of each primer (which corresponded to the 19 bp from position 942 to 960 of the *cruA* gene, and to the 19-bp primer with a sequence complementary to the 3′ end of the NOS terminator) and 1 unit of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.). The programmed cycle for PCR consisted of a denaturation step at 94 °C for 15 s, an annealing step at 45 °C for 15 s, and an extension step at 72 °C for 30 s to 1 min.

Western-blotting analysis

Proteins in seed extracts were separated by electrophoresis in a slab gel that contained 12% acrylamide. The fractionated proteins were electroblotted onto a nitrocellulose filter using a semi-dry blotting apparatus (Bio-Rad Laboratories) in accordance with the manufacturer's recommendations. After the transfer of proteins, the membrane was blocked in TBS (20 mM Tris-HCl, pH 7.5, 0.5 NaCl) which contained 5% non-fat milk powder, for 30 min. The membrane was briefly washed in TTBS (TBS plus 0.05% Tween 20) and then incubated in TTBS that contained the primary antibody, namely, a polyclonal antibody against cruciferin raised in rabbit (kindly provided by Dr. L. Rask), at 25 °C for 1 h. After washing in TBS, the membrane was soaked in TTBS that contained the second antibody, goat antibodies rabbit IgG, conjugated to alkaline phosphatase (Bio-Rad Laboratories), for 1 h at 25 °C. The proteins were visualized by a colorimetric reaction catalyzed by the immobilized alkaline phosphatase.

Amino-acid analysis

Half-seeds of *T1* plants that showed decreased levels of cruciferin in the analysis by SDS-PAGE were used for the analysis of amino acids. Eight half-seeds were homogenized in ice-cold acetone to remove lipids. After centrifugation, the pellet was dried under a vacuum. Total protein was extracted from the pellet with 70% formic acid and brought to dryness under a vacuum. The protein residue was hydrolyzed in 6 N HCL that contained 0.01% phenol and 0.1% DTT for 24 h at 110 °C under a vacuum, and the hydrolysate was dissolved in citrate buffer (pH 2.2; amino-acid analysis grade; Nakarai Chemicals, Kyoto, Japan). The sample was analyzed by HPLC. For determination of cysteine content, the protein was oxidized with performic acid prior to hydrolysis. Data were the average of two separate hydrolyses of two different preparation of total seed protein.

Fatty acid analysis

Total fatty acid was extracted from six half-seeds, with decreased levels of cruciferin in the analysis, by SDS-PAGE using the method of Bligh and Dyer (1959). Total fatty acid extracted from six half-seeds of non-transgenic *B. napus* cv Westar was used as a control. They were analyzed by capillary gas chromatography as described in Kohno-Murase et al. (1994).

Results

Transformation of *B. napus* and the accumulation of seed storage protein in transgenic plants

Cruciferin is composed of six subunits and each subunit consists of a heavy α chain and a light β chain. Electrophoretic analysis of cruciferin showed that four different α chains ($\alpha1$ through $\alpha4$) and four different β chains ($\beta1$ through $\beta4$) migrated during SDS-PAGE as proteins of 29 kDa to 33 kDa and 21 kDa to 23 kDa, respectively. The $\alpha2$ and $\alpha3$ subunits migrated to the same position ($\alpha2/3$) (Rödin and Rask 1990). The $\beta2$ and $\beta3$ subunits also migrated similary during electrophoresis. The antisense construct contained a sequence that was coding complementary to that part of the *cruA* gene

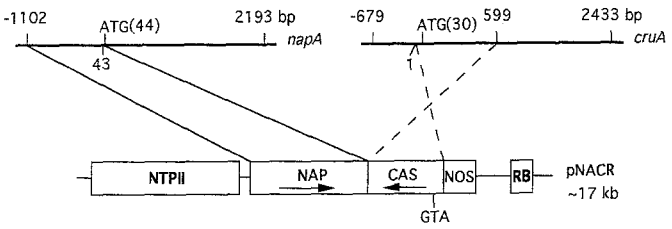

Fig. 1 Construction of the antisense gene for cruciferin. *NAP* the promoter fragment of the gene for napin; *CAS* fragment of the gene for cruciferin in an inverted orientation relative to that of the napin promoter; *NOS* terminator of the gene for nopaline synthase; *NPTII* gene for neomycin phosphotransferase; *RB* right border. The amplified NAP fragment included the region from 10 bp to 682 bp upstream of the site of initiation of transcription

(Fig. 1) corresponding to the α2/3 and β2/3 subunits (Ryan et al. 1989; Rödin and Rask 1990). The promoter region of the gene for napin, used for this experiment, controlled the expression of a gene for β-glucuronidase (GUS) in a seed-specific manner in the seeds of *B. napus* (Kohno-Murase et al. 1994).

The antisense construct was introduced into *B. napus* by *Agrobacterium*-mediated transformation. Three regenerated plants were obtained. All plants were shown by PCR to habor the antisense gene (data not shown) and all produced self-pollinated seeds (*T1* seeds). Extracts of each *T1* seed from transgenic plants were subjected to SDS-PAGE to analyze the effects of the antisense gene on the storage protein in the seeds. The analysis by SDS-PAGE revealed that seeds of all the transgenic plants examined had reduced amounts of cruciferin, and one transgenic plant (CA#3) showed a clear reduction in cruciferin content. *T1* seeds of the CA#3 plant were further analyzed. A survey by PCR showed all the *T1* seeds from this plant had an introduced antisense gene, suggesting that the transformant had integrated more than two antisense genes (data not shown). Analysis by SDS-PAGE of the *T1* seeds revealed that all the *T1* seeds contained reduced levels of α1, α2/3, β1, β2 and β3 chains (Fig. 2). However, the intensities of the bands that corresponded to the α4 and β4 chains were not reduced.

Western-blot analysis confirmed the reductions in the levels of the α1 and α2/3 chains (see Fig. 4A).

The total protein content per seed of the CA#3 plant did not differ significantly from that of seeds of the non-transformant, *B. napus* cv Wester (Table 1). The control plants, *B. napus* cv Westar, were grown beside the transgenic plants in a growth chamber. For the analysis of two other storage proteins, namely, napin and oleosin, aliquots of extracts were fractionated by electrophoresis. SDS-PAGE revealed that all the seeds of the CA#3 plant had increased levels of napin (Fig. 3). However, no changes were observed in the amount of oleosin by Western blotting (Fig. 4B).

The total lipid content of the CA#3 plant did not differ from that of seeds of the non-transformant, *B. napus* cv Westar (Table 2). There was no difference in fatty acid composition between CA#3 and non-transgenic plants (data not shown).

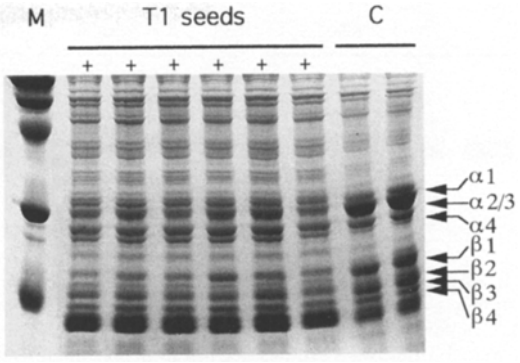

Fig 2 Analysis by SDS-PAGE of seed proteins from the transgenic plants. Total seed proteins from each half-seed of the transgenic plant designated CA#3 were subjected to analysis by SDS-PAGE. Cruciferin was examined on a gel with 12% polyacrylamide. *Plus signs* above lanes indicate that the presence of the antisense gene was confirmed by PCR. *M* molecular-size markers; *T1* seeds, transgenic plant CA#3; *C* non-transformant *B. napus* cv Westar. α1 through 4 and β1 through 4 indicate α and β chains of cruciferin, respectively

Table 1 Total protein content of *T1* seeds from a transgenic plant and control plants

Seed type	Total protein content[a] (mg/seed)
T1 seeds with decreased levels of cruciferin	0.58 ± 0.15
Non-transformed seeds (*B. napus* cv. Westar)	0.63 ± 0.17

[a] Values shown are the means from 100 (transgenic plants) and 34 (*B. napus* cv Westar) separate experiments ± SD

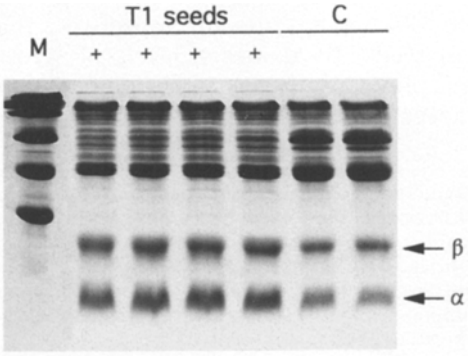

Fig 3 Analysis by SDS-PAGE of seed proteins from the transgenic plants. Total seed proteins from each half-seed of the transgenic plant designated CA#3 were subjected to analysis by SDS-PAGE. Napin was examined on a gel with 20% polyacrylamide. *Plus signs* above lanes indicate that the presence of the antisense gene was confirmed by PCR. *M* molecular-size markers; *T1* seeds, transgenic plant CA#3; *C* non-transformant *B. napus* cv Westar. α and β indicate the α and β subunits of napin, respectively

Amino-acid composition of the transgenic *T1* seeds

Amino acids in the *T1* seeds of the CA#3 plant were analyzed. The results showed that the seeds contained

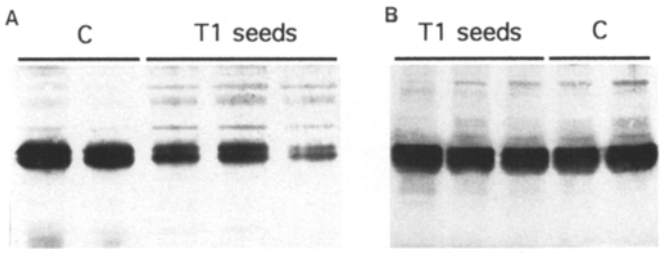

Fig. 4A, B Western-blot analysis of levels of the α chain of cruciferin (**A**) and of levels of oleosin (**B**). Extracts of the seeds with decreased levels of cruciferin were fractionated by SDS-PAGE. The same proteins were blotted on two membranes and one membrane was allowed to react with antiserum against the α1 and α2/3 chains of cruciferin (**A**) while the other was allowed to react with antiserum against oleosin (**B**). *C* non-transformant *B. napus* cv Westar; *T1* seeds, transgenic plant CA#3

Table 2 Total fatty acid content of *T1* seeds from a transgenic plant and control plants

Seed type	Total fatty acid content[a] (% seed weight)
T1 seeds with decrased levels of cruciferin	29.8 ± 6.6
Non-transformed seeds (*B. napus* cv. Westar)	30.6 ± 5.3

[a] Values shown are the means from six separate experiments ± SD

increased amounts of lysine, methionine and cysteine. The transgenic seeds contained 32% more cysteine than non-transgenic seeds, and lysine and methionine contents were 10% and 8% greater, respectively, than those in control seeds. By contrast, levels of tyrosine and asparagine plus aspartic acid were significantly reduced in the transgenic seeds (Table 3).

Table 3 Amino-acid compositions (% mol/mol) of total seed protein from a transformed and a control plant. The column labelled CA#3 contains data from a transgenic plant with a decreased level of cruciferin in seeds, while the column labelled Westar contains data from a non-transgenic plant, *B. napus* cv Westar. The column labelled % shows amino-acid levels as percentages. The level of each amino acid in *B. napus* cv Westar was set at 100%

Amino acid	CA#3	Westar	(%)
Cys	10.8	8.3	132
Lys	5.3	4.8	110
Met	1.3	1.2	108
His	2.9	2.7	107
Pro	7.8	7.4	105
Val	6	5.8	103
Glx	17.9	17.6	102
Thr	4.5	4.5	100
Ser	4.4	4.5	98
Ile	3.7	3.9	95
Gly	7.5	8	94
Ala	6.7	7.1	94
Leu	6.9	7.4	93
Phe	3.2	3.6	89
Arg	4.5	5.1	88
Asx	6.3	7.7	82
Tyr	0.4	0.5	80

Discussion

The data presented here show that inhibition of the accumulation of cruciferin by the introduction of an antisense gene for cruciferin can increase the amount of some essential amino acids in seeds of transgenic *B. napus*. The seeds contain 32% more cysteine and 8% more methionine than do the seeds from control plants. This increase is similar to that obtained by Altenbach et al. (1992) who increased the levels of methionine in seed protein by the introduction of a gene for a methionine-rich protein from Brazil nut. In addition to the increase in levels of sulphur-containing amino acids, we also observed an increase in the lysine content of the seeds. Seeds of *B. napus* contain less lysine than soybean seeds (Norton 1989). Such an increase in the lysine content of rapeseed could lead to enhanced use of these seeds in livestock feed.

While the level of cruciferin was reduced by the antisense method, levels of total protein and oleosin in the seeds were unchanged. In previous studies, we found that reductions in the level of napin by the introduction of an antisense gene for napin were balanced by increases in the level of cruciferin, while the levels of total protein and oleosin remained constant (Kohno-Murase et al. 1994). Napin and cruciferin are synthesized at a similar stage of embryo development and are stored in protein bodies (Crouch and Sussex 1981). By contrast, while oleosin is synthesized at the same developmental stage it is integrated into the membranes of oil bodies (Tzen et al. 1993). Our observations indicate that surplus free amino acids which result from the inhibition of synthesis of a particular storage protein can be used immediately for the synthesis of another storage protein and are not allocated to the synthesis of oleosin.

Introduction of an antisense gene for just one of the subunits of napin was sufficient to completely inhibit the accumulation of napin in *B. napus* (Kohno-Murase et al. 1994). Genes for napin are more than 90% identical over their coding regions (Baszczynski and Fallis 1990), whereas the homology between genes for each subunit of cruciferin is about 60% (Rödin and Rask 1990). The observed incomplete inhibition of the synthesis of cruciferin might have been due to the lower homology between the genes. Although genes for α1β1, α2/3-β2/3 and α4β4 exhibit similar homology to one another, the accumulation of the α1β1 subunit, as well as that of α2/3-β2/3, was decreased, while that of α4β4 was unaffected by the introduction of the antisense gene for the α2/3-β2/3 subunit of cruciferin.

In our previous study we found modifications in the levels of both oleic acid and linoleic acid in seeds with reduced levels of napin (Kohno-Murase et al. 1994). However, we did not observed any modification in the total lipid content and the fatty acid composition in seeds with reduced levels of cruciferin (Table 2). Since *B. napus* is mainly grown for oil content, it is important

that the total storage lipid content was not reduced in seeds of the transgenic plant.

The transformants with a modified amino-acid composition were maintained for two generations without showing any abnormalities. The rate of seed germination of the transformants was similar to that of non-transformants. Thus, the antisense method presented in this report allowed us to generate plants that produced seeds with a modified amino-acid composition without any of the disadvantages often associated with mutant lines.

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