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Cytosine deaminase as a substrate-dependent negative selectable marker in *Brassica napus*

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Abstract The enzyme cytosine deaminase, encoded by the *codA* gene, catalyzes the deamination of the non-toxic compound 5-fluorocytosine (5-FC) to the highly toxic compound 5-fluorouracil (5-FU). Cytosine deaminase activity is not found in higher plants and *Brassica napus* seedlings are unaffected by the presence of 5-FC in the growth medium. In *codA*-transformed *B. napus* seedlings, expression of cytosine deaminase results in a reduction of root and hypocotyl lengths, and a severe suppression of true leaf development. This phenotype is dependent on the presence of the 5-FC substrate and no effects are seen in plants grown in the absence of the substrate or in sibling plants lacking the transgene. The *codA* transformants have been assessed over three generations of growth and in each generation the transgene is stably inherited and confers the same 5-FC-sensitive phenotype. Transfer of 5-FC-sensitive seedlings to soil results in the restoration of normal growth in up to 100% of the seedlings. These results indicate that *codA* is a versatile dominant marker gene that can be used effectively in *B. napus* for substrate-dependent negative selection.

Key words *Brassica napus* · Cytosine deaminase · *codA* gene · 5-Fluorocytosine · Negative selection

Introduction

Negative selection is a process whereby the expression of dominant cytotoxic genes results in cell lethality. These cytotoxic genes can be substrate-dependent (conditional) or -independent (non-conditional). Cytotoxic markers have been employed in several distinct roles. Under the control of tissue specific promoters, they have been used to develop genetic ablation systems in both animals and plants. In plants, genetic ablation has been used to induce male sterility in tobacco and *Brassica* (Mariani et al. 1990) and to elucidate cell interactions during floral development in tobacco and *Arabidopsis* (Day et al. 1995). Cytotoxic genes have also been incorporated into T-DNA elements to use in conjunction with positive markers. Such multiply marked systems permit direct selection for and against individual T-DNA elements in segregating populations (Bancroft et al. 1992) or counter-selection against random T-DNA integration events in gene-targeting experiments. A similar positive/negative selection system has proven to be very effective in achieving gene replacement by homologous recombination in animal systems (Capecchi 1989). Conditional cytotoxins have also been proposed for use in novel genetic screens. These screens are designed to isolate mutants that survive negative selection by turning off the expression of a cytotoxic gene. This approach has yielded insights into the mechanism of gene silencing in plants (Brusslan and Tobin 1995) and should also recover mutants defective in either signal transduction or transacting factors that influence gene expression (Heimer et al. 1995; Day and Irish 1997).

We are developing new genetic marker systems for use in *Brassica* species in order to follow the segregation of introduced T-DNA elements. Thus far, researchers have introduced and tested three classes of negative selectable markers in *Brassica*. These are the RNase genes, *RNase-T1* and *barnase* (Mariani et al. 1990); the ADP ribosylating genes, diptheria toxin A chain (Thorsness et al. 1991) and the exotoxin A chain (Koning et al., 1992); and the amido hydrolase gene, *aux2* (Béclin et al. 1993). With the exception of *aux2*, these markers are non-conditional and their use has been restricted to genetic ablation studies in which the temporal and spatial expression of their toxic phenotypes are strictly a function of their chimaeric promoters.

The *aux2* gene is a conditional marker that converts the substrate naphthalene acetamide into the active auxin naphthalene acetic acid. Overproduction of auxin can be lethal to plants. Béclin et al. (1993) report that in the

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presence of substrate transgenic *Brassica oleracea* plants carrying this gene develop abnormal root systems and grow more slowly than wild-type. However, they also observed reduced growth rates of wild-type *Brassica* germinated on media containing high levels of the *aux2* substrate. Similar observations were made when wild-type *Arabidopsis* seedlings were grown on media containing high levels of naphthalene acetamide (Karlin-Neumann et al. 1991). In addition, Béclin et al. (1993) were unable to distinguish between sensitive and resistant individuals within segregating *Brassica* populations grown on defined medium. They postulate that secretion of auxin into the media by transgenic individuals affected the growth of all individuals, including plants lacking the transgene. As a consequence of these reports, we decided to identify and test an alternative conditional cytotoxic marker that might function more effectively in *Brassica*.

codA is an *Escherichia coli* gene that encodes the enzyme cytosine deaminase. This enzyme deaminates the non-toxic compound 5-fluorocytosine (5-FC) into the highly cytotoxic compound, 5-fluorouracil (5-FU). 5-FU is metabolized to 5-FUTP, which is incorporated into newly synthesized RNA, and to 5-FdUMP, a competitive inhibitor of thymidylate synthetase (Beck et al. 1972). Consequently, both RNA and DNA synthesis is inhibited by 5-FU. Homologues of *codA* are not found in higher eukaryotes and cytosine deaminase activity has not been detected in a wide sample of plant species including wild-type oilseed *Brassica* (Stougaard 1993). To date, the *codA* gene has been introduced into the nuclear genomes of *Arabidopsis*, *Lotus japonicus*, and several species of *Nicotiana* (Perera et al. 1993; Stougaard 1993; Risseeuw et al. 1997). *codA* functions efficiently in each of these species as a conditional negative selectable marker and there are no observable deleterious effects on wild-type plants in the presence of the 5-FC substrate. Recently, Risseeuw et al. (1997) successfully demonstrated the utility of *codA* in increasing the efficiency of gene-targeting in plants through counter-selection of illegitimate recombination (T-DNA integrations). *codA* also confers 5-FC sensitivity when introduced into the plastid genome of tobacco and thus can be used in a negative selection scheme to facilitate the identification of nuclear genes that regulate plastid gene expression (Serino and Maliga 1997).

We have introduced the *codA* marker gene, under the regulation of the CaMV 35S promoter, into *Brassica napus* and have assessed its activity over three generations of growth. This paper describes our findings that *codA*, which encodes cytosine deaminase activity, can be used successfully as a substrate-dependent negative selectable marker in *Brassica*.

Materials and methods

DNA construct

A 2.3-kb *Eco*RI fragment containing the *codA* coding sequence under the regulation of the CaMV 35S promoter and CaMV terminator (Perera et al. 1993) was cloned into the T-DNA binary vector pRD400 (Datla et al. 1992). The resulting plasmid, pTN1 (Fig. 1), was then moved into the *Agrobacterium tumefaciens* strain, GV3101 (pMP90) (Koncz and Schell 1986) by tri-parental mating with the helper strain MM294 (pRK2013) (Ditta et al. 1980).

Transformation and growth of *B. napus*

Fifty cotyledons of *B. napus*, cultivar Westar, were transformed using *A. tumefaciens* bearing the plasmid pTN1, according to the method of Moloney et al. (1989) with modifications (http://molomac1.bio.ucalgary.ca/LabProtocols/Brassica_Protocol.html). Rooted primary transformants (T0) were transferred to soil and placed in a growth chamber (85% relative humidity, temperature 20°C, 16-h day length at a photon flux density of 200 µmol m^{-2} s^{-1}). Three to four weeks later, plants were moved to the greenhouse to allow the production of T1 seeds. T1 seeds were harvested and the T2 and T3 generations were derived through selfing. A combination of fungal contamination and limited seed set precluded genetic analyses in the T1 generation. However, phenotypes conferred by the *codA* transgene were assessed. In the T2 and T3 generations, complete phenotypic characterization as well as complete genetic analyses were done. Data presented in this paper are derived from the analyses of 16-day old seedlings from the T2 generation unless otherwise indicated.

Growth on selective and non-selective media

For all tests, seeds were surface-sterilized in a 1% hypochlorite/0.1% Triton X-100 solution, followed by four washes in ster-

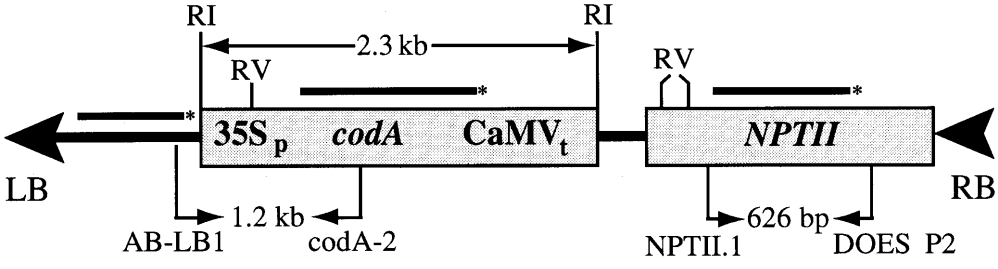


Fig. 1 Schematic representation of the *codA*-containing T-DNA from the binary plasmid pTN1. *LB* left-border, *RB* right-border, *arrowheads* indicate the direction of the R and L borders, *RI EcoR*I, *RV EcoR*V, *codA* cytosine deaminase, *NPTII* neomycin phosphotransferase II, $35S_p$ 35S promoter, $CaMV_t$ cauliflower mosaic virus terminator, lines labeled with an *asterisk* indicate the approximate positions of sequences used as probes, *small arrows* indicate the approximate positions and orientation of DNA primers. Primer names as indicated. Figure not drawn to scale

ile deionized water. Seeds were immediately transferred onto media in a 16×15 mm Petri dish (ten seeds per dish) and the dish was placed uncovered within an inverted magenta box to allow the obstacle-free upright growth of the seedlings. The boxes were incubated at 24°C, at a photon flux density of 200 µmol m^{-2} s^{-1} and a 16-h day length.

The germination frequency and phenotype of seedlings in the absence of selection was determined on germination medium, GM, [1×MS salts (Sigma), 2 g/l glycine, 100 g/l myo-inositol, 0.5 g/l nicotinic acid, 0.5 g/l pyridoxine-HCl, 0.5 g/l thiamine-HCl, 30 g/l sucrose, and 7 g/l tissue-culture grade phytagar, pH 5.8]. Solutions of 5-fluorocytosine (5-FC) (Sigma) and 5-fluorouracil (5-FU) (Sigma) were dissolved in Milli-Q water (Millipore) at 65°C at a concentration of 20 mg/ml. Solutions were filter-sterilized and immediately added to cooled molten media (at approximately 55°C). Wild-type plants were tested for their response to 5-FC and 5-FU on GM containing either 200, 400, 800, or 1600 µg/ml of 5-FC or 10, 25, 50, 100, 200, 250 or 300 µg/ml of 5-FU. *codA*-transformed plants were tested for their response to 5-FC by germinating seeds on GM containing either 100, 250, 500, 750, 1000, 1250 or 1500 µg/ml of 5-FC. Segregation data in the T2 and T3 generations were obtained by germinating seeds on GM supplemented with either 500 µg/ml of 5-FC or 50 µg/ml of kanamycin. Root and hypocotyl lengths were determined by tracing seedlings directly onto 1-cm^2 graph paper and measuring the tracings.

To determine if sensitive seedlings could recover from the toxic effects of 5-FC treatment, 10- and 16-day old sensitive seedlings were transferred directly to soil in the absence of any further selection. The seedlings were maintained in a growth chamber (85% relative humidity, temperature 20°C, 16-h day length at a photon flux density of 200 µmol m^{-2} s^{-1}). After 3–4 weeks survivors were transferred to the greenhouse to allow seed production.

Molecular analyses

Southern and Northern analyses were performed on nucleic acids isolated from the leaves of single plants. Genomic DNA was isolated according to Bernatzky and Tanksley (1986). Ten micrograms of DNA were digested with either *Eco*RI (for verification of the *codA* gene structure) or *Eco*RV (for determination of T-DNA copy number) and fractionated on a 0.8% agarose gel. Total RNA was extracted according to Ausubel et al. (1994) and poly A^+ mRNA was prepared using a Qiagen Oligotex mRNA kit. Two micrograms of poly A^+ mRNA were fractionated on a 1% agarose-formaldehyde gel. Nucleic acids were transferred to GeneScreen Plus membranes and blots were hybridized at 65°C. ^{32}P-dCTP labeled probes were prepared using the Multiprime DNA labeling systems (Amersham). Poly A^+ mRNA isolation, transfer of nucleic acids to the membrane, and the preparation of probes were all done according to the manufacturer's protocol. Blots were washed at 65°C, twice in 0.2×SSC and 0.1% SDS, and twice in 0.1×SSC and 0.1% SDS. The following DNA sequences were used as probes: for determining T-DNA copy number a 626-bp PCR-generated right-border specific probe [NPTII.1: 5´-CGCTCAG AAGAACTCGTCAAGAA-3´, DOES P2: 5´-TTGTCAAGACC GACCTGTCC-3´ (Does et al. 1991)] (Fig. 1) and a 613-bp PCR-generated left-border specific probe (AB-LB3: 5´-GTTGGCTGG CTGGTGGCAGG-3´, AB-LB4: 5´-CGCCTTGCAGCACATCCC CC-3´) (Fig. 1) were used; for assessing *codA* gene expression and structure a 1.2-kb *Pst*I-*Cla*I *codA* specific fragment (Fig. 1) was used; and for Northern analysis a 1.7-kb *Arabidopsis thaliana* actin cDNA was used as a control probe (Huang et al. 1997) after stripping the *codA* probe from the original blot. Quantification of mRNA level was performed using the public domain NIH-Image program (developed at the National Institutes of Health, Springfield, VA., part no. PB95–500195GE1).

For PCR analyses, genomic DNA was prepared from approximately 100 mg of leaf tissue using a scaled-down version of the protocol by Dellaporta et al. (1983) which allowed all manipulations to be performed in a 1.5-ml microfuge tube. The DNA was resuspended in 100 µl of water and 5 µl were used per 100 µl of reaction volume. Amplification of the *codA* sequence was performed with primers AB-LB1 (5´-GATTAAGTTGGGTAACGCC AGGG-3´ and codA-2 (5´-CATCGATCTCATCACAGTGAA CG-3´) (Fig. 1), while amplification of the *NPTII* sequence was performed with primers NPTII.1 and DOES P2 (see above) (Fig. 1). PCR conditions were according to Does et al. (1991).

Results

Response of wild-type seedlings to 5-FC and 5-FU

Wild-type seeds were germinated on media containing various concentrations of the non-cytotoxic compound 5-FC (200–1600 µg/ml) and the cytotoxic compound 5-FU (10–300 µg/ml) to determine if these compounds had any observable effects on the growth and development of *Brassica* seedlings. Relative to control seedlings grown on media without selection, no differences were observed in the growth of cotyledons, hypocotyls, roots or true leaves among 16-day old seedlings grown in the presence of 200, 400, 800, or 1600 µg/ml 5-FC (Fig. 2A). These observations indicate that, at the concentrations tested, 5-FC has no visible effect on wild-type seedlings.

Sixteen-day old seedlings grown on 10 µg/ml of 5-FU appeared to develop normally (Fig. 2B), although some individuals displayed a modest reduction in root growth. As the concentration of 5-FU increased, there was an increasingly severe reduction in hypocotyl and root lengths, and in cotyledon size (Fig. 2B). In addition, at the highest concentration tested (300 µg/ml), cotyledons accumulated anthocyanin on both surfaces. True leaf development was also reduced on all concentrations above 10 µg/ml of 5-FU, and at 300 µg/ml no true leaves were visible (Fig. 2B) even in 30-day old seedlings (data not shown). These results indicate that low concentrations of the compound 5-FU have severe cytotoxic effects on the growth and development of wild-type seedlings.

Plant transformation

In order to test the efficacy of *codA* negative selection in *Brassica*, we introduced the *codA* gene under the regulation of the CaMV 35S promoter into *B. napus*. Four kanamycin-resistant (T-DNA selectable marker, Fig. 1) plants were recovered from the *Agrobacterium*-mediated transformation of 50 wild-type cotyledons. Subsequent Southern analysis of right and left T-DNA borders revealed, however, that three of the four transformants were clonally related (data not shown) and that therefore only two independent transformants were recovered. Selfed-progeny from all three clonally related transformants were analyzed in both the T1 and T2 generations and they displayed similar phenotypes under both selective and non-selective conditions. Consequently, T2-generation data derived from only one of these clonally related transformants, codA4, and from the second independent transformant, codA1, is presented below.

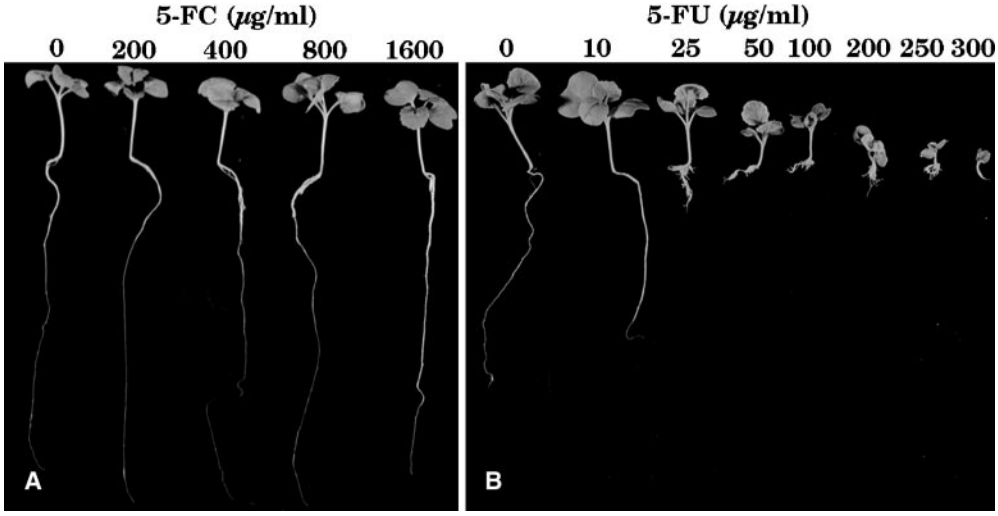


Fig. 2 Phenotypes of 16-day old wild-type *B. napus* seedlings germinated directly on various concentrations of 5-FC-(**A**) and 5-FU-(**B**) containing media. Panels **A** and **B** are shown at the same magnification

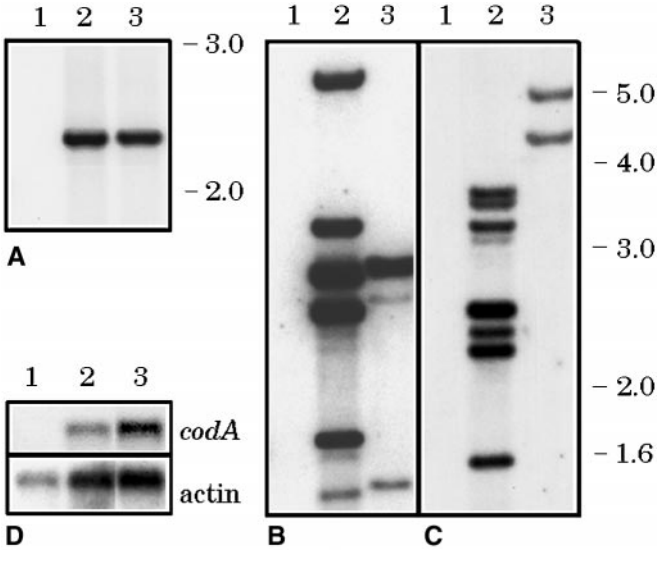


Fig. 3A–D Southern and Northern analysis of wild-type and transgenic *codA B. napus* plants. *Lanes: 1* wild-type, *2* codA1, *3* codA4. *Eco*RI-digested DNA hybridized with a *codA* specific fragment (**A**). *Eco*RV-digested DNA hybridized with a right-border specific probe (**B**) and a left-border specific probe (**C**). Poly A^+ mRNA hybridized with a *codA* specific fragment and *A. thaliana* actin cDNA (**D**). Position of DNA molecular-weight markers (kb) are to the right

Selfed progeny from these two lines were also analyzed in the T3 generation.

Molecular characterization of transgenic lines

The presence of *codA* in the transgenic lines, codA1 and codA4, was verified by Southern analysis. *Eco*RI digested genomic DNA was probed with a *codA*-specific fragment (Fig. 1) and, as seen in Fig. 3A, the probe hybridized to the expected 2.3-kb fragment. As predicted, the probe did not hybridize to wild-type DNA.

T-DNA copy number within the two transgenic lines was determined by Southern analysis of the right and left T-DNA borders. *Eco*RV-digested genomic DNA was probed with an *NPTII* specific fragment for right-border analysis and with a left-border specific fragment for left-border analysis (Fig. 1). Analysis of hybridization profiles (Fig. 3B, C) revealed that codA1 contains multiple T-DNA insertions while codA4 contains three T-DNA elements.

Expression of the *codA* gene was demonstrated by hybridization of poly A^+ mRNA with a *codA*-specific fragment (Fig. 1). Results revealed that the probe hybridized specifically and strongly to a single transcript in the transgenic lines which corresponded to the expected size of about 1500 nucleotides (Fig. 3D). Normalization of these hybridization signals against the control actin signal indicated that the *codA* transcript level is 50% higher in the codA4 plant as compared to the codA1 plant (Fig. 3D). As predicted, the *codA* probe did not hybridize to mRNA from the wild-type plant.

Phenotypic characterization of *codA* transgenic lines

To observe the growth and development of transgenic plants relative to wild-type plants, T2 seeds were germinated on media without any selection. Both transgenic lines produced viable seeds with a high germination frequency comparable to wild-type seeds (Table 1). The codA4 seedlings grew well and were phenotypically indistinguishable from the wild-type seedlings (Fig. 4A, C, and see Fig. 5). codA1 seedlings also showed robust growth (Fig. 4B, and see Fig. 5A) but had a much shorter hypocotyl (see Fig. 5B). T2 seeds were also germinated on media containing kanamycin to determine the segregation ratios of the T-DNA elements in each of the codA lines. All codA1 T2 individuals tested were resistant to

Table 1 Genetic analysis of wild-type and *codA* transgenic lines of *B. napus*

Line	GM[a]	Kanamycin Selection		5-FC Selection	
	% Germination	% Germination	No. of Seedlings[b] (resistant:sensitive)	% Germination	No. of Seedlings[b] (sensitive:resistant)
WT	94	96	0:48 (0:4)	94	0:47 (0:4)
codA1	96	99	138:0 (4:0)	93	124:0 (4:0)
codA4	98	95	73:27 (2.7:1)[c]	98	72:24 (3:1)

[a] 50 seeds plated on GM
[b] Numbers in parentheses are segregation ratios
[c] Not significantly different from the expected ratio of 3:1 based on χ^2 analysis, $P>0.05$

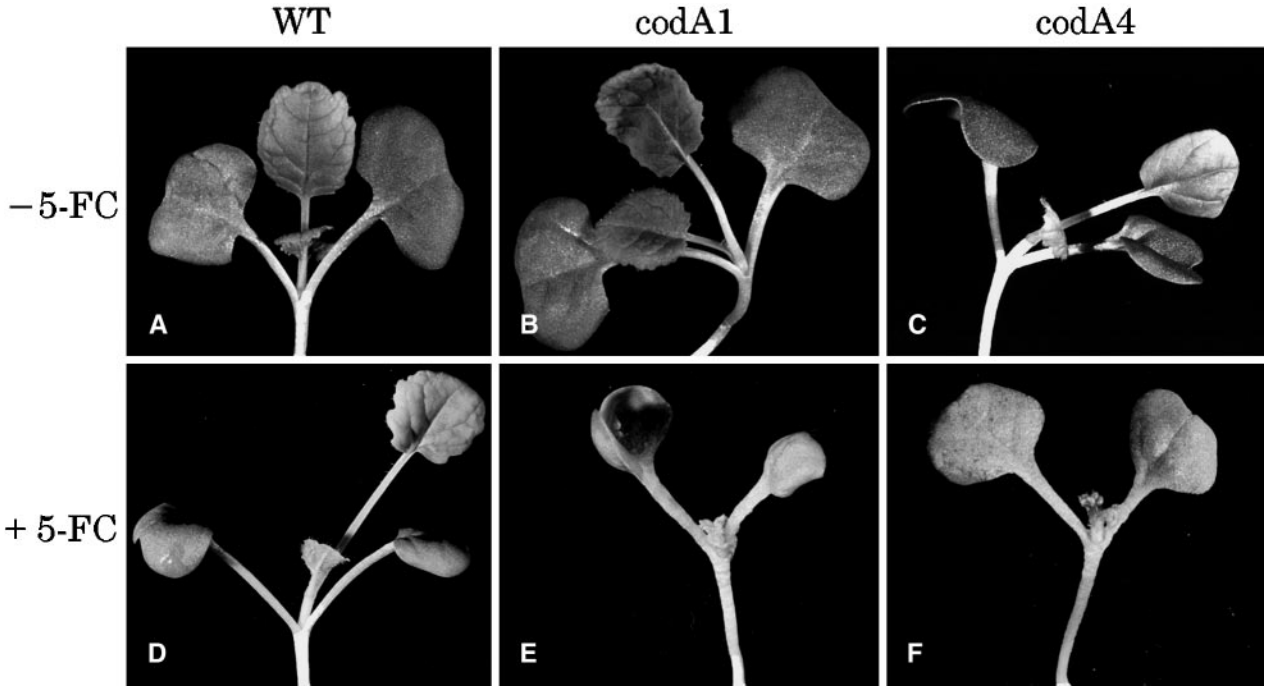
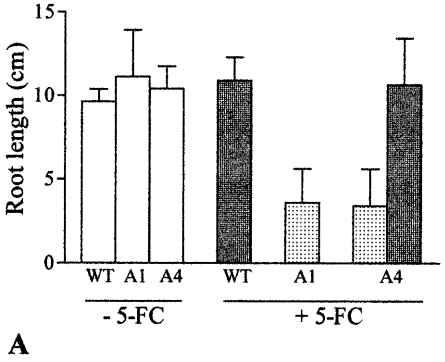


Fig. 4A–F Phenotypes of 16-day old wild-type and transgenic *codA B. napus* seedlings germinated directly on GM (–5-FC) (**A–C**) and on GM containing 500 µg/ml of 5-FC (**D–F**). The codA4 5-FC-resistant phenotype is not shown, but it is indistinguishable from that displayed by the plant in panel **C**. All panels are shown at the same magnification

kanamycin while codA4 segregated both kanamycin-resistant and -sensitive individuals at a ratio of about 3:1 (Table 1). This latter finding indicates that the three T-DNA elements in codA4 are integrated at a single locus in the genome and segregate in the manner predicted for a single dominant locus in the hemizygous state.

To determine if *codA* expression confers a sensitive phenotype to 5-FC, T2 seeds were germinated on media containing various concentrations of 5-FC (100–1500 µg/ml) and compared to wild-type plants after 16 days of growth (data not shown). As described earlier, wild-type plants are unaffected by 5-FC and display complete resistance. Transgenic lines produced seedlings that had a readily identifiable sensitive phenotype and, with increasing concentrations of 5-FC, there was a concomitant reduction in root and hypocotyl length and a dramatic change in true leaf development. The sensitive phenotype was unambiguously scored at 500 µg/ml of 5-FC and a complete description is detailed below. The segregation ratios of sensitive to resistant individuals on 5-FC was in good agreement with the segregation data obtained on kanamycin selection. All codA1 T2 individuals tested were sensitive to 5-FC while codA4 segregated both 5-FC-sensitive and -resistant individuals at a ratio of 3:1 (Table 1). codA4 resistant individuals were indistinguishable from seedlings grown in the absence of 5-FC selection (Fig. 5).

In the transgenic lines, sensitive individuals showed a range of phenotypic responses to 5-FC. The effect of 5-FC on cotyledons was highly variable and could not be used as a consistent indicator of the sensitive phenotype. Some sensitive plants had normal appearing cotyledons, while others produced hyponastic cotyledons (Fig. 4E, F); anthocyanin accumulation was also occasionally observed. When compared to siblings grown in the absence of selection, the average root length of sensitive individuals was reduced by about 67% in both lines (Fig. 5A) while the average hypocotyl length was reduced by 56% for codA1 and 72% for codA4 (Fig. 5B). In all sensitive



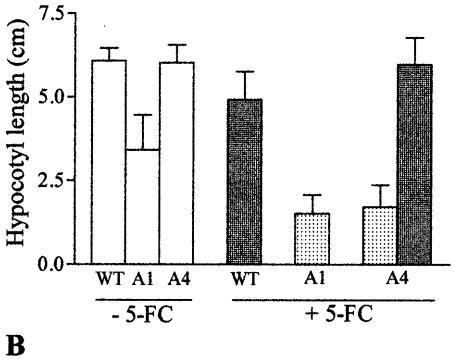


Fig. 5 Averages of root (**A**) and hypocotyl (**B**) lengths of wild-type and *codA* transgenic lines of *B. napus* germinated directly on GM (–5-FC) and on GM containing 500 µg/ml of 5-FC. For 5-FC selection, *heavy stippling* represents 5-FC-resistant individuals and *light stippling* represents 5-FC-sensitive individuals. *Error bars* indicate standard deviation. For seedlings on GM, *n*=23–25; for seedlings on 5-FC, *n*=11 for WT, *n*=27 for codA1 and *n*=62 for codA4. *WT* wild-type, *A1* codA1, *A4* codA4

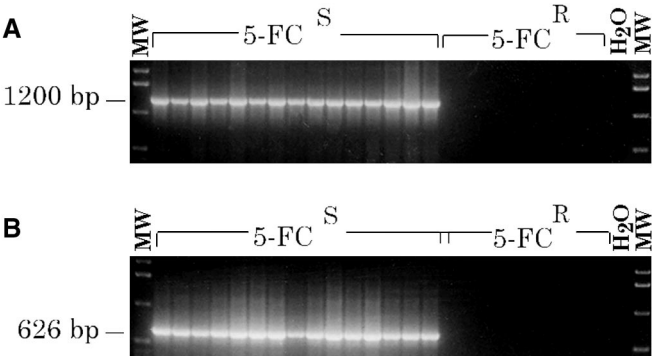


Fig. 6A, B PCR analysis of 5-FC-sensitive and -resistant *B. napus* seedlings. Seedlings were selected from a 16-day old codA4 segregating T2 population germinated directly on GM containing 500 µg/ml of 5-FC. Fifteen 5-FC-sensitive and nine 5-FC-resistant individuals were analyzed for the presence of *codA* (**A**) and *NPTII* (**B**) sequences. *MW*, DNA molecular-weight marker; lanes as indicated

seedlings true leaf development was severely affected. Sensitive codA1 and codA4 individuals developed very small leaves consisting of a rudimentary petiole and an abnormally shaped blade (Fig. 4E, F) that accumulated anthocyanin. In contrast, 16-day old wild-type seedlings produced a pair of green true leaves consisting of an elongating petiole and expanding leaf blade (Fig. 4D). In wild-type plants the leaves continued to develop while in transgenic plants no further growth of true leaves was observed. While we consistently saw a reduction in root and hypocotyl length among the 5-FC-sensitive individuals, the distinction between wild-type and *codA* seedlings was most readily determined by scoring the presence and morphology of true leaves.

PCR analysis of segregating codA4 T2 population

To confirm that the visual identification of 5-FC-sensitive individuals correlated with the presence of the *codA* transgene, the following experiment was performed. Seeds from the segregating codA4 line were germinated on media containing 5-FC (500 µg/ml) and sensitive and resistant individuals were identified based solely on the presence and morphology of true leaves. Genomic DNA was isolated from the individual seedlings and subjected to PCR analyses to assay for the presence or absence of the *codA* and *NPTII* sequences. Since bacteria and fungi, which are both known to carry the *codA* gene, may have been present as contaminants in the DNA preparation, a specific combination of primers was used (AB-LB1, for the T-DNA sequences, and codA-2, for the *codA* open reading frame) (Fig. 1) which ensured that the PCR product could only be amplified from the transgene. All of the 5-FC-sensitive individuals contained both the *codA* and the *NPTII* sequences, while all of the 5-FC-resistant individuals lacked both sequences (Fig. 6). The reciprocal experiment was also performed where kanamycin-selected resistant and sensitive individuals were analyzed for the presence and absence of the *codA* and *NPTII* sequences. As predicted, all of the resistant individuals contained both sequences while all of the sensitive individuals did not (data not shown). These results clearly indicate that selection based on the presence and morphology of true leaves is a reliable indicator of the transgene.

Recovery from 5-FC cytotoxic effects

To determine if *codA*-transformed individuals could recover from the cytotoxic effects of 5-FC, seedlings were removed from the 5-FC selection media and transferred directly to soil in a growth chamber. The following are pooled data from the T1 and T2 generations for codA1 and the three clonally related lines, codA2, codA3 and codA4. In the T1 generation 16 out of 24 5-FC-sensitive individuals showed a restoration of normal growth in soil, and 15 of these were moved to the greenhouse for seed production. In the T2 generation 16 out of 16 5-FC-sensitive individuals recovered from the cytotoxic effects, and five of these were transferred to the greenhouse. All individuals transferred to the greenhouse grew well and produced viable seeds (data not shown).

Stable inheritance of the *codA* phenotype

We have analyzed the *codA* transformants over three generations (T1, T2, T3) of growth. In all three generations, the presence of the *codA* transgene produces the same sensitive phenotype in the presence of 5-FC. While a combination of poor seed set and fungal contamination precluded genetic analyses in the T1 generation, these analyses were performed in the T2 and T3 generations. In both the T2 (Table 1) and T3 (data not shown) generations, all codA1 individuals tested were sensitive to 5-FC and resistant to kanamycin. In the T2 generation (Table 1), codA4 segregated both 5-FC and kanamycin-resistant and -sensitive individuals at a ratio expected for the segregation of a single dominant locus in the hemizygous state. Selfed progeny from two T2 codA4 plants were analyzed in the T3 generation and all individuals tested were sensitive to 5-FC and resistant to kanamycin (data not shown) indicating that the *codA* locus was homozygous in both parents.

Discussion

We have introduced the *codA* gene, which encodes the enzyme cytosine deaminase, into the important crop plant *B. napus*. The resulting transgenic plants are acutely sensitive to 5-FC, the precursor to the cytotoxin 5-FU. *codA*-transformed seedlings grown in the presence of 5-FC have shortened hypocotyls and roots, and are severely suppressed in the development of true leaves. Transgenic plants, however, develop normally in the absence of the substrate. Genetic and molecular analyses of a *codA* segregating population indicate that 5-FC sensitivity is a readily distinguishable dominant trait that cosegregates exclusively with the *codA* transgene. Thus, expression of the phenotype depends on both the presence of the substrate 5-FC and the *codA* gene.

As predicted, wild-type plants, which lack the transgene, grow normally in the presence of 5-FC and display no visible effects even at high concentrations. When 5-FU is included in the media, however, *Brassica* plants do succumb to the highly cytotoxic effects. The resistance of wild-type *B. napus* to 5-FC suggests that this plant species lacks enzymatic activity capable of converting 5-FC to the cytotoxin 5-FU. This conclusion is in agreement with findings from in vitro assays which indicate that cytosine deaminase activity is absent from oilseed *Brassica* (Stougaard 1993).

A combination of Southern and genetic analyses in the T2 generation indicates that our transgenic lines vary with respect to T-DNA copy number. codA1 contains multiple T-DNA elements, and all progeny are sensitive to 5-FC. In contrast, codA4 possesses three T-DNA elements at a single hemizygous locus and segregates sensitive and resistant individuals in the expected Mendelian ratio. Despite having a higher T-DNA copy number, codA1 produced slightly less of the *codA* transcript, yet both codA1 and codA4 individuals have identical 5-FC-sensitive phenotypes.

The sensitive phenotype we observe with transgenic *Brassica* in the presence of 5-FC is similar to what Stougaard (1993) reports in *codA*-transformed *Lotus* and tobacco. It is not, however, as severe as that observed in *Arabidopsis* (Perera et al. 1993; data not shown). Nevertheless, in a segregating *codA* population, the phenotypic difference between sensitive and wild-type *Brassica* individuals is striking and unambiguous, and we are able to reliably identify the sensitive individuals within 2 weeks of germination based on their abnormal leaf development. Our ability to make this clear distinction on defined media is unlike that described by Béclin et al. (1993) for *Brassica* seedlings segregating the *aux2* gene in the presence of naphthalene acetamide. Therefore the metabolite 5-FU, unlike napthalene acetic acid, is probably not secreted into the media and thus wild-type segregants remain unaffected. Since our goal is to develop new genetic marker systems for following the segregation of T-DNA elements in *Brassica* populations, *codA* appears to be the optimal choice for use as a conditional negative selectable marker.

Although 5-FU is a cytotoxic compound, approximately 67% of the sensitive T1 seedlings displayed full recovery after transfer to soil. Furthermore, in the T2 generation, 100% of the seedlings recovered when transferred to soil. We believe that the lower recovery frequency observed among T1 individuals was due to fungal contamination during the 5-FC selection. Stougaard (1993) also reports that plants can recover from the effects of 5-FC negative selection; however, he finds that only 40% survive under his conditions. These results suggest that the effects of 5-FU do not always result in the death of meristematic cells, and thus normal growth can be restored in the absence of selection in a significant number of individuals. The level of recovery observed in *Brassica* indicates that sensitive plants can be identified and subsequently maintained for other experimental manipulations without the aid of a positive marker.

Our results indicate that *codA* is stably inherited over three generations in *B. napus* and acts as an effective marker for 5-FC substrate-dependent negative selection. The clear and unambiguous sensitive response, as well as the insensitivity of wild-type plants to a wide range of 5-FC concentrations, makes *codA* well-suited to many of the potential applications described.

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