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ORIGINAL PAPER

Interspecies compatibility of the anther specific cell wall invertase promoters from *Arabidopsis* and tobacco for generating male sterile plants

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Abstract Histochemical GUS-staining and fluorometric analyses revealed strong tissue specific activities of the cell wall invertase promoters *Nin88* from *Nicotiana tabacum* and *AtcwINV2* from *Arabidopsis thaliana* that are restricted tightly to anthers and pollen, respectively. Both in *A. thaliana* and *N. tabacum* repression of invertase 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. In the case of tobacco, comparable results were also obtained by expressing a proteinaceous invertase inhibitor, whereas this approach was less efficient in *Arabidopis*. The present study revealed that anther specific interference with invertase-activity in order to generate male sterile plants can be applied to members of the two different plant families *Solanaceae* (*N. tabacum*) and *Brassicaceae* (*A. thalaina*) and the strategy seems to be a general tool for practical application in hybrid breeding or as biological safety precautions. To elucidate the compatibility of the isolated promoters beyond plant families, we transferred the regulatory sequences into the respectively heterologous systems, i.e. the *Nin88* promoter into Arabidopsis and the *AtcwINV2* promoter into tobacco. The specificities of both promoters are maintained in the heterologous backgrounds, but their activities are strongly reduced as GUS-stainings of flowers and pollen revealed and fluorometrical quantification confirmed.

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Introduction

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. Different methods can be used to prevent self-pollination of the female line, all of them with special advantages but also with drawbacks. Hand emasculation is labor and cost intensive and thus limited to crops with separate male and female flowers—as is the case with maize. In crops with androgynous flowers this procedure is normally not feasible. The use of gametocides under field conditions did not become widely accepted, since 100% sterile offspring is difficult to achieve and only a few fertile flowers can waste the hybrid with inbred plants (sibs).

Sex inheritance and self incompatibility are limited to certain crops where such genetic systems are available, as in cucumber and cabbage, respectively. Genic (or nuclear) male-sterility (GMS or NMS) is frequently found in different species, usually inherited in a monogenic recessive way. Genic male-sterility plants can at best be propagated by pollination with heterozygous plants, resulting in a segregation of sterile and fertile plants in a ratio of 1:1 in diploid species, so that fertile plants have to be eliminated. Only few systems are described in which environmental factors such as photoperiodism or temperature effects with influence on sterility can be used for propagating genic male sterile lines (Dickson 1970; He et al. 1999; Subudhi et al. 1997; Dong et al. 2000; Latha et al. 2004).

The most frequent used mechanism in hybrid breeding is cytoplasmic male-sterility (CMS), as the maternal transmis-

sion of sterility inducing cytoplasms, specified by mitochondrial mutations, in combination with Mendelian nuclear genes, permits the efficient control of pollination. However, the requirement to maintain three lines (male-sterile, maintainer and restorer) as well as the transfer of these traits to locally adapted varieties is a time consuming process. Problems may occur from instable male sterile lines resulting in the pollution of the hybrid with sibs, or instability of the restorer, with the consequence of decreased pollination and thus fruit or seed setting (reviewed in Engelke et al. 2004a; Roitsch and Engelke 2006). Apart from these special drawbacks that are limited to certain CMS systems, CMS is not available in all crops, due to limitations in mitochondrial genome diversity which can partly be explained with the evolutionary development of the different species (Engelke et al. 2003, 2004b and references therein). Crosses between far related crops by using embryo rescue methods in order to broaden the mitochondrial genome diversity are limited due to missing combining ability of the nucleus with a foreign cytoplasm, and the introgression of restorer loci brought in from the donor species might be associated with meiotic disorders or unwanted genes can be linked to the restorer locus (Bannerot et al. 1977; Menczel et al. 1987; Delourme et al. 1998).

Despite the technical difficulties, breeders tend to favor hybrid breeding due to the advantages over open pollinated varieties. F1-hybrid seeds are obtained by crossing parental inbred lines. Their special importance is due to the uniformity that is based on the resulting genetic homogeneity as well as on heterosis or hybrid vigour that is based on the heterozygous nature of the individuals (Brewbaker 1964; Feistritzer and Kelly 1987). This is also the reason, why the purchase of seeds is necessary every year from the breeding company.

A further important application for male sterility systems, apart from hybrid breeding, is their use as a biological safety method for the increasing number of genetically modified organisms used in field trials and agricultural production. Although the risk of out-crossing to wild type species depends on the individual species, their mode of pollination (self-pollination, out-crossing by wind or insects) as well as the presence of related species in the surrounding ecosystem, the availability and use of such biological safety will help to cope with the increasing public awareness and fears of the potential spread of transgenes.

Biotechnology has added new possibilities to obtain male-sterile plants. Various successful approaches to engineer nuclear-encoded male sterility have been described, although only few systems are ready to be used in agriculture or already in practical use (reviewed in Roitsch and Engelke 2006). Most of them interfere with the tapetum, a cell layer that contributes to the development of pollen by secreting numerous substances into the locular space, such as carbohydrates and proteins that are either important for pollen growth and development or become components of the outer pollen wall (Pacini and Franchi 1991). Since pollen are symplastically isolated, an unloading pathway of assimilates via the apoplasmic space is mandatory. 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 invertase, which is ionically bound to the cell wall. The hexose monomers (glucose and, with a lower preference fructose) are taken up into the sink cell by high-affinity hexose transporters. The importance of cell wall bound invertases during this process was demonstrated in a former study (Goetz et al. 2001). It was shown that the cell wall invertase *Nin88* from tobacco is characterized by a specific spatial and temporal expression pattern in tapetum tissue and developing pollen, pointing out the critical role of this enzyme for carbohydrate supply of the symplastically isolated pollen. Indeed, the tissue-specific antisense repression of *Nin88* under control of the corresponding promoter in tobacco resulted in a block during early stages of pollen development, thus causing male sterility without having any pleiotropic effects.

Since pollen development is a fundamental process that takes place in different seed plants in a more or less analogous manner, it is supposed that the process of inhibiting anther specific cell wall invertase can be adapted to a broad range of species. However, cloning of constructs with the particular sequences from each plant would be a time and labor intensive process. Thus the question arises, if one and the same construct can be used in different plant species, or, in other words, if there is an interspecies compatibility for such constructs. Therefore, three different aspects have to be regarded: (i) Is the antisense sequence of a cell wall invertase from one plant able to induce RNA-interference in another species, (ii) Can the antisense approach be substituted by the anther specific expression of a proteinaceous invertase inhibitor, which might have a functional redundancy and thus would be generally applicable to different species? (iii) Does the promoter exhibit the same expression characteristics (level and pattern of expression) when transferred in a species other than the original? To answer these questions the following experiments were done: An invertase inhibitor from tobacco (*NtCIF*; Weil et al. 1994; Greiner et al. 1998) under the control of the *Nin88*-promoter was expressed in tobacco transformants and the reduction in pollen fertility was compared to the previously described antisense approach. Both constructs were also tested in *Arabidopsis thaliana* and compared to the function of constructs including the homologous sequences from *Arabidopsis*, namely the promoter of *AtcwINV2* (*in silico* data from Genevestigator, Zimmermann et al. 2005) fused to its own antisense sequence and to an

invertase inhibitor from *Arabidopsis* (*AtC/VIF2*; Link et al. 2004). Furthermore, both promoters, *Nin88* from tobacco and *AtcwINV2* from *Arabidopsis*, were compared in the particular homologous and heterologous systems by fusion with the reporter gene *uid*A and detecting *β*-glucuronidase (GUS) enzyme activity using the substrate 5-bromo-4-chloroindoylglucuronide (x-gluc) to determine promoter activity in a histochemical assay and the substrate 4-methylumbelliferyl-glucuronide for a sensitive quantitative assay (Jefferson et al. 1987).

Materials and methods

Nin88 promoter constructs

The reporter construct for identification of tobacco *Nin88* promoter activity (*Nin88* fused to the *uidA* gene) as well as the construct *Nin88::Nin88-antisense* were used, as previously described, in pBI101 binary vector (Goetz et al. 2001). For the inhibitor construct, the *uidA* gene was replaced against the cDNA of *NtCIF*, using *Sma*I and *Xho*I restriction sites.

AtcwINV2 promoter constructs

A 1160 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′-CGAAGCTTGTAAATATGGGGA AGATAC-3′) and Ath2CW-Prom-R (5′-ATGTCGACTG GTGCAAGAGAACTCTATG-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.

For generating the antisense construct, a 1251 bp fragment of exon 3 was amplified by 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*.

For generating the invertase inhibitor constructs, the coding sequence of *AtC/VIF2* (At5G64620) was amplified using the primers Atcwinh-1 (5′-CTGAGGTACCTCG AGCCTGAAATGGCTTCTTCTC-3′) and Atcwinh-2neu (5′-CTGATCTAGAGGGCCCTCATTCAACAAGGCGA TC-3′) as an amplicon of 543 bp as well as the coding sequence of *NtCIF*, using the primers Ntcwinh-F (5′-CTCC TCGAGGTCGACATGAAGAATTTGATTTTC-3′) and Ntcwinh-R (5′-GTTGGATCCGAGCTCTCACAATAAA TTTCTGAC-3′) as an amplicon of 531 bp. Both inhibitors were fused 3′ of the *AtcwINV2* promoter in the binary vector by replacing the *uidA* gene.

Plant transformation

The constructs mentioned above were used for *Agrobacterium tumefaciens* strain LBA4404-mediated transformation. Tobacco leave discs (*N. tabacum* cv. Samsun) were transformed by using standard procedures (Horsch et al. 1985). *A. thaliana* cv. Columbia was transformed using the floral dip method (Clough and Bent 1998). Transformed plants were selected on Murashige-Skoog (MS) medium containing 2% sucrose, 0.3% gelrite, and 100 mg L^{-1} kanamycine. *Arabidopsis* plants were transferred to soil and grown in a climate chamber under short day conditions (9 h day, 15 h night) at 22°C in the light and 18°C in the dark until flowering. Tobacco plants were grown in the greenhouse at 22°C in the light and 18°C in the dark with 12 h light under additional illumination. Transformation was confirmed by PCR, amplifying either the *nptII*-gene or parts of the specific constructs.

Southern hybridization

The copy numbers of the integrated constructs were determined by Southern-analysis. Genomic DNA (15 µg) were restricted with *Bam*HI, *Eco*RI and *Hin*dIII, respectively. After fixation onto a nylon-membrane, the DNA was hybridized with a ^{32}P-labelled probe of the *uidA*- and the *npt*II-sequence.

Seed set, expressing in vivo ability to fertilization

Natural occurring seed set after self-pollination was observed, in order to preselect plants with reduced ability to fertilisation as an indication of reduced pollen germination. In the case of *Arabidopsis*, the development of siliques was macroscopically observed and compared to the wild type, in the case of tobacco at least five seed vessels were collected and the weight was determined just prior opening of the vessels.

In vitro pollen germination and vitality tests

The germination of *Arabidopsis* pollen was tested on sitting drops of media containing 0.4 mM calcium chloride and 0.4 mM boric acid, with 1% (w/v) low melt agarose (according to Hülskamp et al. 1995, with modifications). As carbon source, 440 mM sucrose was added to the medium. 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 26°C overnight. The percentage of germinated pollen was calculated by counting at least 600 pollen per transgenic plant.

The germination of tobacco pollen was tested under the same conditions in a medium according to Shivanna and Sawhney (1995) containing 290 mM sucrose, 1.5 mM calcium nitrate, 1.6 mM boric acid, 1 mM potassium nitrate, 0.8 mM magnesium sulfate, 15 % (w/v) PEG4000, solidified to sitting drops with 1.5 % (w/v) low melt agarose. Pollen germination rates reflect the mean of three independent flowers with at least 100 pollen each.

Pollen number per anther was calculated using a Jessen counter chamber, and pollen vitality was investigated by unspecific staining of the cytoplasm with acteocarmine acid. Results reflect the mean of three independent flowers.

Determination of extracellular invertase activity of pollen

Pollen from single flowers were collected and stored at −80°C. Before determining the invertase activity, pollen was washed twice in 500 µl solution containing 200 mM HEPES (pH 7.5), 3 mM $MgCl_2$,1.0 mM EDTA, 2% Glycerin and resuspended in the same. Pollen-solution (100 µl) was incubated with 30 µl of 0.1 M sucrose solution and 470 µl of buffer (70 mM K_2HPO_4 and 40 mM citric acid, pH 4.5). Samples were incubated at 30°C and after 30, 60, 120, 180, and 240 min, 100 µl were removed and cooled down on ice in a microtiter well. 200 µl GOD-reagent (0,1 M K_2HPO_4, pH 7.0; 0.8 U/ml peroxidase; 10 U/ml glucose-oxidase; 0.8 mg/ml ABTS) was added and after further incubation for 1 h on ice, the intensity of the green color was measured at 595 nm using an Elisa-Reader (MRX-TC Revelation, Dynex Technologies) and converted in ng glucose (glc) using a standard curve. The amount of pollen was determined using a Jessen counting chamber and the invertase activity was expressed as ng glc per 1000 pollen and min. Results reflect the mean of three independent flowers. Three repeats were performed for each flower.

Histochemical GUS staining

Reporter gene activity was histologically determined with (1 mM) 5-bromo-4-chloro-3-indolyl-ß-D-glcuronide (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 against 70% (v/v) ethanol to remove chlorophylls.

Fluorometric assay of GUS activity

GUS activity was assayed according to Jefferson et al. (1987). Pollen were lysed in 250 µl extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM ß-Mercaptoethanol) by freezing in liquid nitrogen and grinding with mortar and pestle, as well as by three times ultrasonication for 5 s each. Cell debris was removed by centrifugation (8,000 rcf; 4°C; 8 min). Aliquots of 50 µl of the supernatant were added to 50 µl of the assay buffer (extraction buffer containing 2 mM 4-methylumbelliferyl-D-glucuronid (4-MUG) as substrate, prewarmed) and incubated at 37°C in the dark. After 60 min of incubation, 50 µl stop solution (200 mM sodium carbonate) were added. The reaction product 4-methylumbelliferone (4-MU) was detected fluorometrically using a Fluoroskan Ascent (Labsystems). Protein concentration was determined by Bradford assay (Bradford 1976). GUS activity was expressed as picomole 4-MU min^{-1} μg^{-1} protein with data from pollen-samples from at least three independent flowers per plant. Three repeats were performed for each sample.

Results

Expression of the invertase inhibitor *NtCIF* as an alternative to antisense repression of tobacco invertase *Nin88* for reduction of male fertility in tobacco

Tapetum and pollen specific antisense repression of extracellular invertase *Nin88* under control of the corresponding *Nin88* promoter has been shown previously to be a highly efficient method to generate male sterile plants (Goetz et al. 2001). To address the question, whether this antisense approach to reduce invertase activity in anthers can be replaced by the expression of a proteinaceous invertase inhibitor, which might have the advantage of a functional redundancy, transgenic plants have been generated that express the inhibitor *NtCIF* under control of the *Nin88*-promoter. Compared to untransformed control plants, the transgenic plants expressing the *Nin88::NtCIF* construct did not show any phenotypic differences with respect to growth rate, height, morphology of vegetative and floral organs and tissues and time of flowering. The effect of *Nin88* mediated expression of *NtCIF* in tobacco on fertility has been compared to Nin88 mediated expressing of Nin88 antisense construct. The ability to produce seeds after self-pollination was reduced in both types of transgenic plants, indicating a lower in vivo pollen germination capacity (Fig. 1a).

Seed vessels of wild type tobacco normally have a weight in the range between 150 and even more than 200 mg (Figs. 1b, 2). The strongest inhibition of seed vessel development was observed for the *Nin88::Nin88-antisense* plant NT102K with 28 mg as mean from nine vessels in the first bloom and 26 mg as mean from seven vessels in the second bloom, as well as for the plant NT102KK that

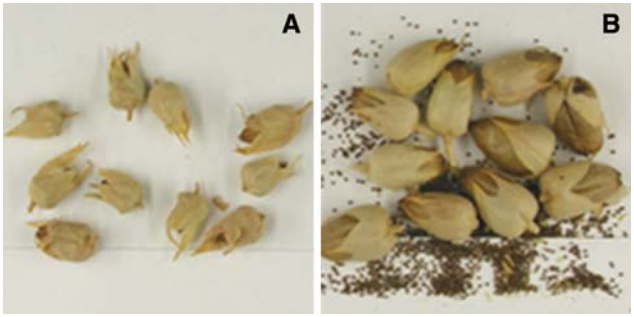

Fig. 1 Reduced seed vessel development of the transgenic *Nin88::Nin88-antisense* plant NT102K (**a**) compared to wild type tobacco SNN (**b**)

was grown as two cuttings from one and the same callus, giving 19 mg as mean from six vessels for the first cutting and 17 mg as mean from ten vessels for the second cutting (Fig. 2). Drastic weight losses of seed vessels to less than 50 mg, nearly without any seeds, were seen in 10 out of 23 (43%) antisense plants (Fig. 2).

In case of the plants expressing the inhibitor, only 1 out of 9 (11%) plants was observed showing a strong phenotype with a seed vessel weight beyond 50 mg (Fig. 2, plant NT105L with 25 mg).

As expected, the inability to produce seeds was correlated to defects of the pollen, expressed by a reduced pollen number, pollen vitality and in vitro pollen germination capacity. The amount of pollen per anther was reduced from ca. 50.000 in the wild type to around 7,500 in NT102K (*Nin88::Nin88-antisense*) and 1,000 in NT105L (*Nin88::NtCIF*). Considering the vitality (examined with acetocarmine acid staining) of the present pollen, this result becomes even more drastic: The pollen of the wild type showed 90% vitality, while NT102K exhibited 26% and NT105L only 14% of vital pollen (Fig. 3a–c). The in vitro pollen germination was reduced from around 80–90% in the wild type, to less than 10% in the total population of pollen in the transgenics (Fig. 3d–f).

To test if the reduced pollen numbers, vitalities and germination rates are correlated with a reduction of the extracellular invertase activity, the extracellular invertase activity was measured in pollen from transgenic plants and compared to the wild type activity (Fig. 4). The extracellular invertase activity is drastically reduced in both, pollen from transgenic *Nin88::antisense* as well as *Nin88::NtCIF* plants, compared to pollen from wild type plants. In addition, the reduction of invertase activity nearly directly matches the results of the vitality staining of the transgenic pollen with a decrease of invertase activity to ca. fourth of the wild type.

The *Nin88*-antisense and *NtCIF*-inhibitor constructs under control of the *Nin88*-promoter are less efficient in *Arabidopsis* in terms of reducing fertility

To check if the constructs that successfully established metabolically induced male sterility in tobacco are transferrable to *Arabidopsis*, transgenic plants were generated using the same constructs as for tobacco transformation, i.e. the *Nin88::Nin88-antisense* and *Nin88::NtCIF* constructs. By screening 70 and 10 primary transformants, respectively, not any plant exhibited a phenotypical change, like reduction of silique growth or prolonged flower life, which would be an indication for missing pollination. Because of that reason three lines each were randomly selected and in vitro pollen germination was determined in the next generation. The T2-lines inheriting *Nin88::Nin88-antisense* showed pollen germinations rates of about 50% and thus were comparable with the wild type with 60%. Likewise, no significant reduction was found in two of the lines inheriting *Nin88::NtCIF*. However, the third line showed a more severe decrease to 24% of germinating pollen. With 38%, this double insertion line showed a high percentage of small and degenerated pollen.

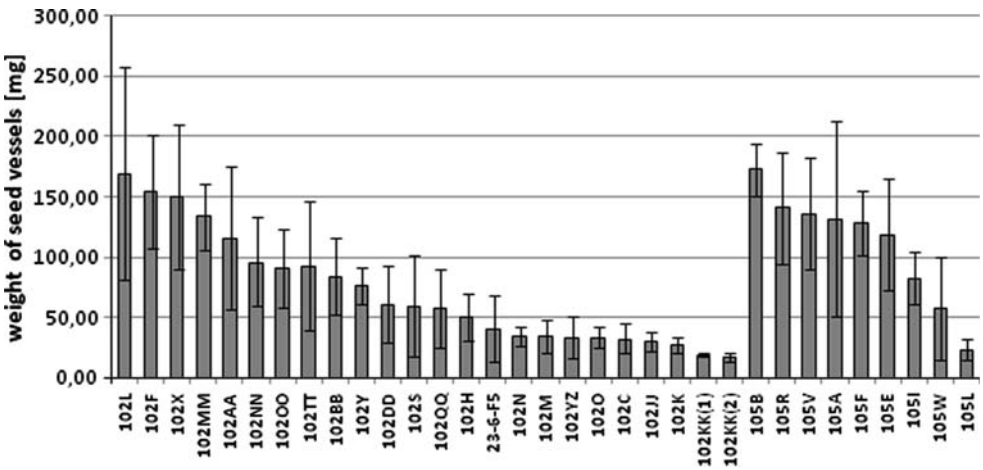

Fig. 2 Weights of seed vessels produced from *Nin88::Nin88-antisense* (line Nr.: 102) and *Nin88::NtCIF* (line Nr.: 105) tobacco plants (mean of at least five vessels ± SD)

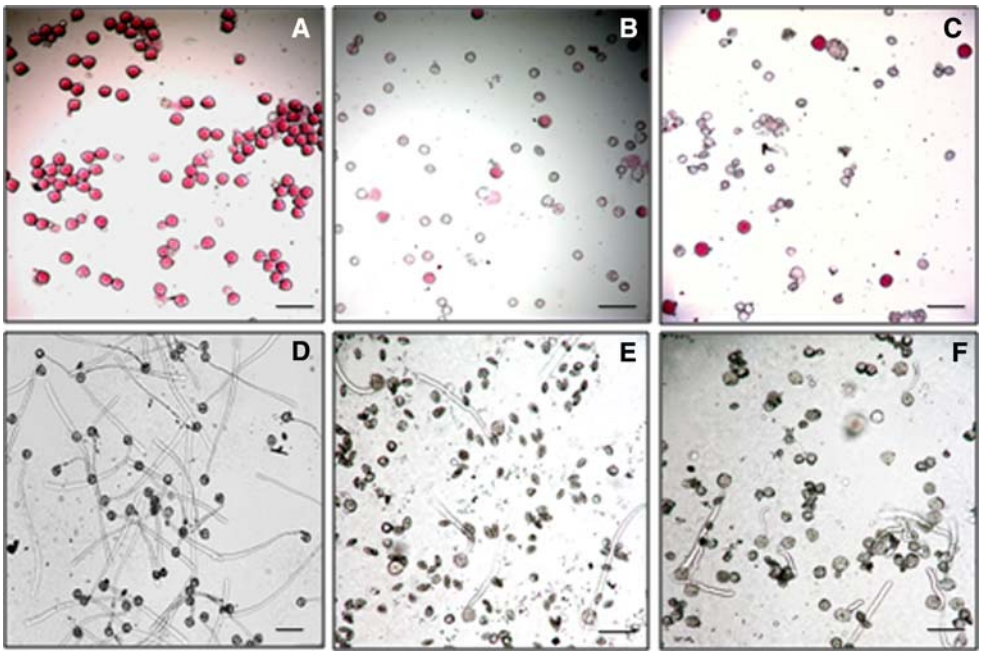

Fig. 3 Pollen viability (acetocarmine acid staining) and in vitro pollen germination of wild type tobacco SNN (**a**, **d**), *Nin88::Nin88-antisense* (**b**, **e** plant NT102K), and *Nin88::NtCIF* (**c**, **f** plant NT105L) (*bars* 100 µm)

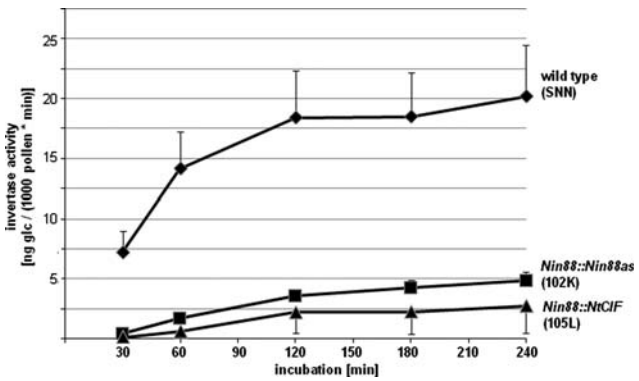

Fig. 4 Invertase activity from pollen of wild type tobacco SNN, transgenic *Nin88::Nin88-antisense* (plant NT102K), and *Nin88::NtCIF* (plant NT105L) (mean of three flowers ± SD)

Comparison of reporter lines expressing the β-glucuronidase (GUS) gene *uidA* under the control of either the tobacco *Nin88*- or the *Arabidopsis AtcwINV2*-promoter in the particular homologous and heterologous plant families reveals a limited interspecies compatibility of both promoters

The fact that *Nin88-antisense* and *NtCIF*-inhibitor constructs under control of the *Nin88*-promoter are less efficient in *Arabidopsis* in terms of reducing fertility raises the question, whether this is due to the insufficient promoter compatibility or due to the expressed tobacco sequences. Therefore we generated transgenic *Arabidopsis* and tobacco plants expressing the GUS reporter gene under control of the pollen specific promoter *Nin88* and its orthologous promoter *AtcwINV2* from Arabidopsis. Both promoters, when used endogenously in the particular system (*AtcwINV2::uidA* in *Arabidopsis*, Fig. 5a, b; and *Nin88::uidA* in tobacco) mediate a very strong GUS staining. However, only a weak expression of the reporter gene was detected, when the constructs were transferred to the respective other plant species (*Nin88::uidA* in Arabidopsis, Fig. 5c, d; and *AtcwINV2::uidA* in tobacco).

Quantification of the reporter gene activity by fluorometric analysis confirmed the results of the histological GUS staining. While the endogenous *AtcwINV2* promoter moderated a reporter activity up to 7.8 pmol MU min^{-1} μg^{-1} in *Arabidopsis* (Fig. 6, line AT37-10), the maximum activity in the corresponding tobacco lines (AT32) was found to be 0.067 pmol MU min^{-1} μg^{-1}, which is two orders of magnitude lower. The fluorometric analysis in tobacco revealed a reporter activity of up to 2.05 mediated by the endogenous promoter *Nin88* (NT40-13-8) while most of the tobacco lines transformed with *AtcwINV2*-promoter (NT123 lines) showed a GUS activity that was almost not distinguishable from the natural background (Fig. 6). However, three out of five analyzed lines (NT123-2-18, NT123-2-25 and NT123-2-30) showed a measurable reporter activity—with NT123-2-18 exhibiting an activity of 1.8, coming close to the maximum of the *Nin88::uidA* plants.

The copy number of insertions was determined by Southern-hybridization and revealed a linkage to the GUS expression. In *Arabidopsis*, the endogenous promoter mediated smaller levels of GUS expression as a single copy (Fig. 6, line AT37/4, /5, and /13), compared to plants with

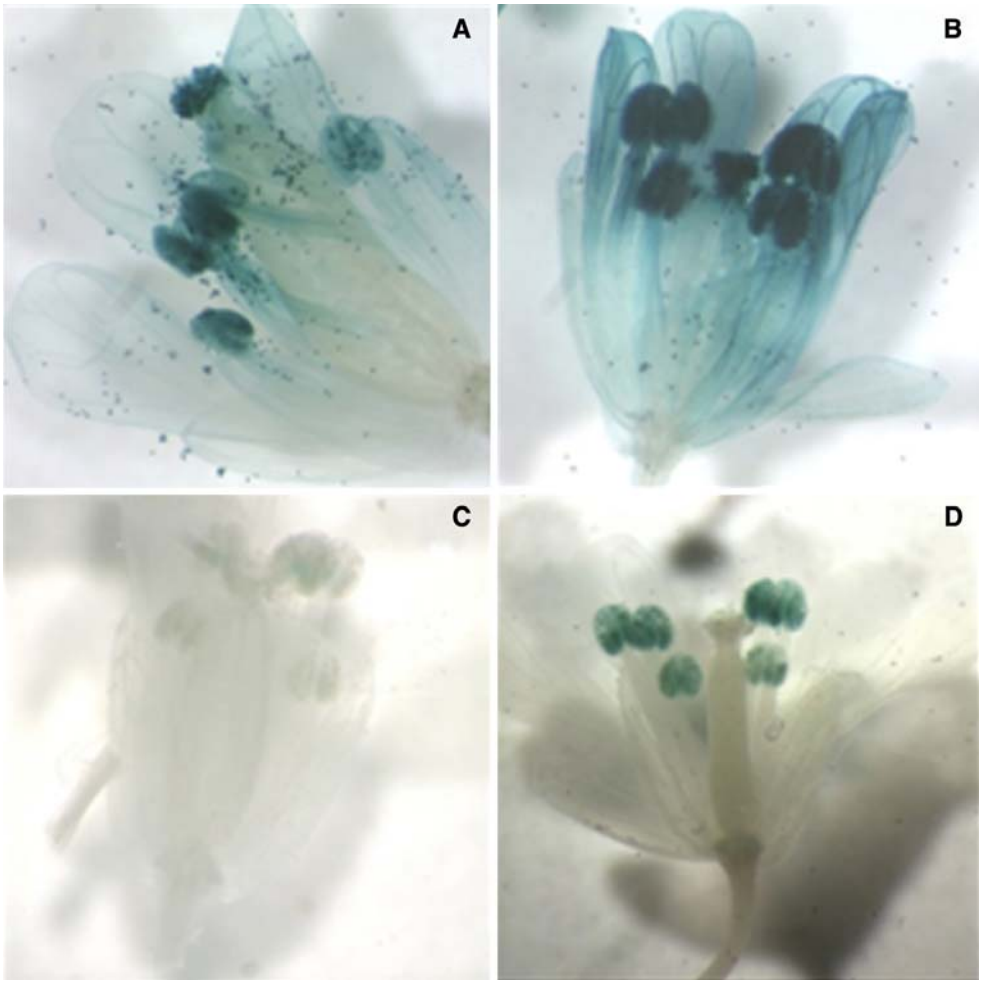

Fig. 5 Histological GUS-staining of *Arabidopsis* flowers expressing *AtcwINV2::uidA* (**a** AT37/13, single copy; and **b** AT37/11, six copies), and *Nin88::uidA* (**c** AT32/2-2, single copy; and **d** AT32/2-1; 4 copies), respectively

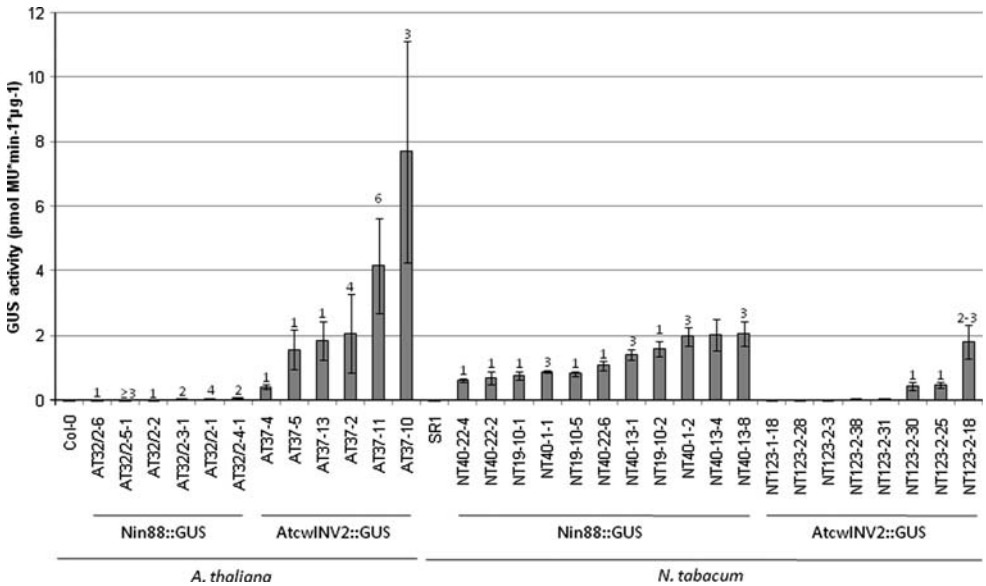

Fig. 6 Fluorometric GUS-assay quantification of *Arabidopsis* flowers and tobacco pollen expressing *AtcwINV2::uidA*, and *Nin88::uidA*, respectively (mean of at least three flowers ± SD)

multicopy insertions (Fig. 6, line AT37/2, /11, and /10 with 4, 6, and 3 insertions, respectively). This tendency holds to be true for tobacco with its endogenous promoter (Fig. 6, line NT19 and 40): within the six lines with lower GUS activity only one inherits a multicopy insertion (NT40/1-1 with three copies), the remaining five are single copies, while the five lines with higher GUS activities all but one (Nt19/10-2) inherit three insertions.

Antisense repression of *Arabidopsis* invertase *AtcwINV2* is more efficient than expression of the invertase inhibitors *AtC/VIF2* and *NtCIF* in terms of reduced seed setting and pollen germination in *Arabidopsis*

Since the constructs containing the tobacco sequences are not that efficient in *Arabidopsis* than in tobacco, the corresponding constructs using the endogenous *Arabidopsis* promoter *AtcwINV2*, fused to either the antisense sequence of *AtcwINV2* or to the inhibitor sequences from *Arabidopsis* (*AtC/VIF2*) and tobacco (*NtCIF*), were transformed into *Arabidopsis*.

Eleven out of 57 plants (20%) transformed with the antisense construct (*AtcwINW2::AtcwINV2-antisense*; line AT34) showed a differential phenotypic alteration concerning fertility. In comparison to the wild type, all of these lines exhibited a more or less impaired silique development and a partially prolonged flower life (Fig. 7a, b). Five plants did not produce any siliques at all and were pollinated with wild type pollen to maintain these lines. Because of limited plant material, pollen germination tests were performed in the T2-generation and confirmed the expected reduction in pollen germination capacity (Fig. 7c, d). Some of the lines were separated in two groups, since silique development was obvious in one part of the plants (indicated with F in Fig. 8), while decreased silique development was observed in another part (indicated with S in Fig. 8). These findings were not unexpected, since segregation of the copies take place in T2-generation. In the main, however, the percentages of germinated pollen in T2 tend to result in a strong correlation to the copy number of the construct determined in T1 (Fig. 8): in plants containing one single insertion, germination efficiencies from 30 to 40% have been measured, while in the line AT34/2-44 with four copies of the transgene the germination rate was reduced to 5% without any silique development.

In a further approach the proteinaceous invertase inhibitors *AtC/VIF2* (AT40) and *NtCIF* (AT41) were specifically expressed in the male tissue of Arabidopsis, in order to evaluate their capacities in reducing invertase activity in comparison to the antisense approach. In 5 out of 9 lines (56%) expressing *AtC/VIF2* and 5 out 19 lines (26%) expressing *NtCIF*, a repression in silique development was observed. While plants expressing the tobacco invertase

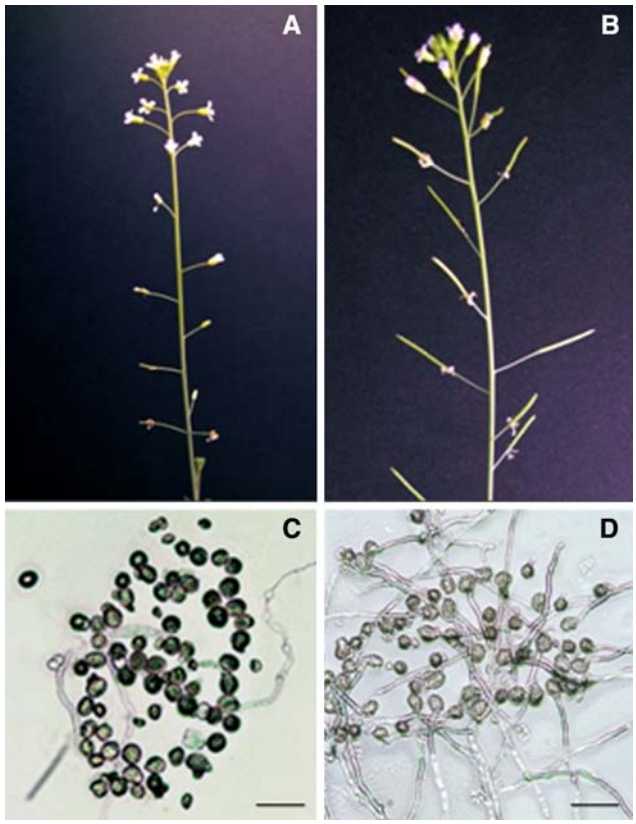

Fig. 7 Impaired silique development, prolonged flower life and reduced pollen in vitro germination in a transgenic *AtcwINV2::AtcwINV2-antisense* plant AT34/2-44 (**a**, **c**) compared to wild type *Arabidopsis* Col-0 (**b**, **d**) (*bars* 100 μm)

inhibitor *NtCIF* only showed a moderate reduction in pollen germination to between 38 and 32%, plants expressing the *Arabidopsis* inhibitor *AtC/VIF2* showed a variation in the germination rate between the lines, that was not directly dependent on the copy number of the inserted constructs (Fig. 8). Plants of the line AT40-1 with one copy did not differ in germination efficiency, compared to the wild type, while the line AT40/2-6F inherited four copies and showed a reduction of the germination rate to 23%. Plants of the lines AT40/2-1 and AT40/2-6 split in the T2-generation into groups with a higher and a considerably lower germination efficiency.

Discussion

The present study supports that generating male sterile plants by anther specific interference with invertase activity is generally applicable to different plant species, and has been proven for members of the two different plant families *Solanaceae* and *Brassicaceae*. The formerly described invertase *Nin88* from *N. tabacum* (Goetz et al. 2001) was compared to *AtcwINV2* from *A. thaliana* by histochemical

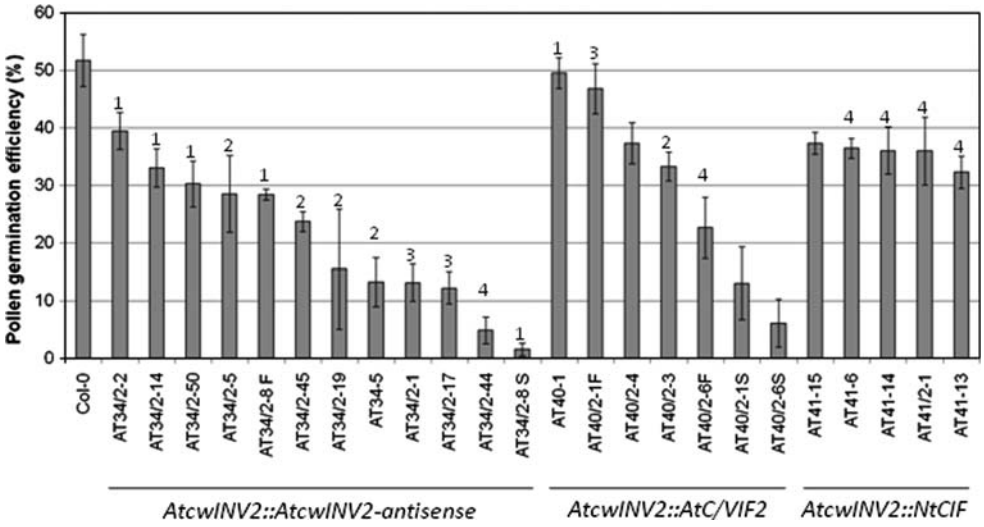

Fig. 8 Pollen germination rates of transgenic *AtcwINV2::AtcwINV2-antisense*, *AtcwINV2::AtC/VIF2* and *AtcwINV2::NtCIF Arabidosis* plants (mean of at least 600 pollen ± SD)

GUS-stainings and fluorometric analyses. Both promoters revealed a strong, tissue specific activity in their endogenous genetic background that is restricted tightly to anthers and pollen. The specific involvement of invertases during anther development was also suggested for other plant species, e.g. *Lilium longiflorum* (Ranwala and Miller 1998; Clément et al. 1996), tomato (Godt and Roitsch 1997), and potato (Maddison et al. 1999), indicating the crucial function of extracellular invertases in providing carbohydrates for the male gametophyte development. It has also been shown that an arrest in pollen development in wheat because of water deficiency, is correlated with alterations in carbohydrate metabolism and a drastic decrease in invertase activity (Dorion et al. 1996).

With regard to a broad usage for hybrid breeding or as a tool for biological safety precautions by using these already as anther specific characterized promoters and thus overcoming the complex cloning of new promoter sequences, we tested the regulatory sequences in the respectively heterologous systems, i.e. we transferred *Nin88* into *Arabidopsis* and *AtcwINV2* into tobacco to elucidate their compatibility beyond plant families. The specificities of both promoters are maintained in the heterologous systems, but their activities are strongly reduced as GUS-stainings of flowers and pollen revealed and fluorometrical quantification confirmed. In particular, the generated transgenic *Arabidopsis* lines inheriting the heterologous promoter *Nin88* expressed GUS at very low levels, compared to the activity reached with the same promoter in tobacco or with the endogenous promoter *AtcwINV2* in *Arabidopsis*. By expressing this latter promoter in tobacco, the majority of the transgenic lines also confer an explicitly weaker expression and only one line with three insertions reached the expression level of *Nin88*. The reduced promoter activities in the heterologous systems have to be considered as the main reason, why only a weak influence on pollen germination efficiency was seen in *Arabidopsis* lines transformed with the *Nin88::Nin88-antisense* (AT30) or *Nin88::NtCIF* (AT31) constructs. In none of these lines any drastic effect on pollen fertility and seed set occurred, on opposite to tobacco plants transformed with the same constructs. Apart from the male sterility approach that requires a strong inhibition of invertase activity, the gradual regulation of genes by using these different promoters might be interesting as a tool for other approaches in basic research, such as manipulating phytohormone levels or to interfere with nutrient supply during microspore development to facilitate the generation of double haploids.

The reduced activities of both promoters in the heterologous systems are most likely caused by the evolutionary distance of both plant families. It has been shown in numerous investigations that a variety of promoters from mono- and dicotyledonous species maintained their temporal and spatial activity (Bäumlein et al. 1991; Takaiwa et al. 1991; Hamilton et al. 1992; Pauls et al. 1994; Tsuchiya et al. 1994; Yamada et al. 1997). However, these promoter studies were mainly used to identify various *cis*-acting elements from promoter fragments, so that comparative studies concerning promoter activity in homo- and heterologous systems are mostly not available. Reporter studies concerning different seed, and anther specific promoters show that also the specificity can be changed in the heterologous system (Gallusci et al. 1994; Stålberg et al. 1998). The activity of flower specific promoters from *B. campestris* differs after transference into tobacco, while specificity remains unchanged in the close relative *A. thaliana* (Sato et al. 1991; Xu et al. 1993). These results show that a differential set of transcription factors is available in the corresponding plant organs and that they do not recognize all *cis*-elements of foreign promoters (Weising et al. 1988; Stålberg et al. 1998). The reduced activities of the promoters *Nin88* and *AtcwINV2* could therefore be caused by an insufficient

recognition of specific *cis*-acting elements in these promoters and by the binding of factors to certain sequences, which are repressive to transcription, respectively.

Even among lines transformed with one and the same endogenous construct, different levels of GUS expression or reduction of fertility were observed. Stronger phenotypic alterations were correlated to the copy number of the transferred constructs. However, this relation is not completely linear and might be influenced by position effect variegation (Henikoff 1990; Peach and Velten 1991; Springer et al. 2003; Lippman et al. 2004) or structural reorganization of the transgene (Rai et al. 2007). Besides this, very high copy numbers could be responsible for silencing-effects, which lead to a reduction in transcript amount (Schubert et al. 2004).

In addition to the missing compatibility of the promoters, RNA interference by using *Nin88-antisense* in *Arabidopsis* is maybe less efficient, since the homology of the *Nin88* and *AtcwINV2* sequences (61%) is too small. As a consequence, not only the available promoters might be unusable in far related crops, but also the antisense-part of the construct has to be generated from the endogenous invertase sequence. To address the question, whether the antisense approach can be replaced by the anther specific expression of a proteinaceous invertase inhibitor, which might have a more general redundant function, transgenic lines expressing the inhibitor *NtCIF* under control of *Nin88*-promoter were compared to the respective antisense construct in tobacco. Though the relative number of strong phenotypes was higher in the case of the antisense repression of the *Nin88*, expressing the invertase inhibitor *NtCIF* turned out to be an alternative in terms of reduced seed setting and pollen germination. However, expressing the inhibitor *AtC/VIF2* under control of the *AtcwINV2*-promoter in *Arabidopsis* was less efficient than the respective antisense construct. Thus, the expression of a proteinaceous inhibitor is not per se as efficient as RNA interference. This conclusion is substantiated by the fact that the endogenous inhibitor *AtC/VIF2* proved to be more effective in *Arabidopsis* than *NtCIF* from tobacco under control of one and the same promoter. So the expression of an orthologous inhibitor might be inappropriate to decrease invertase activity and thus a general function cannot certainly be predicted. The structural difference of invertase *AtcwINV2* in comparison to tobacco extracellular invertases might lead to modified protein interactions which result in a suboptimal inhibition of cell wall invertase activity in *Arabidopsis* (Greiner et al. 1998).

A precondition for the practical use of engineered male sterility is the ability to propagate the male sterile line, without segregation in sterile and fertile plants in the next generation. So far, only one report exists describing the genetic-engineering of CMS (Ruiz and Daniell 2005), since this way is limited due to the lack of efficient methods to transform either mitochondria or chloroplasts, and problems that arise from effective restoration. Concerning engineered nuclear-encoded male sterility-systems, maintaining of sterility in the next generation is a main problem which is the reason why only few systems are ready to be used in agriculture or already in practical use (reviewed in Roitsch and Engelke 2006). To overcome this problem, the sterility has to be restored in an inducible way in order to allow the temporary production of fertile pollen for self-pollination. Possible approaches include the supertransformation of the male sterile individuals with a yeast invertase gene that is not subject to the antisense repression by the plant invertase. Alternatively, the introduction of a sucrose transporter would be a method to bypass the requirement for extracellular sucrose cleavage. The same approaches can also be used for plants, in which fruits or seeds are harvested (e.g. rice, corn, or tomato) to obtain restoration of fertility in the hybrid.

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