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ORIGINAL PAPER

Metabolically engineered male sterility in rapeseed (*Brassica napus* L.)

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Abstract Male sterility is of special interest as a mechanism allowing hybrid breeding, especially in important crops such as rapeseed (*Brassica napus*). Male sterile plants are also suggested to be used as a biological safety method to prevent the spread of transgenes, a risk that is high in the case of rapeseed due to the mode of pollination, out-crossing by wind or insects, and the presence of related, cross-pollinating species in the surrounding ecosystem in Europe. Different natural occurring male sterilities and alloplasmic forms have been tried to be used in rapeseed with more or less success. Due to the difficulties and limitations with these systems, we present a biotechnological alternative: a metabolically engineered male sterility caused by interference with anther-specific cell wall-bound invertase. This is an essential enzyme for carbohydrate supply of the symplastically isolated pollen. The activity of this enzyme is reduced either by antisense interference or by expressing an invertase inhibitor under control of the anther-specific promoter of the invertase with the consequence of a strong decrease of pollen germination ability.

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Introduction

Rapeseed (*Brassica napus* L.) became one of the most important crops, when double low varieties (low erucic acid and low glucosinolate), in Canada known as canola (Canadian Oilseed, Low-Acid), entered the market in the 1980s. Since these days the efforts to create hybrid varieties were intensified. F1 hybrid seeds are obtained by crossing parental inbred lines and breeders tend to favor hybrid breeding due to the advantages over open-pollinated varieties. Their special importance is due to the uniformity that is based on the genetic homogeneity as well as on heterosis or hybrid vigor that is based on the heterozygous nature of the offspring of the parental inbred cross (Brewbaker 1964; Feistritzer and Kelly 1987). This is also the reason, why the purchase of seeds is necessary every year from the breeding company. Hybrid breeding is, compared to other breeding methods, lengthy and difficult and the seeds are thus expensive, since cross-pollination has to be ensured by preventing self-pollination. The most important genetic system to prevent self-pollination of the female line is male sterility.

A further important application for male sterility systems, apart from hybrid breeding, might be their use as a biological safety method in the so-called confinement strategy, since there are already several trials with modified oil rapeseed. For example, see http://www.inspection.gc.ca/english/plaveg/bio/dt/dt_09e.shtml. Due to the mode of pollination (out-crossing by wind or insects) and the presence of related species in the surrounding ecosystem, the potential spread of transgenes is high in the case of rapeseed. The availability and use of such biological safety will help to cope with the increasing public awareness and fears.

Several forms of cytoplasmic male sterility (CMS) have been reported in rapeseed, which include two endogenous

cytoplasm types, *nap* (or *napus*) and *pol* (or *polima*), while a third cytoplasm, *cam*, is not known to confer male sterility. (Shiga and Baba 1971; Thompson 1972; Fu 1981). CMS is normally associated with mitochondrial ORFs that consist of different chimeric aggregates of the mitochondrial genome. A special feature of the rapeseed *orf222* of *nap*-CMS and *orf224* of *pol*-CMS is their high similarity to one another (L'Homme et al. 1997; Brown 1999; L'Homme and Brown 1993; Singh and Brown 1991), and, in addition, the *nap*- and *pol*-CMS restorers, *Rfn* and *Rfp*, represent different alleles or haplotypes of a single nuclear locus (Li et al. 1998). Despite these molecular similarities, the *pol*- and the *nap*-CMS are both sensitive to environmental influences in certain nuclear backgrounds leading to breakdown of sterility, with subsequent contamination of the F1 hybrids with sibs (Fan and Stefansson 1986). Lacking alternatives, the *pol*-CMS became the first CMS system to be extensively utilized for hybrid seed production (Fan et al. 1986; Röbbelen 1991), but due to the instability of this system there is an ongoing process of searching for new options, mostly by creating alloplasmic cybrids. The most prominent example is the introduction of the *ogu* (*ogura*; Ogura 1968) cytoplasm from radish into *Brassica* species by interspecific crosses (Bannerrot et al. 1974) and subsequent modification by protoplast fusion (Pelletier et al. 1983; Bonhomme et al. 1991, 1992; Grelon et al. 1994). Restoration of fertility in *ogura* radish plants has been shown to involve several genes and modifiers (Bonnet 1975). Introgression of the restorer radish nuclear genome into *ogu* male sterile rapeseed allowed the restoration of male fertility, when meiotic disturbances had been overcome by classical breeding methods (Heyn 1976; Pelletier et al. 1987; Pellan-Delourme and Renard 1988; Delourme et al. 1991). The implementation of this system, however, has been hampered by difficulties in the development of restorer lines free of negative agronomic characters that were introduced from radish into *B. napus* along with *Rfo*. In particular, most existing restorer lines have a dominant radish gene that results in elevated levels of seed glucosinolates such that hybrids produced with these lines cannot be approved to be grown as double low varieties. It has been difficult to separate *Rfo* from this and other deleterious radish genes because of the low frequency of recombination between radish and *Brassica* DNA in the *Rfo* region (Delourme et al. 1998). The cloning and characterization of the *Rfo* restorer gene was achieved by Brown et al. (2003). This gene, like most of the known restorer genes from other species, codes for a PPR-containing protein with multiple, in this case 16 PPR domains. The availability of the isolated restorer gene is used for the development of restorer lines that lack the deleterious agronomic characters that accompany *Rfo* when it is introduced from radish into *B. napus* through traditional plant breeding methodologies (Brown et al. 2003).

The current F1 hybrids in oilseed rape are based on the above-mentioned *pol*- and *ogu*-CMS systems, as well as on an alloplasmic CMS, carrying the cytoplasm from *B. tournefortii* (*tour*, Mathias 1985; Stiewe and Röbbelen 1994). Despite their commercial success, synthesis of new and possibly superior CMS systems or other systems permitting hybridization remains an important activity for many *Brassica* breeders, because of certain biological limitations associated with current systems (especially *tour* and *pol*) and the concern for genetic vulnerability in view of the current emphasis on *ogu*-CMS. Recent reports of CMS in *Brassica* include CMS 681A (Liu et al. 2005), 126-1 (Sodhi et al. 2006) and *hau*-CMS (00-6-102A; Wan et al. 2008). Several other CMS systems of alloplasmic origin have also been reported in *B. juncea* containing cytoplasm of several wild species (reviewed in Wan et al. 2008) and, apart from CMS, other hybridization mechanisms were suggested, including the resynthesis of *B. napus* for self-incompatibility that is used in hybrid breeding of the parental species (Esch and Wricke 1995; Möhring et al. 2005; Rahman 2005; Zhang et al. 2008).

Due to the difficulties and limitations with the above-mentioned male sterilities, we present a biotechnological alternative. So far, only one engineered nuclear-encoded male sterility system is ready to be used in agriculture or already in practical use, although various successful approaches to obtain male sterile plants have been described (reviewed in Roitsch and Engelke 2006). In former studies, we demonstrated that carbohydrate supply of developing microspores is dependent on the activity of a cell wall-bound invertase, since microspores are not connected to the phloem via plasmodesmata and thus are symplastically isolated. Therefore, the most ubiquitous transport sugar sucrose is released from the sieve elements of the phloem into the apoplast via a sucrose transporter, where irreversible hydrolysis occurs by an extracellular, cell wall-bound invertase (*cwINV*), which is strongly and tissue-specific expressed in anthers and pollen for this reason in different monocotyl and dicotyl plants (reviewed in Engelke et al. 2010). The hexose monomers (glucose and with a lower preference fructose) are taken up into the sink cell by high-affinity hexose transporters. In the model plants investigated so far, *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Solanum lycopersicum*, repression of *cwINV* activity (*AtcwINV2*, *Nin88*, and *Lin7*, respectively) by anther-specific RNA interference turned out to be an efficient method to circumvent carbohydrate supply of the symplastically isolated pollen with subsequent strong decrease of pollen germination ability and seed setting (Goetz et al. 2001; Proels et al. 2006; Hirsche et al. 2009).

Comparable results were also obtained by expressing a proteinaceous invertase inhibitor.

Rapeseed is a crop with increasing acreage due to the aims achieved by classical breeding methods in the past, i.e. double low varieties, and with intensive transgenic efforts for future modifications. Thus, in the present paper, we demonstrate the proof of concept for producing male sterility using the above-mentioned metabolic engineering in this crop.

Materials and methods

Partial cloning of rapeseed invertases and sequence alignment

Parts of the exon 3 of rapeseed invertases were amplified from genomic DNA or cDNA from anthers using degenerated primers that have been designed by alignment of different known invertase sequences. Primers oin3 (CCTT CACYTNTTYTAYCARTAYAAYCC) and oin4 (CCTTT CRWARAARGTYTTDGWWGCGTA) amplified PCR products of ca. 750 bp in size (Roitsch et al. 1995). In a second approach, a nested PCR was done, using primers KON1-F (TAYCAYYTNTTYTAYCA) and KON5-R (ANYTC BHBVAYNGGCCA) and subsequent KON3-F (GAYCC VWCVACNGCNTGG) and KON4-R (TCRWARAANG WYTTNGANGC), resulting in PCR products of ca. 410 bp in size. The PCR products were ligated in pGEM-T Easy vector and transformed in *E. coli* DH5a, according to the instructions of the supplier (Promega, Madison, USA). Due to the degenerated nature of the primers used for amplification, a mixture of different invertases was expected among the transformed cells. For that reason, single colonies were picked, plasmids were isolated and characterized by test restriction with enzymes having 4-bp recognition sites (*Bsu*RI, *Hpa*II, *Taq*I; MBI Fermentas, St Leon-Rot, Germany). Plasmids with different restriction patterns were sequenced. Putative invertase sequences of *B. napus* were identified, using Blast N program (NCBI, Altschul et al. 1997) and aligned with reported coding sequences (CDS) from other species, using the Clustal W program (Larkin et al. 2007). The p*I* value of the corresponding protein was calculated with Protein Calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html).

Expression analyses by Northern hybridization

15 µg RNA, isolated from leaves, stems, roots, small and big flower buds, flowers, anthers, carpels, and siliques, was blotted onto a nylon membrane and hybridized with ^{32}P-labeled probes of the different rapeseed invertases by standard procedures.

Construction of anther-specific promoter::GUS fusion (*AtcwINV2::uidA*) as reporter for expression analysis in rapeseed

A 1,160-bp fragment 5′ of the coding sequence of *A. thaliana cwINV2* (At3g52600) containing the promoter sequence was amplified by PCR, using the primers Ath2CW-Prom-F (5′-CGAAGCTTGTAAATATGGGGAAGATAC-3′) and Ath2CW-Prom-R (5′-ATGTCGACTGGTGCAAGAGAA CTCTATG-3′), with integrated restriction sites *Hin*dIII and *Sal*I, respectively. The sequence was ligated into the binary vector pBI101, 5′ of the GUS reporter sequence (Hirsche et al. 2009).

Construction of anther-specific promoter::cell wall invertase-antisense fusion (*AtcwINV2::AtcwINV2 antisense*) for generating male sterile rapeseed

For generating the antisense construct, a 1,251-bp fragment of *AtcwINV2* exon 3 was amplified using the primers Ath2CW-Ex3-F (5′-CTGAGCTCGGAGTTTACCATCT CTTCTACC-3′) and Ath2CW-Ex3-R (5′-TAGTCGACC TTGGCGTCAGAGCACATG-3′), with integrated restriction sites *Sac*I and *Sal*I, respectively, and ligated in antisense direction 3′ of the cloned promoter sequence into the binary vector pBI101, replacing *uidA*.

Construction of anther-specific promoter::invertase inhibitor fusion (*AtcwINV2::AtC/VIF2*) for generating male sterile rapeseed

For generating the invertase inhibitor construct, the coding sequence of *AtC/VIF2* (At5G64620) was amplified using the primers Atcwinh-1 (5′-CTGAGGTACCTCGAGCCT GAAATGGCTTCTTCTC-3′) and Atcwinh-2neu (5′-CT GATCTAGAGGGCCCTCATTCAACAAGGCGATC-3′) as an amplicon of 543 bp and fused 3′ of the *AtcwINV2* promoter in the binary vector by replacing the *uidA* gene.

Transformation of rapeseed

The constructs mentioned above were used for *Agrobacterium tumefaciens* strain C58C1-mediated transformation of rapeseed cv ‘Drakkar’, and ‘Mozart’. Seeds were sterilized for 30 min in 70% EtOH and then for 20 min in 5% Na-hypochloride + 0.01% Tween 20, subsequently rinsed five times in sterile water. Seeds were germinated for 7 days (16 h light, Phillips TLD 58W 33-640 and Osram L58 W/76 Natura de Lux; 8 h dark) at 23°C on 1/2 MS222 medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) with 1.5% sucrose and 0.3% gelrite, pH 5.7, in light-covered boxes with transparent lids, in order to get prolonged hypocotyls. 0.8-cm sections of hypocotyls were excised in liquid

callus-inducing CIM-G medium (MS222 medium with 1% glucose, 0.05% MES and phytohormone mix: 1 mg/l Kinetin, 1 mg/l 2.4D, 0.01 mg/l GA_3). The sections were pre-conditioned for 72 h on MS222 medium with 3% sucrose, 0.05% MES, 0.05% PVP K25, 0.3% gelrite, and the above-mentioned callus-inducing phytohormone mix, pH 5.8 (according to Cardoza and Stewart 2003, with modifications). A 3-ml preculture of *A. tumefaciens* was grown overnight in YEB medium at 28°C, transferred to 50 ml MinA medium (46 mM K_2HPO_4, 33 mM KH_2PO_4, 4 mM $(NH_4)_2SO_4$, 2 mM Na-citrate, 1 mM $MgSO_4$, 150 µM acetosyringone, 0.2% glucose, pH 5.5) containing 100 µg/ml antibiotics and grown to an OD_{600} of ca. 1.5. After centrifugation, cells were resuspended in CIM-G supplemented with 150 µM acetosyringone (final OD_{600} was 0.3–0.4). Pre-conditioned hypocotyl segments were collected and given to the *Agrobacterium* culture for 1 h. Suspension was then removed and the sections given to the same CIM-G medium with acetosyringone for 1 day at 22°C. After this co-cultivation, sections were washed three times in CIM-G medium with 400 mg/l carbenicillin. Sections were then placed on medium with antibiotics for preventing growth of *Agrobacterium*, but for the first 2 weeks without selection for transformed cells (MS221 medium with 1 ml/l MS vitamins and 1 ml/l B5 vitamins, Duchefa Biochemie B.V., Haarlem, The Netherlands; 0.5 g/l MES, 6.5 mg/l Fe–EDTA, 20 g/l sucrose, 0.3 g/l myo-inositol, 0.5 g/l PVO K25, 6.5 g/l agar, 5 mg/l $AgNO_3$, 2 mg/l BAP, 0.01 mg/l picloram, 400 mg/l carbenicillin, 50 mg/l cefotaxime, pH 5.8). Selection was started then on the same medium with 25 mg/l kanamycin. When plants started to regenerate, they were further cultivated for shoot elongation on DKW247 medium (Duchefa Biochemie B.V., Haarlem, The Netherlands), with 20 g/l sucrose, 0.5 g/l MES, 6.5 mg/l Fe–EDTA, 0.3 g/l myo-inositol, 6.5 g/l agar, 2 mg/l BAP, 0.01 mg/l IBA, 0.01 mg/l GA_3, 5 mg/l $AgNO_3$, 400 mg/l carbenicillin, 50 mg/l cefotaxime, and 25 mg/l kanamycin for selection, pH 5.7. Selected plants were transferred to root-inducing MS222 medium with 2.2 g/l, 0.3 g/l myo-inositol, 0.5 g/l MES, 5 g/l sucrose, 6.5 g/l agar, 0.1 mg/l NAA, 100 mg/l cefotaxime, and 25 mg/l kanamycin. Plants were transferred to soil and grown in the greenhouse under long day conditions (15 h day, 9 h night) until flowering. Transformation was confirmed by PCR, amplifying either the *nptII* gene or parts of the specific constructs.

Histochemical GUS staining

Reporter gene activity was histologically determined with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) in 50 mM $NaPO_4$ buffer (pH 7.0). Plant material was covered with staining solution and incubated over night at 37°C. The liquid phase was replaced by 70% ethanol to remove chlorophylls.

In vitro pollen germination test

Pollen germination was tested with medium containing 10% sucrose, 5 mM $CaCl_2$, 5 mM KCl, 1.62 mM H_3BO_3 and 1.5% LM agarose (Boavida and McCormic 2007). This was supplemented with 1 mM TRIS pH 8 that efficacy is due in part to the establishment of an optimum pH and also has an "amine effect" (Roberts et al. 1983). Furthermore, 15% PEG4000 was added that increases pollen tube elongation due to osmotic effects (Ferrari and Wallace 1975; Hodgkin 1983; Shivanna and Sawhney 1995). After heating to 65°C, droplets of the medium were placed on microscope slides. Pollen of two flowers per droplet was applied to the solidified medium by dipping the flowers on the surface. Germination was performed in a humid chamber at 22°C overnight. Pollen germination rates reflect the mean of three independent flowers with at least 100 pollens each.

In situ staining of invertase activity

Anthers were collected, frozen in liquid nitrogen and stored at −80°C. Before staining, 500 µl buffer (200 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA and 2% glycerin) was added to the anthers, giving a pollen suspension from which anthers were removed using a pipette tip. Pollens were centrifuged for 5 min at 8,000×*g*, and the pellet was resuspended in 500 µl buffer. This washing step was repeated to remove sugars which might give false-positive results. Washed pollen was resuspended in 40 µl buffer and each sample was separated into two incubation tubes. Invertase activity was determined according to a modified protocol of Doehlert and Felker (1987). The staining method uses sucrose as substrate. The glucose liberated by invertase activity is oxidized by glucose oxidase (GOD), thereby reducing phenazine methosulfate (PMS) and finally nitroblue tetrazolium salt (NBT). The reaction yields an intensely blue insoluble formazan which is easily visible microscopically, while Nitro BT itself is soluble and yields a practically colorless (slightly yellow) solution (Dahlqvist and Brun 1962). The incubation medium contained 70 mM K_2HPO_4 + 40 mM citrate, resulting in pH 4.5, 20 units/ml GOD (Boehringer), 0.014% PMS, 0.024% NBT, and 0.5% sucrose. 1 ml of this reaction mixture was added to pollen and incubated for 20 h at 26°C in the dark with shaking (600 rpm). In the control reactions, sucrose was omitted. Subsequent to centrifugation for 5 min at 8,000×*g*, the incubation medium was replaced against 500 µl 70% EtOH. For light microscopic investigation of the staining, pollen was centrifuged again and resuspended in 100 µl water. Pictures were taken with 100-fold magnification.

Table 1 Sequence alignment of partial *B. napus* invertases with corresponding Arabidopsis genes

Nomenclature, GenBank accession no.	Amplified from		Highest homology to Arabidopsis	
	DNA	RNA (anthers)	Identicalness (%)	Gene[a]
BncwINV1 HM246667	+ (410)	–	97	*AtcwINV1* At3g13790
BncwINV2a[b] HM246668	+ (750)	+ (750)	88	*AtcwINV2* At3g52600
BncwINV2b HM246669	+ (410)	–	98	
BncwINV3a (FEH?) HM246670	+ (750)	–	87	*AtcwINV3* At1g55120
BncwINV3b (FEH?) HM246671	+ (410)	+ (410)	98	
BncwINV4 HM246672	+ (410)	–	97	*AtcwINV4* At2g36190
–	–	–	–	*AtcwINV5* At3g13784
BncwINV6 (FEH?) HM246673	+ (750)	–	77	*AtcwINV6* At5g11920
Bnβfruct3 HM246674	+ (950)	–	87[c]	*Atβfruct3* At1g62660
Bnβfruct4 HM246675	–	+ (410)	86	*Atβfruct4* At1g12240

'+' positive amplification, in blankets: size of the amplified product, according to the primer used; '–' no amplicons found

[a] Nomenclature according to Sherson et al. (2003)

[b] a and b: two similar but not identical sequences

[c] Partial sequence alignment, regardless of a putative intron

Results

Phylogenic relationship of the invertase gene family from *B. napus* and other species

A part of exon 3 of invertases was amplified from rapeseed DNA or cDNA from anthers, using either primers oin3 and oin4 with amplicons of ca. 750 bp or KON1-F and KON5-R and nested KON3-F and KON4-R with amplicons of ca. 410 bp. Subsequent characterization by restriction profiles resulted in different sequences, which could be clearly aligned to the known sequences from *A. thaliana* (Table 1). Six putative genes coding for *cwINVs* have been identified in the *A. thaliana* genome. Two of them originally referred to as *Atβfruct1* and *Atβfruct2* (Tymowska-Lalanne and Kreis 1998) were renamed as *AtcwINV1* and *AtcwINV2*, respectively, by Sherson et al. (2003). We decided to adapt this nomenclature for the rapeseed invertases. For instance, *BncwINV1* corresponds to *AtcwINV1*, etc. Homologous sequences in rapeseed were detected for five out of the described six *cwINVs* from Arabidopsis by this approach with homology values between 77 and 97%. Two highly homologous but not identical sequences were found in the case of *BncwINV2* and *BncwINV3*, respectively. The subgroups are indicated by letters a and b. This finding can be explained by the amphidiploid nature of *B. napus*: the subgroups most probably represent the sequences of the parental species *B. rapa* and *B. oleracea*.

It should be mentioned that *AtcwINV3* and *AtcwINV6* have lower p*I* values of 5.5 and 4.8 compared to 8.1–9.7 for the remaining *cwINVs*. Both these Arabidopsis genes turned out to be fructan exohydrolases (*FEHs*) that split one terminal fructose unit from a longer fructan chain, instead of sucrose cleavage (de Coninck et al. 2005). To accommodate these findings, we added (*FEH?*) behind the corresponding genes in rapeseed that have to be assumed to be also FEHs rather than invertases (Table 1).

Besides the *cwINVs*, two vacuolar (*vacINVs*) were described in Arabidopsis *Atβfruct3* and *Atβfruct4*, and homologous sequences have also been found in rapeseed, designated as *BnvacINV1* and *BnvacINV2* (Table 1). In the case of *BnvacINV1*, the sequence seems to be interrupted from a ca. 200-bp intron that is absent in the corresponding sequence of Arabidopsis.

BncwINV2 has an organ and tissue-specific transcript profile comparable to *AtcwINV2*

Transcript levels were determined using the 750-bp amplicon of *BncwINV2* as probe in Northern hybridization against RNA that was isolated from leaves, stems, roots, small and big flower buds, flowers, anthers, carpels, and siliques from rapeseed (Fig. 1). The probe showed a highly specific transcript profile that is limited to big flower buds and open flowers. More precisely, transcription was evident especially in the anthers, while carpels RNA revealed only a minor transcription level (Fig. 1). To compare this result with the data for *AtcwINV2* in Arabidopsis, we added in Fig. 1 the mean values of *AtcwINV2*-transcript levels as given by the reference expression database for the meta-analysis of transcriptomes, Genevestigator V3 (Hruz et al. 2008).

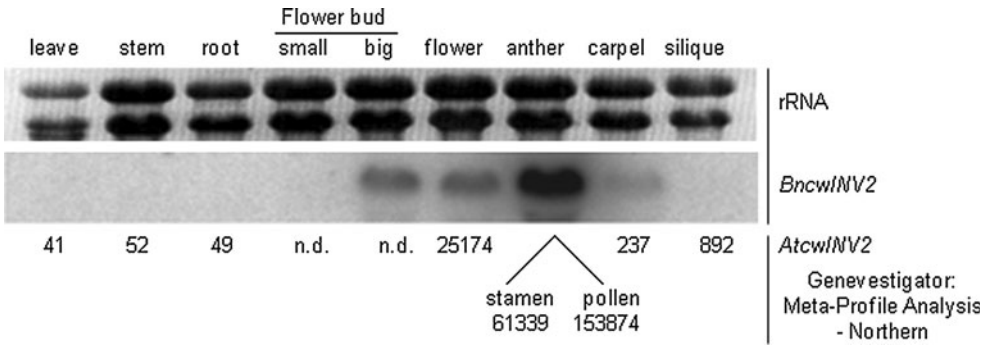

Fig. 1 Northern analysis of *BncwINV2*, probed against 15 µg RNA isolated from different tissues (rRNA is given as loading control: the leaves RNA has a slightly lower intensity) in comparison to *AtcwINV2*-transcript levels as given by the reference expression database for the meta-analysis of transcriptomes, Genevestigator V3

Reporter lines verify the interspecies compatibility of the Arabidopsis *AtcwINV2* promoter and *B. napus* by expressing the *β*-glucuronidase (GUS) gene *uidA*

Promoter compatibility between different species cannot be definitely predicted. For instance, incompatibility of the promoter *cwINV2* from Arabidopsis in tobacco and of the corresponding, orthologous tobacco promoter, *Nin88*, when transferred to Arabidopsis was found in a former study (Hirsche et al. 2009). Despite the closer relationship of *A. thaliana* and *B. napus*, the interspecies compatibility of the *AtcwINV2* promoter had to be checked by transferring the reporter construct *AtcwINV2::uidA* to rapeseed.

The rapeseed transformation was optimized using different protocols. According to our experiences, a pre-conditioning of the hypocotyl segments prior to transformation as described by Cardoza and Stewart (2003) was helpful to increase the transformation efficiency. Furthermore, the usage of a hypervirulent *Agrobacterium* strain (C58C1) seems to be a pre-condition for a successful transformation. The regeneration process started for the first 2 weeks on a regeneration medium without selection pressure, followed by cultivation on the same medium with selection on kanamycin. Developing shoots were transferred to another medium enhancing shoot elongation and finally on a root-inducing medium (for details, see "Materials and methods").

The transfer of the reporter fusion *AtcwINV2::uidA* to rapeseed verified the interspecies compatibility of the *AtcwINV2* promoter and *B. napus* by expression of the *β*-glucuronidase (GUS) gene *uidA* (Fig. 2). The construct turned out to maintain both its anther specificity and activity level in rapeseed.

Pollen germination is strongly reduced in consequence of antisense interference with *cwINV2* or by expressing a proteinaceous invertase inhibitor

In Arabidopsis, the repression of *cwINV2* activity by anther-specific RNA interference turned out to be an efficient method to circumvent carbohydrate supply of the symplastically isolated pollen with subsequent strong decrease of pollen germination ability and seed setting (Hirsche et al. 2009). Comparable results were also obtained by expressing the proteinaceous invertase inhibitor *AtC/VIF2* under the control of this promoter. Due to the close relationship of *A. thaliana* and *B. napus*, the similarities of the coding sequences of invertases and the orthologous

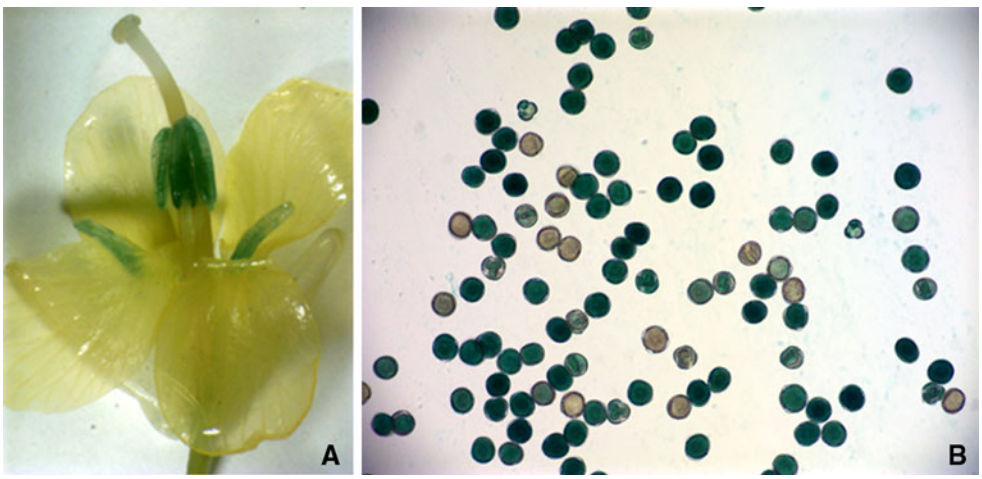

Fig. 2 Histological GUS staining of *Brassica napus* flowers (**a**) and pollen (**b**) expressing *AtcwINV2::uidA*

promoter function established in the course of this study (see above), we decided to use the same constructs for rapeseed transformation, whose functionality to interfere with pollen maturation in Arabidopsis was already proven in our former study (Hirsche et al. 2009).

Among the regenerated rapeseed plants from transformation with *AtcwINV2::AtcwINV2 antisense* or the proteinaceous invertase inhibitor *AtcwINV2::AtC/VIF2*, pollen germination was initially evaluated by light microscopy and plants with obviously reduced pollen germination rates were preselected (Fig. 3). From these plants, pollen germination rates were quantitatively determined (Table 2). Among these lines, a variation of germination rates was observed with a reduction to ca. 5% of visible pollen in the strongest phenotypes. In comparison, the germination rates in the empty vector control or wild types reached 66–77%.

In situ staining confirms the reduced invertase activity in pollen with reduced germination rates (Fig. 4). While pollen with normal germination rates show a dark blue-black staining in incubation medium, pollen of male sterile plants largely failed to be stained. Even in the control, where sucrose as the substrate is omitted, pollens of male sterile plants do not show the reddish staining normally observed in fertile pollen. This verifies the degradation of the pollen in the male sterile plants.

Discussion

The acid invertase gene family from *B. napus* in comparison to other species

In plant cells, invertases (beta-fructofuranosidases EC 3.2.1.26) are found in the cell wall (*cwINV*), vacuole (*vacINV*) and cytoplasm (*cytINV*). *cwINVs* and *vacINVs* are both acid invertases with optimum pH about 5.0 and their amino acid sequences are more closely related to each other than to the *cytINV* (alkaline/neutral, optimum pH between 6.5 and 8.0). The origin of *cytINVs* is believed to be in cyanobacteria (Sturm 1999; Sturm and Tang 1999; Vargas et al. 2003) and recent reports point to a subcellular location with targets to mitochondria and chloroplasts (Ji et al. 2005; Murayama and Handa 2007; Vargas et al. 2008).

Six putative *cwINV* genes have been identified in the *A. thaliana* genome (*AtcwINV1-6*; Sherson et al. 2003), two of them, *AtcwINV3* and *AtcwINV6*, later turned out to be fructan exohydrolases (*FEH*) that split one terminal fructose unit from a longer fructan chain, instead of sucrose cleavage (de Coninck et al. 2005). They possess lower p*I* values of 5.5 and 4.8 compared to 8.1–9.7 for the remaining *cwINVs*. Due to high sequence homologies between the *B. napus* invertases that have been isolated in our investigations and the *A. thaliana* invertases, we

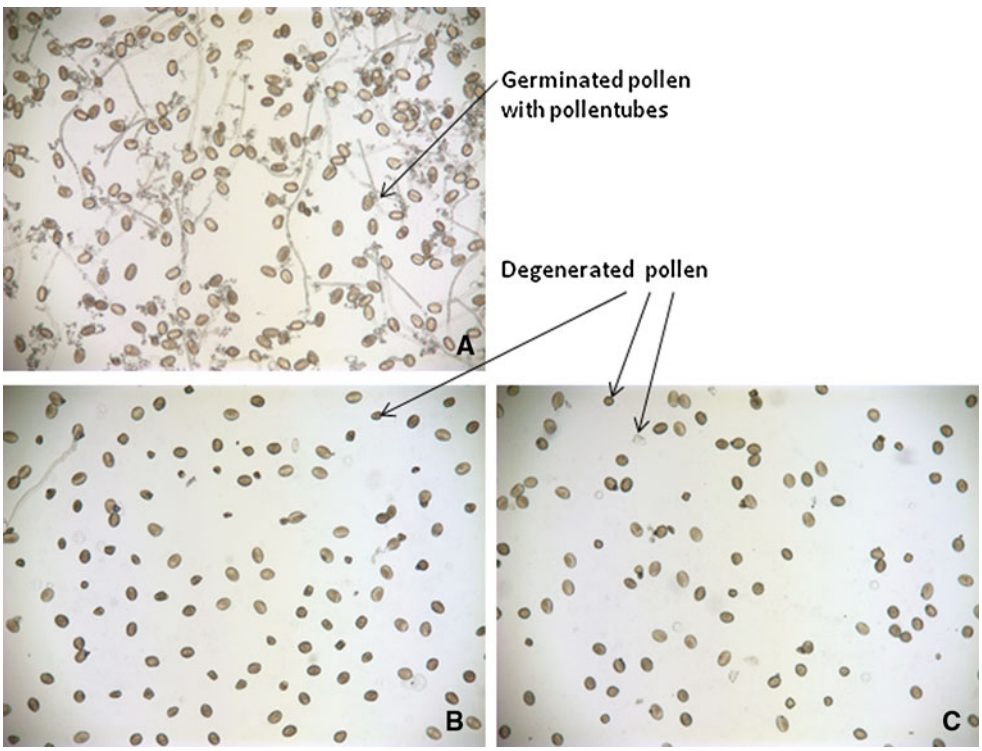

Fig. 3 In vitro pollen germination of *Brassica napus* transformed either with the empty vector (**a**) or with the invertase antisense (*AtcwINV2::AtcwINV2 antisense*) (**b**) and the invertase inhibitor (*AtcwINV2::AtC/VIF2*) construct (**c**), respectively

Table 2 In vitro pollen germination rates of *B. napus* transformed with *AtcwINV2::AtcwINV2-As* or with *AtcwINV2::AtC/VIF2* in comparison to an empty vector control and to wild-type plants

Construct	Plant no.	Germination rate (%)
AtcwINV2::AtcwINV2-As	D_2/20-10	5
	D_2/20-21	17
	M_6/3-4	13
	M_6/5-4	13
	M_6/5-11	17
AtcwINV2::AtC/VIF2	D_3/13-18	6
	D_3/13-23	38
	D_3/13-29	7
	D_3/13-41	29
	D_3/14-3	25
	M_7/2-4	18
	M-7/2-5	18
	M_7/3-7	19
	M_7/3-8	24
	M_7/3-10	9
pCambia2000 (empty vector)	M_8/2-3	66
Wild type	M_5	74
	M_7	77

D cv. 'Drakkar', *M* cv. 'Mozart'

decided to adapt the nomenclature from Sherson et al. (2003) for the *cwINVs* from rapeseed, but to accommodate the new findings concerning the *FEH* function, we added (*FEH?*) behind the suggested nomenclature of *B. napus* (Table 1). Interestingly, a comparable set of genes seems to be present in tobacco: Among the group of six *cwINVs* in tobacco, two possess lower p*I* values and have most probably to be considered as *FEHs* (Engelke et al. 2010). As in *A. thaliana* and *B. napus*, and as predicted for *N. tabacum* also other species like sugar beet (*Beta vulgaris*) possess *FEHs*, but apparently lack endogenous fructan substrates. The most plausible function for a specific *6-FEH* in these non-fructan plants would be to degrade (and/or prevent the formation of) exogenous levan-type fructans from bacterial origin (van den Ende et al. 2003). The close relationship between the gene functions of *FEHs* and *cwINVs* was shown by Le Roy et al. (2007): a single amino acid exchange in *AtcwINV1* is sufficient to switch the invertase function to *FEH* function. Both types of enzymes, *cwINVs* and *FEHs*, partly do not cluster in different branches of the phylogenetic tree, indicating that they evolved rather from each other than from different ancestors (Engelke et al. 2010).

vacINVs have lower p*I* values of ca. 5–6, compared to *cwINVs* with p*I* values of 8–9 and can be distinguished from another by a single amino acid difference in their cysteine catalytic sites (WEC-P/V-DF): *cwINVs* have a proline residue in the sequence motif and *vacINVs* possessing a valine residue (Goetz and Roitsch 1999). In Arabidopsis, two *vacINVs* are known (Haouazine-Takvorian et al. 1997; Tymowska-Lalanne and Kreis 1998) and have been referred to as *Atβfruct3* and *Atβfruct4* (also by Sherson et al. 2003). Though both rapeseed vacuolar invertases isolated during our investigations are highly homologous to the Arabidopsis sequences, we decided to name them *BnvacINV1* and *BnvacINV2*, since both *cwINVs* and *vacINVs* are beta-fructofuranosidases, and the numbers 3 and 4 in Arabidopsis are somehow misleading, because two *vacINVs* are present only. Again, the same number of *vacINVs* was found in tobacco (Lauer 2006; Engelke et al. 2010) as well as in the monocotyl species rice (Ji et al. 2005). Thus, the set of *vacINVs* seems to be somehow conserved in monocotyl and dicotyl plants, though the sequences do not group in the same cluster of phylogenetic analyses (Engelke et al. 2010).

Identification of an anther-specifically expressed *cwINV* in rapeseed and related genes in other species

BncwINV2 showed a highly specific transcript profile that is limited to the flowers. Transcription was evident in RNA isolated from big flower buds and open flowers, and especially in the anthers, while carpels RNA revealed only a minor transcription level, maybe as a result from previous pollination. The comparison to data from Genevestigator revealed a similar transcription profile of the corresponding gene *AtcwINV2* in Arabidopsis.

The anther-specific expressed isoenzymes indicate the crucial function of extracellular invertases in providing carbohydrates to the male gametophyte in different species, namely in Arabidopsis (*AtcwINV2*, Hirsche et al. 2009), carrots (*DcInvDc2*, Lorenz et al. 1995), potato (*StinvGF*, Maddison et al. 1999), tobacco (Goetz et al. 2001; Hirsche et al. 2009) tomato (*ScLin7*, Godt and Roitsch 1997; Fridman and Zamir 2003; Proels et al. 2006), and rapeseed (*BncwINV2*, present paper). Interestingly, all these anther-specific *cwINVs* from dicotyl plants form a distinct phylogenetic cluster among invertases and related genes. In monocotyl plants, on the other hand, most investigations of the involvement of invertases in pollen development have been done in wheat and rice, considering abiotic stresses, drought and cold (Koonjul et al. 2004; Saini 1997; Oliver et al. 2005; Cho et al. 2005). These *cwINVs* from monocotyl species group in a phylogenic cluster differ from the one with the anther-specific invertases from dicots (Engelke et al. 2010). Thus, in both groups of plants, monocotyls and dicotyls, there seems to be a kind of co-evolution, but no recent common ancestor of anther-specific *cwINVs* (Engelke et al. 2010).

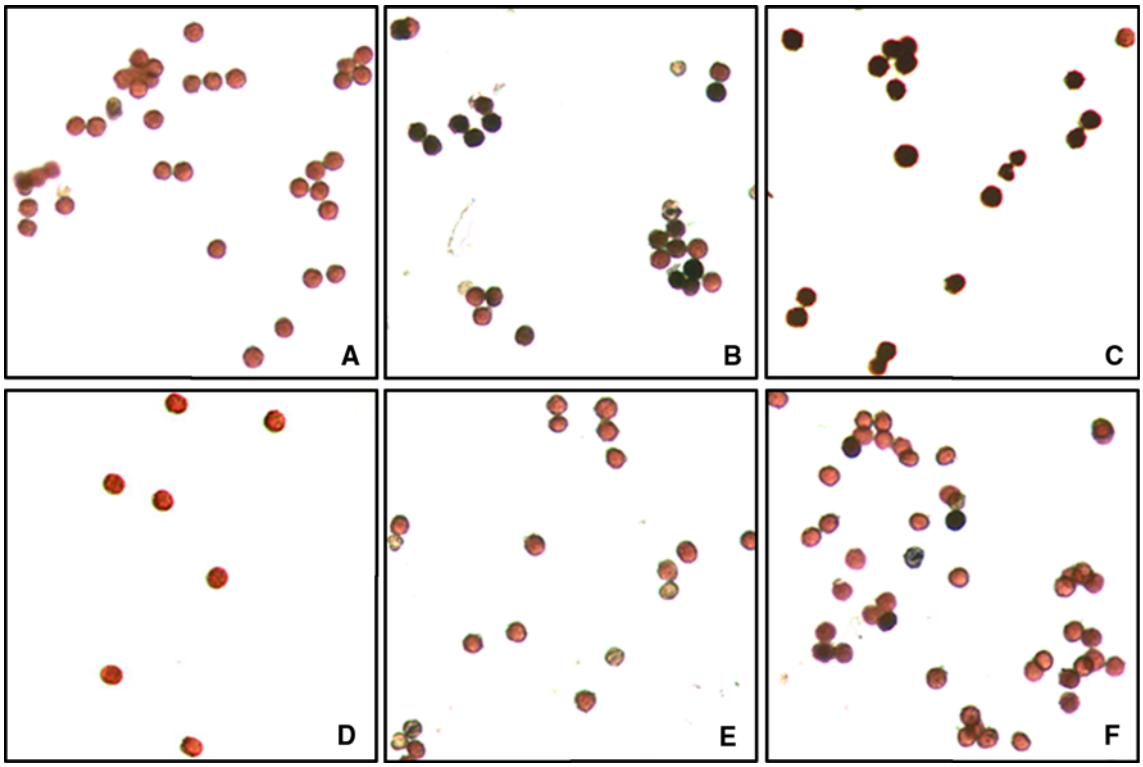

Fig. 4 In situ stain of invertase activity of a plant transformed with the empty vector and male sterile plants transformed either with *AtcwINV2::AtcwINV2 antisense* or *AtcwINV2::AtC/VIF2*. **a** Empty vector (M_8/2-3), control omitting sucrose in incubation medium. **b**, **c** Empty vector (M_8/2-3), dark staining indicates sucrose cleavage (two examples of the same treatment). **d** *AtcwINV2::AtcwINV2 antisense* (D_2/20-10), control omitting sucrose in incubation medium. **e** *AtcwINV2::AtcwINV2 antisense* (D_2/20-10), failure of dark staining indicates missing sucrose cleavage. **f** *AtcwINV2::AtC/VIF2* (D_3/13-29), failure of dark staining indicates missing sucrose cleavage

Promoter compatibility of *AtcwINV2* and rapeseed

In spite of the close relationship among the anther-specific *cwINVs* within one cluster, a promoter compatibility of these invertases cannot necessarily be assumed for far-related species, as our former studies have revealed for Arabidopsis and tobacco (Hirsche et al. 2009). On the other hand, a promoter may maintain its specificity and activity level between closely related species, as it was the case with the *AtcwINV2* promoter in *B. napus* in the present study. This was verified by the GUS reporter line (*AtcwINV2::uidA* in rapeseed) when compared to the reference expression database for the meta-analysis of transcriptomes and to our former investigations of this reporter construct in Arabidopsis (Hirsche et al. 2009).

Generating male sterile rapeseed plants by anther-specific interference with invertase activity

Despite the hard-won success of the transfer of the *ogu*-CMS from Raphanus to rapeseed with its long history (cf. "Introduction"), the synthesis of new and possibly superior systems permitting hybridization remains an important activity for many *Brassica* breeders, not least due to the concern for genetic vulnerability in view of the current emphasis on *ogu*-CMS. In order to reach this goal, additional interspecific crosses have been done with biological limitations associated with current systems. Biotechnology has added new possibilities to obtain male sterile plants and the present study supports that generating male sterile plants by anther-specific interference with invertase activity is applicable not only to the model plants Arabidopsis, tobacco and tomato, but also to agronomic important crops like rapeseed.

Rapeseed plants transformed with *AtcwINV2::AtcwINV2 antisense* or the proteinaceous invertase inhibitor *AtcwINV2::AtC/VIF2* showed the expected reduction of invertase activity by in situ staining and drastic reduction of pollen germination ability with strongest phenotypes to ca. 5% of visible pollen. It should be mentioned that the total amount of pollen is reduced in these plants, and thus the remaining pollination ability is overestimated when compared to the situation in wild-type plants (Hirsche et al. 2009).

Toward the practical application of the described male sterility in hybrid breeding, the system has to be extended for a mechanism that allows the multiplication of male sterile plants without segregation in the offspring (maintaining) as well as for a restorer mechanism, since seeds are

harvested from rapeseed. To reach these goals, a strategy was recently devised in tobacco by replacement of the downregulated natural plant invertase activity by expressing a distantly related isoenzyme (Engelke et al. 2010). Restoration of fertility was successfully achieved by substituting the downregulated endogenous plant invertase activity by a yeast (*Saccharomyces cerevisiae*) invertase fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (*PiII-ScSuc2*), under the control of the anther-specific invertase promoter *Nin88* from tobacco. The chimeric fusion *PiII*-ScSuc2 is known to be N-glycosylated and efficiently secreted from plant cells leading to its apoplastic location. Furthermore, the *Nin88::PiII-ScSuc2* fusion does not show effects on pollen development in wild-type background. Thus, such plants can be used as paternal parents of a hybrid variety, thereby the introgression of *Nin88::PiII-ScSuc2* to the hybrid is obtained and fertility is restored (Engelke et al. 2010). Propagation of the male sterile line can be achieved by coupling the construct that interferes with natural plant invertase activity (antisense or inhibitor) to an herbicide resistance gene. This linkage would permit the identification of the male sterile line before flowering. Another possibility would be the fusion of the construct used for restoration (*PiII-ScSuc2*) under the control of an inducible promoter. This would allow a reversible suppression of the sterility by an inducing agent and thus permit self-pollination of the male sterile line.

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